Angiotensin potentiates excitatory sensory synaptic transmission to medial solitary tract nucleus neurons

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The nucleus tractus solitarii (nTS) in the dorsal medulla comprises rostrocaudally oriented columns of diverse neurons and fiber tracts that lie lateral to the walls of the fourth cerebral ventricle and join above the central canal caudal to the obex (4, 73). This complex structure, with bidirectional connections to medullary and forebrain nuclei that subserve autonomic functions, plays a major role in the regulation and integration of visceral systems, including normal and impaired cardiovascular mechanisms (60, 61, 82). Anatomic studies (25, 26, 43, 52, 85) and in vivo recordings (91) verify that a substantial proportion of the arterial baroreceptor sensory fibers that originate from receptors in the aortic arch and carotid sinus regions course through the solitary tract (TS) into the nTS and make their first synapse on neurons in the dorsal medial region of the nucleus. Neurons in this region also receive synaptic inputs from other visceral and brain autonomic nuclei via the TS (79). The nTS is a primary central site at which angiotensin (ANG) II influences cardiovascular function. Within the brain, the anatomic distributions of ANG II and its receptors and their mRNAs correlate reasonably well with the probable functions of the peptide (2, 57). However, the cellular mechanisms responsible for the cardiovascular effects of ANG II mediated by the nTS remain unclear.

Microinjection of femtomole doses of ANG II into the dorsal medial region of the nTS produces a transient fall in blood pressure and heart rate very similar to the response to activation of the cardiovascular baroreflex in anesthetized rats (41, 71, 78) and evokes a comparable bradycardia in an in vitro beating heart-brain stem rat preparation (75). Paradoxically, however, higher doses evoke pressor responses, creating dose-response relationships that are biphasic. ANG II injection into the nTS also generates dose-dependent inhibition of baroreceptor reflex bradycardia in anesthetized and conscious rats (21, 64, 69) and attenuates baroreflex inhibition of cardiac sympathetic outflow in the rat heart-brain stem preparation (17, 75). Because antagonists to the ANG II type 1 (AT1) receptor injected into the nTS potentiate baroreceptor reflex responses, whereas ANG II type 2 receptor antagonists are ineffective (64, 65), endogenous ANG II may attenuate baroreflex function via AT1 receptors in this region. Cardioinhibitory and neuronal actions of low-dose ANG II in the dorsal medial nTS region are also mediated by AT1 receptors (16, 41, 53).

The neuronal pathways and mechanisms responsible for these contrasting actions of ANG II within the nTS have not been established. Several reports have documented that short-latency sensory synaptic transmission onto nTS neurons is mediated by non-N-methyl-D-aspartate (non-NMDA) ionotropic excitatory amino acid receptors (5, 46, 58, 83, 89, 90), whereas longer-latency responses to synaptic activation of these cells
appear to be mediated by NMDA receptors (8). Others have shown that low doses of substance P (SP) given in the dorsal medial nTS region evoke decreases in arterial pressure and heart rate similar to those produced by ANG II (49, 55, 63). Because the distributions of receptors for ANG II and SP are strikingly overlapped in the nTS (1, 31, 50), these peptides may act on a common subset of nTS neurons. Indeed, extracellular recordings suggest that many neurons in the dorsal medial nTS region that respond to ANG II are also sensitive to the baroreceptor reflex (23, 27), in contrast to the baroreflex inhibition attributed to ANG II.

Recent in vivo studies have begun to examine the interactions between ANG II and SP in the nTS. Prior injection of an SP antagonist blocked the hypotension and bradycardia evoked by ANG II, but not by Glu, microinjected into the medial nTS region (32, 33). In the rat heart-brain stem preparation, potentiation by ANG II of the chemoreflex bradycardia was blocked by an SP antagonist, whereas the fall in heart rate evoked by the baroreflex was unchanged (75). Related studies in rat brain slices suggested that nTS neurons that differ in baseline firing rate also vary in their responses to ANG II (53); cells with spontaneous action potentials responded to TS stimulation with excitatory postsynaptic potentials (EPSPs) that increased after ANG II, whereas the peptide decreased TS-evoked EPSPs or had no effect in silent nTS neurons.

The present studies examined the cellular mechanisms of the modulation by ANG II of short-latency excitatory synaptic transmission from TS afferent fibers onto neurons in the dorsal medial nTS region that were excited by ANG II, Glu, and SP. Our previous results suggest that ANG II activates different neuronal groups within this nTS region via separate mechanisms (77). The reported cardioinhibitory effects of ANG II in the nTS are consistent with the presynaptic facilitation by ANG II of Glu released from sensory axons onto a group of dorsal medial nTS region neurons, which we previously designated group A (28). However, we also identified another group of neurons in this region activated by ANG II and SP via postsynaptic receptors on these cells, which we designated group B.

METHODS

Dorsal Medulla Horizontal Slice Preparation

All experiments followed the guiding principles for the care and use of animals in research of the American Physiological Society (3). Protocols were approved by the Institutional Animal Care and Use Committees at Oregon Health and Science University and the Cleveland Clinic Foundation. Male Sprague-Dawley rats (150–250 g, n = 55; Harlan, Indianapolis, IN, or B & K Universal Northwest, Kent, WA) were deeply anesthetized with halothane or ether and decapitated. The hindbrain was removed, submerged in cold buffered artificial cerebrospinal fluid (aCSF, 2°C) bubbled with O2, and prepared for cutting horizontal medulla slices as described previously (6, 77). The medulla was trimmed into a block centered on the calamus scriptorius and tilted so that 400-µm horizontal slices cut with a Vibratome under cold, oxygenated aCSF contained maximal lengths of the TS. Slices containing the commissural and dorsal medial regions of the nTS surrounding the tracts were perfused with aCSF (34–37°C, flow rate 1.5–2 ml/min) under a warm, humidified stream of 95% O2-5% CO2 in an interface-type slice chamber for ≥1 h before recording was initiated. The aCSF contained (in mM) 120 NaCl, 5 KCl, 1.2 K2HPO4, 2 MgSO4, 2 CaCl2, 25 NaHCO3, and 10 D-glucose and was continuously bubbled with 95% O2-5% CO2. Unless specified otherwise, chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Two types of electrical recordings were made from nTS neurons in these slices. All recordings were obtained from cells located between 400 µm rostral and 200 µm caudal to the obex and medial to the TS, in the dorsal medial region of the nTS, which has been reported to receive a high density of aortic baroreceptor afferent projections (25, 26, 43, 52, 85). In initial survey experiments, neurons were impaled with sharp electrodes to determine whether ANG II modulated the synaptic responses of the cells to stimulation of the TS. The positive findings of these studies prompted us to design a more extensive series of experiments that used whole cell patch-clamp recordings to investigate whether the responsiveness of a neuron in the dorsal medial nTS region to ANG II and Glu or SP was related to the influence of ANG II on the short-latency excitatory synaptic responses of the cell to stimulation of TS axons.

Sharp Intracellular Recordings

Glass microelectrodes were fabricated with a Flaming-Brown horizontal micropipette puller (model P-97, Sutter, Novato, CA) filled with 4 M potassium aspartate; tip resistance of the electrodes was 50–120 MΩ. Microelectrodes were placed within the dorsal medial nTS region under direct observation with a stereomicroscope. Criteria for a successful neuronal impalement included ≥15 min of recording with a stable membrane potential of greater than ∼45 mV, overshooting action potentials, and neuron input resistance >60 MΩ (Axoclamp 2A, Axon Instruments, Foster City, CA).

Whole Cell Patch-Clamp Recordings

Whole cell recordings were made with patch electrodes, pulled from thin-walled borosilicate filament glass (TW150F-4, WPI, Sarasota, FL) using a Flaming-Brown puller (model P-97, Sutter), tip resistance of the electrodes was 4–8 MΩ. The patch pipette solution contained (in mM) 130 potassium gluconate, 10 EGTA, 10 HEPES, 1 MgCl2, 1 CaCl2, and 5 K2-ATP. The solution was adjusted to pH 7.2–7.4 with KOH and had an osmolality of 290–295 mOsm. The voltage offset between the patch pipette and the reference electrode was zeroed when the pipette tip touched the aCSF perfusate in the chamber. When a tight seal (>5 GΩ) had been obtained, the membrane patch was ruptured. Whole cell recordings were obtained in current-clamp mode from neurons in the dorsal medial nTS region with a stable membrane potential greater than ∼45 mV, overshoot action potentials, and input resistance >200 MΩ (Axopatch 200A, Axon Instruments). Stable recordings were maintained for ≥45 min and frequently lasted >1 h.

Experimental Protocols

Microdrop agonist application. Agonist [ANG II or SP (Bachem Bioscience, King of Prussia, PA) or Glu] or the aCSF
vehicle was applied as a microdrop to the slice, as described previously (16, 77). Application of the agonists by the microdrop procedure, rather than in the chamber perfusate, was essential to restrict their effects to the immediate vicinity of the recorded neuron, thus preventing lasting desensitization of the cell. In the initial sharp intracellular recording studies, ANG II was given as a dose of 5 pmol (500 nl of 10 μM), which is within the range that evokes depressor responses. In subsequent whole cell recording experiments, the doses of ANG II (500 or 200 fmol, 500 or 200 nl of 1 μM) were selected from the middle of the range that decreased arterial pressure and heart rate when microinjected in vivo into the dorsal medial nTS region, as reported by us (29, 41) and others (32, 78). These doses were selectively blocked by the AT1 receptor antagonist losartan (16, 41). SP (1 μM) was also given as a 500- or 200-fmol dose (68 or 27 pg), which is within the range that produced hypotension and bradycardia when microinjected into this nTS region (23, 55). Glu (100 μM) was given as a 500-pmol dose. However, because the applied concentrations of the agonists were diluted by the aCSF that perfused the surface of the slices, the actual doses that reached the membrane were likely to be somewhat lower.

The properties of EPSPs evoked by TS stimulation were reevaluated. ANG II was given as a dose of 5 pmol (500 nl of 10 μM) and Glu (100 μM) were selected to test simple EPSPs. We isolated the EPSPs from action potentials by hyperpolarizing the neurons with current injection to maintain membrane potential between −80 and −60 mV. Stimulation protocols were triggered by a computer coupled with a programmable stimulator (Master 8, AMP, Jerusalem, Israel). Responses were digitized on-line at 8–18 kHz (Experimenter’s WorkBench software, DataWave Technologies, Longmont, CO) for analysis after the experiment. Whenever possible, waveforms of four successive responses were signal averaged for each neuron. In addition, membrane resistance was assessed by measuring the changes in membrane potential in response to injection of small hyperpolarizing current steps. To differentiate postsynaptic responses from action potentials by hyperpolarizing the neurons with current injection to measure depolarization and the return of the membrane potential to the resting potential of the neuron was between 56 and 52 mV, hyperpolarizing current was injected to maintain membrane potential between −57 and −60 mV during TS stimulation. Threshold currents for evoking EPSPs and action potentials were established in each neuron. Responsiveness of the cell to ANG II, Glu, and SP was determined by monitoring membrane potential, input resistance, and the rates of spontaneous action potentials before and after administration of each of the peptides (500-fmol doses) and Glu. After return of neuronal properties to baseline after aCSF wash, 10–15 EPSPs evoked by TS stimulation with current just above the threshold for eliciting an EPSP were recorded from 26 ANG-responsive cells before and after application of ANG II or SP (200-fmol doses). Then non-NMDA synaptic transmission was blocked with the antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10–100 μM; Research Biochemicals International). Blockade of TS inputs was evaluated with maximal-intensity TS stimulation and the effects of ANG II or SP on the amplitude of TS-evoked EPSPs, neuronal membrane properties, and rates of spontaneous action potentials were reexamined. Agonist responses were retested after aCSF wash and reversal of non-NMDA receptor blockade in all cells in which DNQX blocked or attenuated the response to the agonist. In eight neurons, the responses to TS stimulation, ANG II, and SP were also examined before and after synaptic blockade with 100 μM tetrodotoxin (TTX). Computer-controlled protocols triggered current injection and data acquisition to measure membrane potential, input resistance, excitability, and other properties utilizing pCLAMP software (Axon Instruments).

The membrane voltage signal was also digitized (model VR-100-8, Instruteck, Larvik, Norway) and stored on videotape for off-line analysis of the properties of TS-evoked postsynaptic potentials, action potential characteristics, and firing rates.

**Data Analyses**

The location of the cell within the dorsal medial nTS region was determined in relation to standard slice landmarks. We measured the perpendicular distance in micrometers of the recording electrode from three markers easily visible in the transilluminated horizontal slice, i.e., rostrocaudal midline, TS, and dorsal motor nucleus of the vagus, with a calibrated eyepiece micrometer at the completion of recording from each neuron. At the end of each experiment, the slices were immersion fixed with 30% sucrose in 10% formalin, frozen sectioned at 50 μm, stained with neutral red, and examined microscopically to verify the location of the recording electrode within the dorsal medial region of the nTS (77). Off-line analyses were performed with pCLAMP or DataWave software routines. To determine whether a whole cell recorded neuron was responsive to an agonist, the rate of action potentials after application of the substance was compared with the 95% confidence interval generated from the distribution of the cell’s instantaneous spike rate during the 60-s period before administration of the agonist using the binomial probability distribution described previously (16). Obvious increases in rate or amplitude of spontaneous EPSPs were identified visually from the videotaped membrane potential with an oscilloscope.

The properties of EPSPs evoked by TS stimulation were analyzed off-line from membrane voltage signals digitized at 11 kHz using DataWave parameter extraction functions to measure the onset latency, area under the EPSP, peak voltage, peak latency, and rise time constant (dV/dt, where V is voltage and t is time) of each potential. Onset latency was defined as the time between the stimulus artifact and the beginning of membrane depolarization. The area under the EPSP was calculated by Simpson’s rule between the onset of depolarization and the return of the membrane potential to baseline. The peak latency was measured between the stim-
trodes, application of ANG II (5 pmol, 500 nl of 10
M) was measured as the maximum slope of the rising EPSP between its onset and the inflection before the peak. Membrane potential records lasting several minutes were sampled at 200 Hz–4 kHz before and after application of ANG II, SP, or Glu to document action potentials and changes in membrane potential or input resistance evoked by these agonists.

Statistical analyses were performed with GraphPad Prism (GraphPad Software, San Diego, CA). Differences between neuron subgroups in quantitative measures of responses were evaluated with Student’s t-test or one-way ANOVA followed by post hoc comparisons of means using Bonferroni’s correction for multiple comparisons. Equality of variances was tested with the two-sample F test. Values are means ± SE. P < 0.05 was the criterion for significance for all tests.

RESULTS

Sharp Intracellular Recording Studies

In the horizontal slice, electrical stimulation of the TS activates short-latency EPSPs (Fig. 1) that have high following frequencies (>100 Hz) and are sensitive to non-NMDA receptor-selective antagonists such as CNQX. If the stimulating electrode is moved out of the visible TS, EPSPs are no longer generated, even at very high stimulation intensities. Thus these responses are consistent with EPSPs evoked by selective activation of sensory afferent axons within the tract, with no contribution from local interneurons (6).

In this initial survey with sharp intracellular electrodes, application of ANG II (5 pmol, 500 nl of 10 μM) to the surface of the slice rapidly increased the amplitude of TS-evoked EPSPs in about one-third of the neurons in the dorsal medial nTS region with short-latency TS responses (Fig. 1). These findings agree with previous reports that between one-third and one-half of the neurons in the dorsal medial nTS region of the dog and rat are responsive to ANG II (12–14). Responsive neurons showed increases in EPSP amplitude ranging from 20% to >200% after application of the peptide (Figs. 1 and 2; 12 of 35 neurons). No decreases in EPSP amplitude were observed in response to ANG II. Membrane properties were unchanged in the 12 ANG-responsive neurons after application of ANG II, with membrane potential of −51.4 ± 4.9 and −52.5 ± 4.6 mV in control and ANG II-treated neurons, respectively (P = 0.207), and input resistance of 77.3 ± 11.6 and 77.8 ± 16.3 MΩ in control and ANG II-treated neurons, respectively (P = 0.856). When ANG II was applied to 5 of these 12 neurons after blockade of TS synaptic transmission with CNQX, the peptide had no effect on EPSP amplitudes (results not shown).

Whole Cell Patch-Recording Studies

Whole cell recordings were obtained from 64 neurons in the dorsal medial nTS region with TS-evoked EPSPs. Twenty-six of these neurons (41%) were excited by ANG II. As in our previous studies (12, 15, 16, 77), ANG II did not inhibit activity in any recorded cell. Responsiveness to ANG II was not related to the depth of the neuron within the slice: the 26 cells activated by ANG II were located an average of 53 ± 4 μm below the surface compared with the 38 neurons that did not respond to ANG II located 52 ± 2 μm below the surface (t = 0.84, P = 0.44). Resting membrane potential in these neurons averaged −54 ± 0.59 mV (range −49 to −60 mV), and input resistance averaged 416 ± 19 MΩ (range 218–617 MΩ). These passive membrane property values were similar to those reported by others (18, 39, 54, 84). The onset latency of EPSPs evoked in these 26 ANG-responsive neurons by stimulation of the TS was 2–6 ms and averaged 3.4 ± 0.2 ms. The distance between the stimulating electrode and the recorded neuron was 2–3 mm. Although the initial re-
spontaneous EPSPs in all 26 neurons, an IPSP was observed after the EPSP in 2 cells. These ANG-responsive neurons exhibited spontaneous EPSPs and action potentials at their resting membrane potential. Visual comparison of the membrane potential recordings on an oscilloscope before and after administration of ANG II also revealed an obvious increase in the frequency and amplitude of spontaneous EPSPs after administration of the peptide.

Application of ANG II (500 fmol, 500 nl of 1 μM) generated action potentials in all 26 neurons. Each cell was also tested for responsiveness to Glu (50 pmol) and SP (500 fmol). The 26 neurons were separated into two groups on the basis of their responsiveness to ANG II and SP. Twelve cells, activated by ANG II but unresponsive to SP, corresponded to a class we previously designated group A (28). All 12 group A neurons were also excited by Glu. The other 14 cells, responsive to ANG II and SP, were designated group B. Group B cells were divided into two subgroups on the basis of their responsiveness to Glu: eight cells were excited by Glu as well as by ANG II and SP, and the other six neurons were responsive only to ANG II and SP. About 75% of the 26 ANG-responsive neurons were excited by Glu, as reported previously (12).

Several characteristics of their synaptic responses to TS stimulation differentiated group A from group B neurons (Table 1). Overall, the onset latencies of TS-evoked EPSPs were significantly shorter in group A neurons than in either subset of group B neurons. Furthermore, the synaptic response latencies of group A neurons were considerably more uniform than those of group B neurons, because the sample variance of the onset latency for TS-evoked EPSPs was significantly lower in group A neurons responsive only to ANG II (0.136 ms²) than in group B cells responsive to ANG II and SP (0.605 ms²; 2-sample test for equal variances: F = 4.45, P < 0.01). Within group B, the latency to the peak voltage of the EPSPs was longer in the subset of group B cells responsive to both peptides and Glu than in the neurons activated by ANG II and SP but unresponsive to Glu (Table 1).

Differential Effects of ANG II and SP on Group A and Group B Neurons

In group A neurons, the area under the curve and the peak amplitude of the TS-evoked EPSPs were significantly increased by application of ANG II (200 fmol, 200 nl of 1 μM; Fig. 3, Table 1, group A). After administration of the peptide, the area under the EPSP was more than doubled in these cells. The maximal dV/dt was also more than doubled after application of ANG II, from 2.3 ± 0.22 to 5.1 ± 0.46 mV/ms (t = 5.39, P < 0.001), and the onset latency of the EPSPs was significantly decreased (Table 1, group A).

**Table 1. Area, onset latency, peak voltage, and peak latency of EPSPs evoked by TS stimulation in medial nTS neurons of group A or group B before and after application of ANG II**

<table>
<thead>
<tr>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Responsive to ANG and Glu only</strong></td>
<td><strong>Responsive to ANG, Glu and SP</strong></td>
</tr>
<tr>
<td>(n = 12)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>Area under EPSP, V-s·10⁻⁶</td>
<td>Control</td>
</tr>
<tr>
<td>123 ± 33</td>
<td>327 ± 85⁎</td>
</tr>
<tr>
<td>Onset latency, ms</td>
<td>3.1 ± 0.1b</td>
</tr>
<tr>
<td>Peak voltage, mV</td>
<td>12.7 ± 3.7</td>
</tr>
<tr>
<td>Peak latency, ms</td>
<td>8.7 ± 0.61</td>
</tr>
</tbody>
</table>

Values are means ± SE. EPSP, excitatory postsynaptic potential; TS, solitary tract; nTS, nucleus tractus solitarius; Glu, L-glutamate; SP, substance P. ⁎P < 0.02. a⁴P < 0.05, F = 4.32 for group A, compared with both subsets of group B neurons by ANOVA; post hoc comparisons were made after 1-way ANOVA using t values adjusted by Bonferroni’s method. cP < 0.01. dP < 0.05 for group A, ANG II compared with control by paired t-test. *P < 0.05, F = 4.40 for control for group B neurons responsive to ANG II, Glu, and SP compared with control for group B neurons responsive only to ANG II and SP by 1-way ANOVA and post hoc t values using Bonferroni’s method.

Fig. 3. ANG II increased area under the curve and peak voltage of EPSPs evoked by stimulation of the TS during whole cell patch recording from a group A medial nTS neuron. Superimposed EPSPs evoked by TS stimulation (12 μA, threshold current 7 μA) are shown. Control: 6 TS-evoked EPSPs before application of ANG II. Mean onset latency was 3.5 ms; mean peak voltage was 11 mV. Baseline membrane potential was −60 mV. ANG II: 6 EPSPs produced by TS stimulation 30 s–2 min after microdrop application of ANG II (200 nl of 1 μM, 200-fmol dose). Mean onset latency was 2.8 ms; mean peak voltage was 23 mV. Baseline membrane potential was −60 mV.

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ANG II (500 fmol) evoked a small depolarization in group A cells (4.8 ± 0.5 mV, n = 12, P < 0.001) that lasted 20–30 s and was accompanied by a modest decrease in input resistance (−33.0 ± 4.1 MΩ, P < 0.001; Fig. 4A, left). Peak firing rate after ANG II averaged 2.3 ± 0.2 spikes/s compared with <1 spike/s before application of the peptide. Firing continued for >2 min after administration of ANG II, and increased variability of the membrane potential after ANG II was also obvious (Fig. 4A). Administration of Glu evoked a characteristic brief depolarization with a decrease in input resistance and a burst of high-frequency action potentials in group A neurons (Fig. 4B, left).

In contrast to group A cells, TS stimulation evoked some action potentials as well as isolated EPSPs in group B neurons after application of 200 fmol of ANG II. However, the properties of TS-evoked EPSPs in group B neurons were unchanged after ANG II application (Table 1, Fig. 5). Furthermore, in the two group B cells that responded to TS stimulation with an EPSP-IPSP complex, the peptide had no effect on either type of postsynaptic potential. ANG II produced substantially greater membrane depolarization in group B than in group A neurons (21.5 ± 1.3 vs. 4.8 ± 0.5 mV, P < 0.0001) and generated high-frequency action potentials sustained for >2 min (Fig. 6A). The peak firing rate after application of ANG II was much higher in group B than in group A neurons (6.7 ± 0.8 vs. 2.3 ± 0.2 spikes/s, P < 0.0001), and the decrease in input resistance was also larger in group B than in group A cells (−51.3 ± 4.3 vs. −33.0 ± 4.1 MΩ, P < 0.005). SP also evoked prolonged depolarization, high-frequency action potentials, and a decrease in input resistance in group B neurons (Fig. 6B). However, TS-evoked EPSPs were unaltered by SP (200 fmol, 200 nl of 1 μM) in the five group B cells (Fig. 5) and two group A neurons tested (data not shown).

Non-NMDA Receptor Blockade Prevents Excitation Produced by ANG II in Group A but not in Group B Medial nTS Neurons

Blockade of non-NMDA ionotropic excitatory amino acid receptors with DNQX eliminated the EPSPs evoked by TS stimulation in all nine group A cells tested (Fig. 7). This blockade was reversed after ∼30 min. One group A neuron exhibited an IPSP after the short-latency EPSP evoked by TS stimulation. Although DNQX eliminated the EPSP in this cell, the IPSP remained and was clearly augmented compared with control (data not shown). In group A neurons, DNQX also blocked the high-frequency action potentials induced by ANG II (Fig. 4A, right). In contrast, the depolarization and action potentials evoked by Glu persisted after DNQX (Fig. 4B, right), verifying the presence of other types of postsynaptic receptors for

**Fig. 4.** Effects of ANG II and glutamate (l-Glu) on membrane potential of a group A neuron before and after administration of 6,7-dinitroquinoxaline-2,3-dione (DNQX). Single records of the membrane potential of this neuron sampled at 200 Hz–4 kHz are shown. Downward deflections at 2-s intervals are current injections (100 ms, −50 pA) used to monitor input resistance. Arrows, application of ANG II or Glu. A, left: before DNQX application, ANG II (200 fmol) evoked high-frequency action potentials, brief depolarization (6 mV), and a decrease in input resistance (66 MΩ). Rapid firing and increased variability of the membrane potential persisted beyond this 2-min record, but average membrane potential was almost unchanged. Baseline membrane potential was −58 mV. A, right: 100 μM DNQX blocked action potentials, depolarization, and decreased input resistance evoked by ANG II, suggesting that ANG receptors were presynaptic on Glu-containing afferent fibers. Baseline membrane potential was −57 mV. B, left: 50 pmol Glu evoked its characteristic rapid, brief depolarization (18 mV), decrease in input resistance (41 MΩ), and high-frequency action potentials. Baseline membrane potential was −57 mV. B, right: after administration of DNQX, Glu continued to elicit brief depolarization (16 mV), a fall in input resistance (38 MΩ), and high-frequency action potentials. Baseline membrane potential was −57 mV.

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Glu on these neurons. Subsequent blockade of synaptic transmission with 10 μM TTX in four group A neurons abolished the EPSPs evoked by TS stimulation and the action potentials produced by ANG II (data not shown). In six of eight group B neurons tested, DNQX eliminated the EPSPs evoked by TS stimulation, indicating that these EPSPs were generated by Glu that activated postsynaptic non-NMDA receptors. In contrast, TS-evoked EPSPs were attenuated by DNQX in one cell responsive to ANG II, SP, and Glu and were not altered in another neuron responsive to ANG II and SP but not to Glu. ANG II and SP continued to induce depolarization and high-frequency action potentials during non-NMDA receptor blockade in all eight cells tested (Fig. 6), consistent with the presence of postsynaptic receptors for ANG II and SP on group B neurons. Finally, after aCSF wash and time to permit recovery of TS-evoked EPSPs from non-NMDA receptor blockade, in four group B neurons responsive to all three agonists and three cells excited by ANG II and SP but unresponsive to Glu, the agonists continued to produce sustained membrane depolarization and EPSPs after blockade of synaptic transmission and TS-evoked EPSPs by TTX (Fig. 8).

The effects of blocking synaptic transmission with DNQX or TTX described above suggest that ANG II receptors are located on afferent terminals presynaptic to group A nTS neurons but are not present on the postsynaptic cell membrane (Fig. 9, top). In contrast, these observations reinforce the presence of postsynaptic receptors for ANG II, Glu, and SP on group B cells (Fig. 9, bottom). Because the group B neuron in which TS-evoked EPSPs were attenuated but not eliminated by non-NMDA receptor blockade was responsive to Glu and SP, its response to TS stimulation might have been mediated by release of Glu and SP from TS afferent terminals.

Further support for the disparate synaptic locations of ANG II receptors in group A and group B neurons was obtained in separate experiments that examined the ability of DNQX or TTX to block responses to ANG II, Glu, SP, or TS stimulation in 24 additional neurons in the dorsal medial nTS region with short-latency TS-evoked EPSPs. Although the capacity of ANG II or SP to alter the properties of TS-evoked EPSPs was not determined in these cells, they were classified as group A or group B on the basis of their responsiveness to ANG II, Glu, and SP by the same criteria used for the 26 neurons in the present study. In eight neurons responsive to ANG II and Glu, but not to SP (group A), TTX blocked TS-evoked EPSPs and ANG II-induced excitation, but not activation by Glu, consistent with ANG II receptors presynaptic to the recorded neuron (Fig. 9, top). In contrast, TTX eliminated the TS-evoked EPSPs, but not responsiveness to ANG II or SP in all 16 group B cells. DNQX and TTX were tested in one group B cell excited by ANG II, Glu, and SP in which non-NMDA receptor blockade attenuated, but did not abolish, TS-evoked EPSPs without altering the responses to either peptide or Glu. Subsequent application of TTX eliminated the TS-evoked EPSPs, but not the responses to the agonists. These data reinforce the intriguing possibility raised above that Glu and SP may contribute to TS-evoked EPSPs in some group B neurons (Fig. 9, bottom).

DISCUSSION

The present studies provide new evidence for multiple actions of ANG II within the dorsal medial nTS region. These disparate effects of ANG II appear to be associated with different types of neurons within the visceral pathways that regulate autonomic function. In the set of dorsal medial nTS neurons with the shortest latencies (group A), ANG II consistently facilitated glutamatergic synaptic transmission from TS afferent inputs via presynaptic receptors, because these responses were blocked by non-NMDA receptor antagonists or TTX, a characteristic feature of responses to sensory inputs. Group A cells were also unresponsive to SP. In contrast, ANG II did not alter TS-evoked
EPSPs in the other class of ANG-responsive dorsal medial nTS neurons (group B). Instead, ANG II and SP evoked intense excitation and sustained depolarization of group B cells. Because neither non-NMDA receptor antagonists nor TTX blocked these responses to the peptides, our findings imply that postsynaptic ANG receptors are present in group B cells. The longer latencies and greater variability of group B responses to TS stimulation are consistent with polysynaptic links to the TS that provide indirect visceral afferent activation of these cells via local interneurons. Thus our findings suggest that the disparate responses reported for ANG II microinjected into the dorsal medial nTS region may reflect preferential activation of second-order vs. higher-order neurons in visceral pathways of the nTS.

ANG II ENHANCES TS-EVOKED EPSPs IN THE nTS

Fig. 6. Effects of ANG and SP on membrane potential of a group B neuron before and after administration of DNQX. A single 2-min record of membrane potential of this neuron sampled at 200 Hz–4 kHz is shown for each condition. Arrows, application of ANG II or SP. A, left: before DNQX application, 200 fmol of ANG II evoked sustained depolarization (22 mV), a substantial fall in input resistance (86 MΩ), high-frequency action potentials, and increased variability of membrane potential that continued for >2 min. Baseline membrane potential was −58 mV. A, right: changes in membrane properties and action potentials evoked by ANG II persisted after administration of 100 μM DNQX, suggesting the presence of postsynaptic ANG receptors on the recorded cell. Baseline membrane potential decreased 20 mV; input resistance fell 75 MΩ. Baseline membrane potential was −57 mV. B, left: 200 fmol of SP produced its characteristic depolarization (18 mV), decreased input resistance (58 MΩ), and high-frequency action potentials. Baseline membrane potential was −58 mV. B, right: after administration of DNQX, SP continued to produce depolarization (16 mV) and a fall in input resistance (55 MΩ) and generate high-frequency action potentials sustained for >2 min. Baseline membrane potential was −59 mV.

Fig. 7. Blockade of non-N-methyl-D-aspartate (non-NMDA) receptors with DNQX abolished EPSPs evoked by stimulation of the TS in group A medial nTS neuron shown in Fig. 3. Control: 5 TS-evoked EPSPs (12 μA) before application of DNQX. Baseline membrane potential was −58 mV. Mean onset latency was 3.4 ms; mean peak voltage was 12 mV. DNQX: 2 min after microdrop application of 100 μM DNQX, maximal-intensity TS stimulation failed to evoke EPSPs. Baseline membrane potential was −57 mV. EPSPs reappeared after 30 min later, when receptors had recovered from non-NMDA blockade.

EPSPs in the other class of ANG-responsive dorsal medial nTS neurons (group B). Instead, ANG II and SP evoked intense excitation and sustained depolarization of group B cells. Because neither non-NMDA receptor antagonists nor TTX blocked these responses to the peptides, our findings imply that postsynaptic receptor mechanisms for both peptides are present in group B cells. The longer latencies and greater variability of group B responses to TS stimulation are consistent with polysynaptic links to the TS that provide indirect visceral afferent activation of these cells via local interneurons. Thus our findings suggest that the disparate responses reported for ANG II microinjected into the dorsal medial nTS region may reflect preferential activation of second-order vs. higher-order neurons in visceral pathways of the nTS.

ANG in the nTS: Integrative Role and Cellular Actions

Interest in the renin-angiotensin system, originally identified through its role in renal control of salt and water homeostasis, has expanded to include a variety of central nervous system actions (2). ANG II influences blood pressure control and fluid homeostasis via the brain and has been implicated in several forms of hypertension (57, 76). In the medulla, neurons in the dorsal medial nTS region are overlaid by dense concentrations of ANG II receptors and commingle with the afferent and efferent components of autonomic regulatory pathways (2, 35, 60, 79). A substantial portion of these ANG II receptors appear to be located on afferent
terminals presynaptic to intrinsic nTS neurons (2, 30, 68, 86). Although there is considerable evidence for generation of ANG II within the central nervous system by enzymatic pathways similar to those of the peripheral renin-angiotensin system, blood-borne ANG II also appears to modulate neuronal activity in specialized brain regions. Some neurons in the area postrema and subfornical organ, which have a minimal blood-brain barrier, are influenced by increased levels of circulating ANG II (36, 47, 74).

Femtomole doses of ANG II microinjected into the dorsal medial nTS region evoke depressor responses and bradycardia, whereas high picomole doses produce reflex increases in blood pressure (41, 71, 78). However, whether these divergent responses at higher concentrations arise from activation of additional neurons within the same region or indicate recruitment of more distant neurons with different properties is unknown. Some nTS neurons that respond to a rise in arterial pressure are activated or inhibited by peripheral administration of ANG II (51). Heterogeneity of actions mediated by AT1 receptors is also implied by their association with different second messengers in distinct classes of neurons and disparate responses (excitation, inhibition, or both responses in succession) in particular types of neurons. Most sensory neurons that send their central axons into the nTS via the TS are contained in the nodose ganglion. Furthermore, somatic calcium currents were inhibited by ANG II acting at AT1 receptors in a subset of acutely isolated nodose sensory neurons that innervate the nTS via the TS, whereas these currents were augmented by ANG II.

Fig. 8. Blockade of synaptic transmission with tetrodotoxin (TTX) failed to prevent the response to ANG II in group B medial nTS neurons. A 30-s record of the membrane potential of this neuron (sample rate 200 Hz–4 kHz) is shown for each condition. Arrows, application of ANG II. ANG II: before TTX application, 200 fmol of ANG II evoked high-frequency action potentials, brief depolarization (23 mV), and a decrease in input resistance (81 MΩ). Baseline membrane potential was –59 mV. TTX: after microdrop application of 10 μM TTX, brief depolarization (20 mV) and decreased input resistance (76 MΩ) evoked by ANG II persisted, accompanied by increased frequency and magnitude of EPSPs, suggesting the presence of postsynaptic ANG receptors on the recorded cell. Baseline membrane potential was –58 mV.

Non-NMDA receptor antagonists block TS-evoked EPSPs and the response to ANG II, whereas other postsynaptic Glu receptor types, indicated by Glu*, continue to mediate activation of the cell by Glu. Projections of this subset of medial nTS neurons could modulate heart rate or blood pressure by enhancing activity of cardiovascular neurons in the dorsal motor nucleus of the vagus or ventrolateral medulla (VLM). Group B neuron: medial nTS neurons excited by ANG II and SP via postsynaptic receptors on the recorded neuron, but 1 subset is unresponsive to Glu. Baroreceptor afferent fibers in the TS evoke short-latency EPSPs via non-NMDA receptors on the recorded cell, indicated by [Glu*], or directly, by activating SP receptors on a recorded cell that lacks Glu receptors. Non-NMDA receptor antagonists block or attenuate TS-evoked EPSPs in cells activated by ANG II, SP, and Glu but do not alter TS responses in neurons responsive only to ANG II and SP. Non-NMDA antagonists do not prevent responses to ANG II or SP in either type of group B neurons. Ap, Area postrema.
via other receptor mechanisms in different groups of nodose cells (9). It is intriguing to speculate that group A neurons in which ANG II enhanced TS-evoked EPSPs in the present study may be innervated by axons of nodose ganglion cells in which ANG II increased these calcium currents. Additional direct evaluation of variations in receptor transduction pathways is needed to clarify the different mechanisms utilized by the subsets of neurons we have identified in the dorsal medial nTS region.

Responses to TS Stimulation of Group A and Group B Neurons

Previous studies using extracellular recordings in horizontal slices documented that ANG II consistently increased firing rates in a substantial portion of neurons in the dorsal medial nTS region (12, 14, 16, 77). ANG II did not inhibit spontaneous firing in any of the nTS cells recorded in the present study. Using sharp (5, 22, 40) or whole cell (20, 44, 45) intracellular recordings, others reported that TS stimulation evoked EPSPs in almost all neurons in this nTS region. In the present experiments, we cut our slices in the horizontal plane so that they contained substantial lengths of the TS, separating the stimulating electrode from the soma of the recorded neuron by 2–3 mm to promote selective activation of sensory afferent inputs to the cell. The latency range of TS-evoked EPSPs in group A neurons (2.2–3.6 ms) is consistent with monosynaptic inputs from TS axons. The onset latencies of successive TS-evoked EPSPs in group A neurons were also very consistent, considered evidence for monosynaptic innervation by TS afferent axons (34, 70). Moreover, these onset latencies in group A neurons were also significantly shorter and less variable than those in group B cells. The longer onset latencies and higher variability of group B synaptic responses (Table 1) suggest that these neurons may receive polysynaptic inputs from TS afferent fibers. The different characteristics of the response to TS stimulation of group A and group B neurons in the present study resemble the findings of our previous extracellular recording studies (77). However, the neurons designated group A in the present study are clearly a subset of the second-order visceral neurons in the dorsal medial nTS region, since more than half of the cells with these short-latency synaptic responses (23 of 35, 65%) did not respond to ANG II.

Differential Effects of ANG II and SP on Group A and Group B Neurons

The most striking difference between the two groups of neurons we have identified is the substantial augmentation by ANG II of EPSPs in group A, but not in group B, neurons. Although the mechanism of the enhancement by ANG II of TS-evoked EPSPs in group A is unknown, blockade of this increase by DNQX argues for presynaptic modulation by ANG II of Glu release (5, 28, 41, 46). Furthermore, group A neurons displayed only minimal depolarization (5 mV) and a relatively modest fall in membrane resistance (~33 MΩ) in response to ANG II. In contrast, group B neurons exhibited substantial depolarization (22 mV) and a much larger decrease in membrane resistance (~51 MΩ) that lasted several minutes. Because blockade of synaptic transmission failed to prevent depolarization of group B neurons by ANG II or SP, receptors for both peptides must be located postsynaptically on the recorded group B cell.

ANG II consistently depolarized neurons in a variety of brain regions, but input resistance changes were variable: increased in cultured spinal cord neurons (56) but decreased in supraoptic neurons (87) and CA1 hippocampal neurons (48). These findings imply that diverse response mechanisms operate in different types of neurons. Subtype-specific ANG II receptor antagonists and blockade of synaptic transmission indicated that the depolarizing responses were mediated by postsynaptic AT1 receptors on paraventricular magnocellular neurons (59) and supraoptic neurons (87). However, in one subgroup of hippocampal CA1 neurons, ANG II increased the rate of EPSPs and action potentials without depolarizing the membrane, whereas other CA1 cells exhibited substantial depolarization and decreased input resistance after ANG II application (48). This dichotomy of responses resembles the contrasting observations in group A and group B neurons found in the present study.

Potential Interactions Between ANG II and SP in the nTS

Previous in vitro studies identified a subset of ANG-responsive neurons in the dorsal medial nTS region that were also excited by SP (12, 77) and reported that ANG II evoked release of SP from perfused coronal medulla slices (12). Because these slices included dorsal and ventral medulla for several millimeters rostral and caudal to the fourth ventricle, the release of SP induced by ANG II may have included cells in the dorsal motor nucleus of the vagus or ventrolateral medulla as well as the nTS. Others reported that the acute hypotension and bradycardia produced by microinjecting femtomole doses of ANG II into the dorsal medial nTS region were blocked by a non-subtype-selective neurokinin receptor antagonist in anesthetized Sprague-Dawley rats, whereas similar cardiovascular responses to Glu injections were unchanged (32). The biphasic changes in blood pressure evoked by microinjection of ANG II into the same nTS region of renin transgenic rats with increased medullary tissue levels of ANG II were also prevented by a neurokinin 1 (NK1) receptor antagonist (33). However, neither study evaluated the capacity of ANG II antagonists to alter these responses. The authors suggested that the mechanisms by which SP contributes to the acute cardiovascular effects of ANG II in the dorsal medial nTS region are unrelated to those responsible for attenuation by ANG II of the baroreflex, because SP has been shown to enhance this reflex (23). Although their findings are
consistent with participation of ANG II-induced release of SP in acute responses to the peptide, they commented that the antagonist may have acted at nTS interneurons or spread to adjacent vasomotor neurons. Thus our group B cells, activated by both ANG II and SP and with synaptic response characteristics consistent with interneurons, may contribute to the acute responses to microinjection of low-dose ANG II into the dorsal medial region of the nTS.

Differential responsiveness of nTS neurons to ANG II and SP is also supported by recent studies in a working heart-brain stem in vitro preparation. These investigators reported that ANG II microinjected into the nTS attenuated baroreflex-induced bradycardia but potentiated the bradycardic response to peripheral arterial chemoreceptor activation (75). Both effects were mediated by AT1 receptors. The chemoreflex enhancement was reversed by nTS injection of an AT1 receptor antagonist or an NK1 receptor antagonist that also blocked the vasodepressor response to SP (81). In contrast, NK1 receptor blockade had no effect on depression by ANG II of baroreflex-induced bradycardia. Thus SP appears to contribute selectively to chemoreflex-evoked bradycardia, but not to baroreceptor-mediated decreases in heart rate (75). Both identified chemoreceptor afferent terminals and axons of intrinsic neurons throughout the nTS contain SP and could provide the anatomic substrate for ANG II to enhance chemoreflex bradycardia by releasing SP (26, 42). However, whether these findings indicate a potential role in modulation of the chemoreflex for our group B neurons responsive to ANG II and SP via postsynaptic receptor mechanisms is unclear. The antagonist volumes injected into the nTS by these investigators have been reported previously to spread into adjacent cardiovascular structures, including the dorsal motor nucleus of the vagus (41), raising the possibility that inhibition of the chemoreflex bradycardia by ANG II or SP may have occurred at higher-order synapses outside the nTS, perhaps on cardiac vagal motor neurons. Moreover, the nTS pathways that mediate chemoreceptor responses are predominantly caudal to the recording sites in the present study, which targeted the dorsal medial nTS region, where aortic baroreceptor inputs are densely concentrated (25, 26, 43, 52, 85).

Related investigations in coronal medulla slices indicated that ANG II can augment excitatory synaptic transmission in spontaneously firing dorsal medial nTS region neurons with very short TS latencies (53), comparable to our group A cells. In contrast, these investigators reported that ANG II diminished TS-evoked EPSPs in “silent” neurons with much longer latencies and EPSP-IPSP complexes, whereas the IPSPs were enhanced. Because ANG II did not alter responses in these neurons to iontophoretic application of GABA, facilitation by ANG II of the polysynaptic GABAergic TS-evoked IPSPs appeared to arise from a site presynaptic to the recorded cells (53). Notably, this report differs from the present results, in that ANG II failed to modify the IPSPs in our two group B cells that responded to TS stimulation with an EPSP-IPSP complex. However, in the present study, all group B cells displayed spontaneous action potentials at their resting potential. Additionally, the more extensive rostral-caudal region from which nTS neurons were sampled in the prior report (53) may also contribute to differences between their findings and the present study, which targeted those areas of the dorsal medial nTS region that contain the majority of ANG II receptors associated with aortic and carotid sinus baroreceptor afferent fibers (25, 26, 43, 52, 85).

**Perspectives**

ANG II within the brain or derived from plasma tonically inhibits the cardiac baroreflex in animals and humans (10, 38). Attenuation of the baroreflex via nTS pathways appears to contribute to the development and/or maintenance of hypertension and could provide the mechanism through which ANG-converting enzyme inhibitors work so effectively to treat this disease (37). The site within the nTS and the cellular mechanisms that mediate this baroreflex attenuation have not been established. However, evidence also suggests that, at physiologically relevant concentrations, ANG II potentiates neurotransmission at baroreceptor synapses in the dorsal medial nTS region (7, 19). The functional implications of this enhancement of synaptic transmission are unclear but depend on the properties of the neurons and the pathways that they innervate. It seems plausible that ANG II might participate in the beat-to-beat regulation of cardiovascular function by potentiating activity in a subset of those nTS neurons with baroreceptor sensory inputs. Anatomic evidence indicates that neurons in the dorsal medial nTS region adjacent to the area postrema receive substantial arterial baroreceptor synaptic innervation (25, 26, 43, 52, 85).

We do not know whether or how group A nTS neurons differ from group B cells in functional significance. Because microinjection of ANG II into the dorsal medial nTS region modulates reflex control of heart rate and arterial pressure without altering respiratory function, the peptide appears to be associated selectively with cardiovascular regulation in this region (41). Thus we speculate that group B neurons may be inhibitory interneurons and could participate in the baroreflex depression reported after microinjection of higher doses of ANG II into this nTS region. In contrast, SP influences respiratory and gastric as well as cardiovascular function (10, 24, 66, 72). Furthermore, the localization of our group B neurons responsive to ANG II and SP in the dorsal medial nTS region (77) is congruent with the distribution of vagal afferent projections from the esophagus and stomach (62, 80). Clearly, additional investigations of the cellular mechanisms, anatomic pathways, and functions of these different neuronal groups in the dorsal medial region of the nTS are required to determine the significance of the presynaptic vs. postsynaptic locations of their ANG II receptors.
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