Functional Interactions Between Angiotensin II and Substance P in the Dorsal Medulla

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Low doses of either angiotensin (Ang) II or substance P (SP) microinjected into the medial nucleus tractus solitarii (NTS) produce hypotension and bradycardia, mimicking activation of the baroreceptor reflex. Anatomical evidence suggests that Ang II binding sites in the medial NTS are located presynaptically on vagal afferent fibers that may contain SP and are codistributed with SP binding sites located postsynaptically on intrinsic medial NTS neurons. To evaluate whether the similar cardiovascular effects of Ang II and SP in the medial NTS could involve Ang II-evoked release of SP, we compared the effects of these peptides on the spontaneous activity of medial NTS neurons recorded in vitro and determined whether Ang II evoked release of SP from rat medulla slices. Both Ang II and SP (1 μM in artificial cerebrospinal fluid) excited 11 of 40 medial NTS neurons. In these cells, the peak response latency was significantly longer to Ang II than to SP (59.5 ± 4.7 versus 26.5 ± 2.4 seconds, p < 0.0001). When rat medulla slices were perfused with Ang II (2 μM in Krebs' bicarbonate), release of SP immunoreactivity was increased by 400% over control perfusion with Krebs' solution alone (p < 0.05). We have provided the first evidence for an excitatory action of Ang II on neurons in the NTS of the rat and for excitation by both Ang II and SP of a subset of neurons in the medial NTS. Moreover, we have shown for the first time that Ang II can stimulate the release of SP immunoreactivity from the brain. The longer latency of the Ang II-induced neuronal excitation is consistent with an indirect action of this peptide, such as the release of SP from afferent fibers. These observations suggest that at least a portion of the cardiovascular effects elicited by NTS injections of Ang II may involve an interaction with SP, and they have revealed a functional interaction between Ang II and SP in the dorsal medulla. (Hypertension 1991;17:1121-1126)

The dorsal medulla is a primary site of the cardiovascular actions of angiotensin (Ang) II. Microinjections of femtomole doses of Ang II into the medial nucleus tractus solitarii (NTS) elicit hemodynamic effects that mimic activation of the baroreceptor reflex. Ang II also causes excitation of neurons in the medial NTS. Recently, we observed a striking coincidence between the distributions of both Ang II and substance P (SP) specific binding sites and immunoreactivity in the medial NTS. Furthermore, nodose ganglionectomy causes the loss of Ang II binding sites and reduces SP immunoreactivity in this structure. However, SP binding sites in the medial NTS are not affected by this procedure. Therefore, Ang II binding sites may be located presynaptically on SP-containing vagal afferent fibers, whereas SP binding sites are likely to be associated with intrinsic medial NTS cells. Moreover, recent studies reveal that SP has cardiovascular actions within the NTS, because femtomole injections of SP into this brain region produce hypotension and bradycardia similar to that elicited by Ang II. The mechanisms of neurotransmission and receptor interactions accounting for the similar actions of Ang II and SP in the NTS are not understood. However, the findings described above suggest that Ang II might cause the release of SP from vagal afferent fibers. We have used two approaches to test this possibility. First, we compared the effects of Ang II and SP on the spontaneous activity of medial NTS neurons recorded in vitro from slices of the rat dorsal medulla. Second, we determined whether Ang II stimulated the release of SP from rat medulla slices.

Methods

In Vitro Recording Studies

Sprague-Dawley rats (250–275 g; Harlan Sprague Dawley, Inc., Indianapolis, Ind.) were anesthetized with halothane and decapitated. The dorsal medulla...
was removed and prepared as horizontal slices for in vitro recording. A block extending from approximately 3 mm caudal to 6 mm rostral to the calamus scriptorius and containing the nucleus gracilis, area postrema, NTS, solitary tract, dorsal motor nucleus of the vagus (dMNX), and the hypoglossal nucleus was cut into 400-μm slices with a vibratome (OTS 3000, Frederick Haer, Brunswick, Me.) under oxygenated artificial cerebrospinal fluid (aCSF, 2°C). The slices were oriented in the horizontal plane parallel to the solitary tract, which provides the major afferent innervation of the dorsal medulla. The slices were perfused with aCSF at 35°C in a custom-made interface-type slice chamber for 1 hour before recordings were begun. The aCSF solution contained (mM) NaCl 124, KCl 5, Na₂HPO₄ 1.25, MgSO₄ 2, CaCl₂ 2, NaHCO₃ 26, and D-glucose 10 and was bubbled with 95% O₂–5% CO₂ and warmed to 35°C before use.

Ang II or SP (both 1 μM in aCSF, pH 7.4), L-glutamate (0.5 mM in aCSF), or aCSF were applied to the surface of the slice as microdrops (200–500 nl) ejected from pipettes with air pressure, as described previously. To compensate for occasional linear trends in baseline firing rate, the order of Ang II and SP application was reversed for successive neurons recorded during an experiment. Whenever possible, each peptide was given twice to each cell in either counterbalanced or alternating order. In addition, aCSF was given before and after the delivery of each peptide. Application of L-glutamate was used to verify the responsiveness to stimulation of neurons that did not respond to either Ang II or SP.

Extracellular recordings were made with micropipettes filled with 0.9% NaCl saturated with Fast Green dye (resistance, 5–15 MΩ). The signal was amplified ×100, filtered (1 Hz–1 KHz), and passed through a window discriminator (DIS-1, BAK Electronics, Germantown, Md.). The multiplexed neuronal signal and window outputs were displayed on an oscilloscope (5111A, Tektronix, Quevail, Ohio). The window acceptance pulse was sent to a computer (386/20, Everex, Fremont, Calif.) so that successive interspike intervals could be computed and displayed as instantaneous frequencies during recording. Neuronal action potentials also were digitized (VR-100-8, Instrutek Corp., Mineola, N.Y.) and stored on videotape for further analysis. Plots of the neuronal discharges as a function of time were generated for each cell (Figure 1), with times of application of peptides or aCSF indicated on the plot. After recording from a cell, the location of the electrode was marked by iontophoresing Fast Green from the electrode tip (–25 μA for 2–5 minutes). At the end of an experiment, the slices were immersion-fixed (20% formalin), frozen-sectioned at 50 μm, and counterstained with Neutral Red. The anatomical localization of recorded neurons in all experiments was identified by microscopic visualization of Fast Green dye marks.

To determine whether a neuron was responsive to a peptide, a 95% confidence interval was generated from the cell’s instantaneous firing rates during a 2-minute baseline period immediately before application of the peptide, using the binomial probability distribution. If the instantaneous firing frequency of the neuron fell outside the confidence interval for at least 10 seconds after peptide application, the cell was classified as responsive to the peptide. Differences in the proportion of neurons excited by Ang II as compared with SP were evaluated with Fisher’s exact test. Peak response latencies are expressed as mean±SEM. Differences between response latencies to Ang II and SP were assessed with paired or unpaired Student’s t tests. A value of p=0.05 was required for significance.

In Vitro Release Studies

The medulla of a separate group of 24 male Sprague-Dawley rats (250±5 g; Harlan Sprague Dawley) was removed after decapitation and placed in ice-cold Krebs’ bicarbonate buffer. The portion of the medulla extending from 1 mm caudal to the calamus scriptorius to 2 mm rostral was chopped into 0.5×0.5-mm pieces using a tissue chopper (Brinkmann Instruments, Inc., Westbury, N.Y.) and placed in a chamber with an internal volume of 1 ml. Each chamber contained the tissue from one animal and was perfused at a flow rate of 0.42 ml/min with 37°C oxygenated Krebs’ bicarbonate solution containing (mM) NaCl 135, KCl 3.5, MgSO₄ 1, NaHCO₃ 20, and CaCl₂ 2.5, as well as bovine serum albumin 0.5%, 20 μM bacitracin, 3.3 mM dextrose, and 6 μM dithiothreitol according to the method of Pang and Vasko. The solution was bubbled continuously with 95% O₂–5% CO₂ to achieve a pH of 7.4. After a 16-minute equilibration period, samples were collected every 6 minutes for the next 24 minutes. The first sample was used for determination of basal release of immunoreactive SP (irSP). The second 6-minute sample (designated experimental) was collected during continued perfusion with the Krebs’ solution (time control; n=10) or Krebs’ containing 0.2 μM (n=6) or 2 μM (n=8) Ang II. The next two 6-minute samples were collected during perfusion with the Krebs’ solution alone (recovery 1 and 2). Samples were collected on ice and acidified with 0.14 ml glacial acetic acid, with 0.1 ml 10% L-cysteine solution added before storage at –80°C.

Samples were prepared for radioimmunoassay by lyophilization and reconstitution in the radioimmunoassay buffer. After sonication, the samples were centrifuged and the supernatant was decanted and adjusted to pH 7.0 with 1N NaOH. A set of standards was lyophilized and treated identically to the samples. Samples and standards were assayed for SP according to the method of McGregor and Bloom using antisera no. 4892 kindly provided by Dr. Vay L.W. Go and iodine-125–labeled SP tracer obtained from Amersham, Arlington Heights, Ill. Cross-reactivity of the antibody was 100% with SP.
amide, SP-(2–11), and SP-(4–11). There was less than 0.1% cross-reactivity with SP-free acid, SP-(1–9), eledoisin, substance K, and kassinin, and 1.1% cross-reactivity with physalaemin. Ang II showed less than 0.002% cross-reactivity with the SP antisera. The intra-assay variability was approximately 9%, and the interassay variability was 10%.

Data are expressed as percent of basal release, where basal release was defined as the amount of SP immunoreactivity present in the first 6-minute sample, collected immediately before the experimental period. Values are presented as mean±SEM. Differences among groups (time control, 0.2 μM Ang II, and 2 μM Ang II) were assessed using analyses of variance followed by the least significant difference test for contrasts between two means. A value of p<0.05 was considered significant.

Results

Neuronal Actions of Angiotensin II and Substance P

Histological localization of Fast Green dye marks documented that 55 neurons from which stable extracellular recordings were obtained were located in the medial NTS. All of these cells displayed spontaneous spike activity and were unresponsive to aCSF.

The effects of both Ang II and SP (1 μM) were evaluated in 40 of the 55 medial NTS neurons. No inhibitory effects were seen with either peptide. In this group of 40 medial NTS neurons, Ang II excited 18 cells (45%), whereas SP activated 26 cells (65%). Although there was a tendency for more neurons to be responsive to SP than to Ang II, the difference was not significant (Fisher’s exact test, p=0.115). Four patterns of response to the two peptides were observed (Table 1). Eleven neurons were excited by both Ang II and SP, seven cells were excited by Ang II but not by SP, and 15 cells were activated by SP but not by Ang II. The other seven medial NTS neurons did not respond to either peptide but were activated by L-glutamate. Figure 1A shows the time course of the firing rate plotted as spikes per 30 seconds for a medial NTS neuron excited by both Ang II and SP. Application of Ang II increased the firing rate of this cell from a baseline of approximately 15 spikes/30 sec to a peak of 135 spikes/30 sec with a latency of 60 seconds. Next, two successive applications of SP evoked peak firing rates of more than 200 spikes/30 sec, with latencies of approximately 20 seconds. A second application of Ang II produced excitation.

Table 1. Response Patterns of 40 Medial Nucleus Tractus Solitarii Neurons Given Both Angiotensin II and Substance P

<table>
<thead>
<tr>
<th>Response pattern</th>
<th>No (%) of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both excitatory</td>
<td>11 (28)</td>
</tr>
<tr>
<td>Ang II excitatory, SP unresponsive</td>
<td>7 (17)</td>
</tr>
<tr>
<td>Ang II unresponsive, SP excitatory</td>
<td>15 (38)</td>
</tr>
<tr>
<td>Both unresponsive</td>
<td>7 (17)</td>
</tr>
<tr>
<td>Total</td>
<td>40 (100)</td>
</tr>
</tbody>
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Ang II, angiotensin II; SP, substance P.
similar to the first dose. Figure 1B plots the activity of a medial NTS neuron from the same experiment as Figure 1A. This cell was activated repeatedly by Ang II but was unresponsive to SP. Because the SP solution excited the cell shown in Figure 1A, it was documented to be active. Figure 1C illustrates the firing pattern of a medial NTS neuron that was excited twice by SP but was not responsive to Ang II.

The time course of the responses to Ang II and SP in the 11 neurons that were excited by both peptides suggested that the latency to the peak response evoked by SP was shorter than the latency of the peak excitation generated by Ang II. Thus, the latency to the peak firing rate (computed over 10-second intervals) was determined for all 33 neurons that responded to at least one of the peptides. For the 11 neurons excited by both Ang II and SP, the average peak response latency for SP was 26.5±2.4 seconds, compared with 59.5±4.7 seconds for Ang II (p<0.0001). Response latencies to Ang II and SP also were compared for the 22 neurons activated by only one peptide. A shorter latency was found for SP compared with Ang II, but this did not reach statistical significance (37.7±6.2 seconds versus 62.9±14.5 seconds, p=0.0778).

**Angiotensin II-Evoked Release of Substance P**

Basal release of irSP averaged 16.8±4.4 pg/ml in the first 6-minute sample collected from 0.11±0.004 g medullary tissue blocks (n=24). Continued perfusion with the Krebs' buffer during the subsequent experimental and recovery periods was associated with a tendency for a decline in irSP levels to approximately 50% of basal in the time control studies (Figure 2). In contrast, Ang II (2 μM) added during the experimental period significantly increased irSP in the perfusate to a level approximately 400% above that observed during the same period in the time control experiments (p<0.05). The irSP values tended to remain elevated during the first recovery period with the high dose of Ang II but returned toward those obtained in the time control studies during the second recovery period. There was a tendency for the lower dose of Ang II (0.2 μM) to maintain irSP release at basal levels for the duration of the study. However, the values were not significantly different from those in the time control group during any time period.

**Discussion**

The present study provides the first evidence for an excitatory action of Ang II on neurons in the NTS of the rat and for excitation by both Ang II and SP of a subset of neurons in the medial NTS. Thus, we have uncovered a common neuronal substrate for the similar cardiovascular effects of Ang II and SP given into the medial NTS. Moreover, these experiments have produced the first demonstration that Ang II can stimulate the release of irSP from the rat medulla oblongata, thus revealing a functional interaction between Ang II and SP. These findings provide new significance for the anatomical congruence of the patterns of Ang II and SP binding sites and immunoreactivity in the NTS.

The present observations that Ang II activated 45% of the neurons recorded in the medial NTS of the rat are in agreement with our previous reports that Ang II excites half of the neurons recorded in the canine medial NTS. Although other investigators have reported neuronal effects of Ang II both in vivo and in vitro in rats and cats, only our previous in vitro experiments in the dog and two in vivo studies in dog and cat have investigated dorsal medullary neurons. Carpenter et al reported a long-latency, prolonged activation of 31 of 68 neurons in the canine area postrema by Ang II. Sessle and Henry iontophoresed Ang II onto "respiratory neurons" or "reflex interneurons" in the feline ventrolateral NTS. Eight of 27 inspiratory respiratory neurons and five of eight interneurons responded repeatedly to Ang II with a current-dependent, slow, prolonged excitation. This response pattern is similar to the excitation of rat medial NTS neurons produced by Ang II in the present experiments.

SP excites neurons recorded from in vitro slices or dissociated cell cultures from rats. Cheeseman et al reported dose-dependent activation of locus coeruleus neurons in pontine slices perfused with SP (10 nM to 1 μM), with excitation that peaked approximately 1 minute after response onset and returned to baseline over 5–10 minutes. This response pattern is similar to the time course of the activation of medial NTS neurons by SP seen in the present study. Nakajima and colleagues found SP excitation of cultured locus coeruleus, basal forebrain, or globus pallidus neurons. Ogata and Abe also reported...
excitation by SP of guinea pig hypothalamic neurons. Recently, Plata-Salaman et al.\textsuperscript{22} superfused rat coronal medulla slices with SP (1 μM). Although they did not record from NTS neurons, intracellular recordings from the dmN showed depolarization with increased input resistance in 18 of 43 neurons; five dmN cells exhibited hyperpolarization with decreased input resistance. Both actions of SP persisted after blockade of synaptic transmission, supporting postsynaptic location of SP receptors in the dmN. Two in vivo studies in the cat have examined the neuronal effects of SP in the NTS. Morin-Surun et al.\textsuperscript{23} reported excitation by SP in 66 of 86 NTS cells. Henry and Sessle\textsuperscript{24} tested the effects of SP on respiratory neurons or reflex interneurons in the ventrolateral NTS. Seventeen of 27 inspiratory respiratory cells and eight of nine interneurons were excited by SP with a current-dependent, prolonged excitation similar to the effects of Ang II on NTS neurons that they reported in a later study.\textsuperscript{17} This response pattern closely resembles the effects of SP on medial NTS neurons of the rat seen in the present study.

In medial NTS neurons that were excited by both Ang II and SP in the present study, the latency to the peak excitation produced by SP was significantly shorter than the peak latency for Ang II. Furthermore, in parallel studies we found that perfusion with Ang II also significantly increased the release of SP from medulla slices. Both of these observations are consistent with our model for a subset of intrinsic NTS neurons that are excited by both Ang II and SP. Ang II may activate these NTS neurons indirectly, by a presynaptic action of Ang II on SP-containing vagal afferent fibers. A critical test of the model involves perfusion of the slices with reduced concentrations of Ca\textsuperscript{2+} to block synaptic transmission from the vagal afferents to the intrinsic NTS cells. The model predicts that this treatment should block the excitation produced by Ang II but should not prevent excitation by SP. These future studies will provide more definitive evidence for a functional interaction between Ang II and SP in the medial NTS.

Previous studies demonstrated that potassium depolarization increases the efflux of irSP from the dorsal medulla in a calcium-dependent manner.\textsuperscript{25} Capsaicin also evoked an increase in release of irSP, providing evidence that at least part of the released SP is from primary sensory afferent fibers within this brain region.\textsuperscript{25} Although the magnitude of the increase in irSP evoked by Ang II in the present study was similar to that seen with capsaicin (i.e., 221% above basal for Ang II versus 224% above basal for capsaicin), the source of the irSP released in response to Ang II in the present experiments is not known. Our previous anatomical evidence\textsuperscript{4} supports the localization of Ang II receptors on vagal afferent fibers in the NTS and on vagal motor neurons within the dmN. Thus, it is likely that Ang II evokes the release of irSP from both sensory afferent fibers and intrinsic central nervous system neurons within the dorsal medulla. Future studies will be necessary to distinguish between these possibilities.

An additional consideration is whether the production of either neuronal excitation or release of SP by Ang II might be due to its primary metabolic product, Ang-(1–7).\textsuperscript{26} Although the present experiments did not evaluate whether local conversion of Ang II to Ang-(1–7) contributed to these responses, several observations suggest that it is unlikely. First, we have shown that in medial NTS neurons that were excited by both Ang II and Ang-(1–7), the latency to peak excitation was similar for both Ang peptides.\textsuperscript{3} Thus, it is unlikely that the longer latency to peak excitation for Ang II than for SP represented conversion of Ang II to Ang-(1–7). Second, in studies just completed, Diz and Pirro\textsuperscript{27} found that although both Ang II and Ang-(1–7) released SP from hypothalamic slices, Ang-(1–7) was not effective in stimulating release of SP from medulla slices. Therefore, it is unlikely that local conversion of Ang II to Ang-(1–7) is responsible for the stimulatory actions of Ang II in the NTS.

In summary, we have identified a subset of the neurons in the rat medial NTS that are excited by both Ang II and SP and exhibit a significantly longer response latency to Ang II than to SP. Ang II also increased the release of irSP from slices of the dorsal medulla. The longer latency of the Ang II–induced neuronal excitation in neurons responding to both peptides is consistent with an indirect action of this peptide on the cells, such as the release of irSP from afferent fibers. We and others have found that injections of Ang II into the medial NTS evoke dose-dependent hemodynamic effects that are indistinguishable from those produced by SP.\textsuperscript{1,9–11,28} Coupled with the anatomical coincidence of Ang II receptors and SP immunoreactivity in vagal sensory afferent fibers,\textsuperscript{4,25} the present observations suggest that at least a portion of the acute cardiovascular effects elicited by NTS injections of Ang II may involve an interaction with SP. Furthermore, because stimulation of the aortic depressor nerve has been documented to evoke release of SP in the NTS,\textsuperscript{29} an interaction between Ang II and SP also may play a role in the effects of endogenous Ang II in the NTS on baroreceptor reflex regulation of blood pressure, as we demonstrated previously.\textsuperscript{30}

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References


KEY WORDS: angiotensin II; substance P; brain; nucleus tractus solitarni; hormones; peptides.
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