Neuronal Responses to Angiotensin II in the In Vitro Slice from the Canine Medulla

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SUMMARY The present studies utilized the in vitro slice preparation of the canine dorsomedial medulla, which we have recently developed, to obtain direct evidence for the effects of angiotensin II (Ang II) on the activity of single neurons in this region. Horizontally oriented slices (300 μm) containing the area postrema, nucleus tractus solitarii (NTS), and dorsal motor nucleus of the vagus were perfused with oxygenated artificial cerebrospinal fluid. The effects of microdrop application of Ang II and its antagonist [Sar1,Thr8]Ang II on spontaneous firing rate were determined in 27 extracellularly recorded neurons. Ang II substantially increased the firing rate of 13 neurons located in the medial NTS, but it did not alter the spontaneous activity of the remaining 14 neurons. In most cases Ang II elicited a slowly developing, prolonged excitatory response. The effects of both Ang II and [Sar1,Thr8]Ang II were tested in 13 neurons. [Sar1,Thr8]Ang II produced a short latency, brief excitation in three neurons, marked inhibition of spontaneous firing in two cells, and no effect on the other eight neurons. Administration of [Sar1,Thr8]Ang II blocked the excitatory effects of subsequent administration of Ang II in three neurons. To our knowledge, these observations provide the first evidence for direct actions of both Ang II and [Sar1,Thr8]Ang II on neurons in the canine NTS and for the specificity of the neuronal effects of Ang II as documented by blockade of the excitatory response to Ang II by [Sar1,Thr8]Ang II. (Hypertension 11: 680–684, 1988)

KEY WORDS • angiotensin II • in vitro brain slice • dorsomedial medulla • neural recording

Our previous investigations of the canine dorsomedial medulla (DMM) have provided substantial evidence that the area postrema (AP) and nucleus tractus solitarii (NTS) exert reciprocal influences on both tonic and reflex control of blood pressure. Initial studies revealed that small amounts of angiotensin II (Ang II) given into the vertebral arteries produce a pressor response mediated by the AP.1 Subsequent investigations documented that the AP contains a neural pressor pathway that counterbalances the vasodepressor actions of the baroreceptor relay in the adjacent NTS.2–4 A role for these structures in the tonic regulation of the cardiovascular system has been underscored by the observations that lesions of either the AP or the NTS result in lasting alterations of cardiovascular function.5–7 Recently, we have shown that microinjection of Ang II into the AP or medial NTS of the dog elicits increases in blood pressure and heart rate.8 In addition, the discovery of a high density of Ang II binding sites in the canine DMM that depend on the vagal innervation of these structures reinforces the cardiovascular importance of Ang II in this region.9

The present experiments have begun to address the question of whether angiotensin peptides regulate transmission in neuronal pathways related to cardiovascular function by determining the capacity of Ang II to alter neuronal activity in the canine DMM. The in vitro slice preparation of the canine medulla that we have recently developed has provided direct evidence for the neuronal effects of angiotensin peptides in this region.

Materials and Methods

In Vitro Slice Preparation
In dogs (8- to 12-kg mongrels) anesthetized with halothane, loose ligatures were placed around the vertebral and carotid arteries and the dorsal medulla was exposed as described previously.2 While the medulla was cooled with ice-cold oxygenated artificial cerebrospinal fluid (aCSF), the carotid and vertebral arteries were ligated. The brainstem was transected at the pon-
tomedullary and spinomedullary junctions, removed, and immersed in ice-cold oxygenated aCSF for approximately 1 minute. The brainstem was placed on a cold dissection stage, and the pia and arachnoid were carefully stripped from the dorsal surface of the medulla. The medulla was cut into a horizontal block about 6 mm square and 4 mm thick, centered on the midline and containing the DMM from approximately 1 mm caudal to 5 mm rostral to obex. The tissue block contained the nucleus gracilis, the AP, the NTS, the caudal portion of the solitary tract (TS), the dorsal motor nucleus of the vagus (DMNX), and the hypoglossal nucleus. The blocked DMM was cut, while submerged in ice-cold aCSF, with a vibrating tissue slicer (Frederick Haer OTS 3000, Brunswick, ME, USA) into 300-μm-thick slices oriented in the horizontal plane to enable inclusion of the TS, which provides the major afferent innervation of the DMM. Slices containing the visually identifiable TS were placed in the recording chamber of an interface type slice chamber and perfused with aCSF. The elapsed time from ligation of the arteries to placing the slices in the chamber was 7 to 8 minutes. The slices were left undisturbed for at least 1 hour before electrophysiological procedures were begun.

The slice chamber (Oslo interface chamber, custom-made at IBM Watson Research Center, Yorktown Heights, NY, USA) had an outer water jacket that was maintained at 36°C. A mixture of 95% O₂, 5% CO₂ was bubbled through the water bath and into the inner recording chamber, over the surface of the brain slices. The aCSF perfusate passed through a tube in the outer water bath and was warmed before it entered the recording chamber at a flow rate of 0.3 ml/min. The slices rested on a tightly stretched nylon mesh at the interface of the aCSF and the warm, humid oxygenated atmosphere in the recording chamber and were transilluminated from below. Electrodes and drug pipettes were positioned under stereomicroscopic visual guidance using precision micromanipulators.

**Application of Peptides**

Angiotensin peptides and aCSF were applied to the surface of the slice by microdrops ejected from pipettes (tip diameters, 40-60 μm). A triple micromanipulator (Model HMT-3, Narishige, Tokyo, Japan) held three independently movable pipettes just above the slice near the recording electrode. Solutions were ejected from the pipettes by gentle pressure, while the microdrop formed on the pipette tip was visually observed. The tip was gently lowered onto the surface of the slice within 100 μm of the recording electrode to apply the drop, then lifted off the slice surface. We have shown with calibrated pipettes that 0.1 to 0.5 μl of solution is ejected by this technique. The three pipettes contained Ang II (8 pmol/μl in aCSF, pH 7.4), [Sar², Thr⁴]Ang II (1 nmol/μl in aCSF, pH 7.4), and aCSF for control ejections. The aCSF contained 124 mM NaCl, 5 mM KCl, 1.25 mM Na₂HPO₄, 2 mM MgSO₄, 2 mM CaCl₂, 26 mM NaHCO₃, and 10 mM D-glucose and was bubbled with 95% O₂, 5% CO₂ before use.

**Electrophysiological Recordings**

Extracellular recordings were made with pipettes containing 0.9% NaCl saturated with fast green dye (resistance, 5-15 MΩ; tip diameters < 10 μm). The signals were amplified 100 times, filtered (1-300 Hz), and displayed on a digital oscilloscope. An IBM PC with 16-channel data acquisition and analysis system (Armonk, NY, USA) was used to count action potential frequency during 30-second periods, with successive counts obtained every 40 seconds. Drug applications were made during the 10-second time-out periods to avoid introducing artifactual counts. Plots of the action potential frequency as a function of time were displayed on the computer monitor. The time of application of aCSF or peptides was indicated on the plot. The original amplified analog signal was stored on FM magnetic tape for further analysis. Constant current electrical stimuli (1-5 mA; 0.1 msec pulses) were delivered to the TS through a bipolar concentric stainless steel electrode (Model MCE-100, Rhodes, Woodland Hills, CA, USA) positioned visually on the rostral TS. The location of the recording electrode was marked by iontophoresing fast green dye from the electrode tip (−50 μA for 2-5 minutes). Intracellular recordings were made with 50- to 150-MΩ pipettes filled with 3 M potassium acetate, using an active bridge balance current clamp amplifier (Model 8700, Dagan, Minneapolis, MN, USA). At the end of an experiment, the slices were immersion-fixed with 30% sucrose in 10% perfused with 0.9% NaCl saturated with fast green dye and was bubbled through a tube in the outer water bath and was warmed before it entered the recording chamber at a flow rate of 0.3 ml/min. The slices rested on a tightly stretched nylon mesh at the interface of the aCSF and the warm, humid oxygenated atmosphere in the recording chamber and were transilluminated from below. Electrodes and drug pipettes were positioned under stereomicroscopic visual guidance using precision micromanipulators.

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**Results**

Extracellular recordings with an excellent signal to noise ratio (>10:1) and long duration (45-120 minutes) were routinely obtained from the relatively small neurons of the medial NTS and surrounding regions. The physical stability of the preparation also facilitated intracellular penetrations of good quality and long duration (at least 30 minutes) and application of peptide solutions without disrupting recordings.

Stable extracellular recordings were obtained from 27 neurons in horizontally oriented slices of the DMM. Histological localization of fast green dye marks confirmed that 24 neurons were located in the medial NTS, two cells were in the lateral NTS, and one neuron (recorded intracellularly) was in the DMNX. The effects of microdrop application of both Ang II and aCSF on spontaneous firing rate were determined in all 27 neurons. The neurons were generally unresponsive to aCSF applied to the brain slice, as shown in Figure 1A. Application of Ang II (0.2 μl, 8 pmol/μl) substantially increased (to at least 1.5 times baseline) the firing rate of 13 of the 24 neurons located in the medial NTS, but it did not alter the spontaneous firing of the remaining 11 medial NTS cells. Ang II had no effect on the activity of the two lateral NTS neurons or the DMNX cell studied. The lack of responsiveness to Ang II of these cells could not be attributed to differ-
ences in spontaneous firing rates. No cells were inhibited by Ang II. Figure 1A displays the effects of two successive applications of Ang II on the firing rate of a neuron in the medial NTS. In most cases, the neuronal firing rate increased within 15 seconds after applying the Ang II microdrop to the slice, peaked 1 to 2 minutes after peptide application, then returned slowly to baseline over the next 5 to 10 minutes. The Ang II-induced excitation usually could be repeated without apparent tachyphylaxis.

The effects of both Ang II and its antagonist analogue [Sar1, Thr8]Ang II (1 nmol/μl) were tested in 13 of the 27 neurons. [Sar1, Thr8]Ang II produced a short latency, brief excitation in three neurons. In contrast, marked inhibition of spontaneous activity by [Sar1, Thr8]Ang II was observed in two other cells. The Ang II antagonist did not alter the spontaneous firing of the other eight neurons tested. Administration of [Sar1, Thr8]Ang II blocked the excitatory effects of subsequent administration of Ang II in three of five cells tested. Figure 1B shows that [Sar1, Thr8]Ang II given alone increased the firing rate of this neuron during two successive applications. Administration of Ang II 80 seconds after the second dose of the antagonist did not produce any further excitation of the cell. However, repeated application of Ang II 6 minutes later produced a small excitatory effect, suggesting that the neuron had partially recovered from the antagonist blockade. In Figure 1C, [Sar1, Thr8]Ang II repeatedly inhibited the spontaneous activity of this neuron. However, administration of Ang II 40 seconds after the antagonist (recording time, 45 minutes) produced nearly the same increase in firing rate as was previously evoked by Ang II alone (recording time, 35 minutes). This neuron also demonstrated the apparent rise in baseline firing rate seen in some cells following several doses of Ang II.

The effects of TS stimulation were tested in 10 of the 27 DMM neurons recorded. Currents above 1 mA were used to ensure stimulation of the smallest TS axons. The brief pulse duration (0.1 msec) favored stimulation of TS axons rather than neuronal cell bodies. TS stimulation evoked action potentials in eight of the 10 cells. However, several of the TS-responsive neurons in both the medial and lateral NTS did not increase their spontaneous firing rate following application of Ang II.

Discussion

To our knowledge, the present study has provided the first evidence for the direct actions of both Ang II and its antagonist [Sar1, Thr8]Ang II on neurons in the NTS of the dog. These experiments also documented the appropriateness of the in vitro slice preparation of the canine DMM that we have developed for electrophysiological studies. Our findings suggest that about half of the spontaneously firing neurons in the medial NTS are excited by Ang II. No inhibitory effects of Ang II on the spontaneous activity of any DMM cells were seen. In contrast, [Sar1, Thr8]Ang II given alone produced both excitatory and inhibitory effects on neurons in the medial NTS; these opposite effects of the Ang II antagonist could not be attributed to either the location of the neurons within the NTS or the baseline firing rates of the cells. These studies also provide new evidence for specific excitation of neurons in the canine DMM by Ang II, as documented by the blockade of the excitatory effects of Ang II with an Ang II antagonist in three neurons. It seems likely that Ang II acted directly on either the recorded neuron or a closely adjacent cell, rather than on a remote neuron providing synaptic input to the recorded cell. If a microdrop of Ang II failed to contact the recording electrode as it penetrated the slice, no response was elicited from a neuron previously demonstrated to be Ang II-responsive.
Although previous studies have reported the effects of Ang II on neurons recorded both in vivo and in vitro, none of the in vitro experiments and only two in vivo studies have investigated neurons in the dorsomedial medulla. Most in vivo reports demonstrated neuronal excitation in diencephalic structures following iontophoresis of Ang II. In the rat, Ang II excited 35% of cells in the lateral septum but inhibited the firing of 15% of neurons scattered throughout the septum. Thornton et al. reported that, of 36 hypothalamic neurons responsive to a fall in blood pressure, 10 were excited by iontophoresed Ang II and 19 were activated by the peptide given into the cerebrospinal fluid. Takak et al. showed that most lateral hypothalamic neurons projecting to the subfornical organ were excited by Ang II, but that cells lacking subfornical organ targets were unresponsive to the peptide. Excitation of subfornical organ neurons by iontophoresed Ang II has been seen in rat and cat. When saralasin or [Sar^1, Ile^8]Ang II was used, the antagonist blocked most excitatory responses to Ang II.

In the medulla, Carpenter et al. reported a long-latency, prolonged activation of 31 of 68 neurons in the canine AP by iontophoresed Ang II. Sessle and Henry have iontophoresed Ang II onto "respiratory neurons" or "reflex interneurons" in the ventral or ventrolateral NTS of the cat. Eight of 27 respiratory neurons and five of eight interneurons responded repeatedly to Ang II with a current-dependent, slow, prolonged excitation. Neither study evaluated blockade of these responses with an Ang II antagonist. The pattern of Ang II–induced excitation reported by Sessle and Henry is similar to the present observations in the canine DMM in vitro slice preparation. Possible explanations for the relatively slow onset of neuronal excitation by Ang II include cleavage of the octapeptide to a smaller active peptide fragment, as suggested by Harding and Felix, or neuromodulatory actions such as disinhibition or facilitation of synaptic inputs.

Several investigators have tested the responsiveness to Ang II of neurons recorded in vitro from rat forebrain slices. An early study, performed by one of us on cells of the organum vasculosum of the lamina terminalis, reported dose-dependent excitation by iontophoresed Ang II in 14 of 22 neurons. The Ang II antagonist saralasin given alone excited nine of 15 cells and inhibited spontaneous firing in four neurons. Blockade of Ang II–induced excitation by saralasin was not tested. The findings by Knowles and Phillips of neuronal excitation by Ang II and both excitatory and inhibitory direct actions of the Ang II antagonist in rat forebrain are similar to the results of the present study in canine medulla slices. In addition, because Knowles and Phillips iontophoresed the peptides, while the present investigation applied the peptides by microdrops on the surface of the slice, the similar findings in both studies suggest that neither application technique produces nonspecific effects on neuronal activity.

Two studies have assessed the neuronal mechanisms involved in the actions of Ang II on CA1 pyramidal neurons in rat hippocampal slices. Haas et al. reported that microdrop application of Ang II depolarized the neuron and increased membrane conductance; these effects were not altered by synaptic blockade with a reduced calcium-high magnesium perfusate. Palovcik and Phillips also reported Ang II–induced excitation of CA1 neurons that was not changed by synaptic blockade. Future studies will determine whether the excitatory effects of Ang II on neurons in the canine DMM are mediated by receptors on the recorded neurons or whether actions of the peptide on presynaptic terminals are involved.

The obvious drawback of the in vitro slice preparation is the absence of many of the afferent and efferent connections of the neurons with other central nervous system structures and the periphery. However, the vertically oriented slice that we have developed includes the major portion of the TS, which contains the central processes of the afferent neurons from the carotid sinus nerve and the cervical vagus, including the aortic depressor nerve. These projections provide the major peripheral innervation of neurons in the NTS. Our previous neuroanatomical studies of the afferent projections of the cervical vagus and the carotid sinus nerve have demonstrated that in the dog these nerves provide substantial inputs to the AP as well as the NTS. Hence, the horizontal slice preparation retains substantial afferent input to DMM neurons. Electrical stimulation of the rostral TS suggested the presence of synaptic input from axons in the TS to eight of 10 DMM neurons tested in the slice.

In summary, these experiments have provided new information about the direct neuronal actions of angiotensin peptides in an in vitro slice preparation of the canine medulla. This study has taken a novel approach to begin to address the question of whether angiotensin peptides influence neuronal transmission in pathways of the DMM thought to relay cardiovascular information.

References

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