Adrenomedullin in the rostral ventrolateral medulla increases arterial pressure and heart rate: roles of glutamate and nitric oxide

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Submitted 19 March 2004; accepted in final form 31 May 2004

Xu, Yong, and Teresa L. Krukoff. Adrenomedullin in the rostral ventrolateral medulla increases arterial pressure and heart rate: roles of glutamate and nitric oxide. Am J Physiol Regul Integr Comp Physiol 287: R729–R734, 2004. First published June 3, 2004; 10.1152/ajpregu.00188.2004.—This study was done to investigate the effects of microinjections of adrenomedullin (ADM), a vasoactive neuropeptide, in the rostral ventrolateral medulla (RVLM) on mean arterial pressure (MAP) and heart rate (HR) in urethane-anesthetized rats, and to assess the potential roles of glutamate and nitric oxide (NO) in these effects. Unilateral injections of ADM (0.01 or 0.1 pmol) into the RVLM significantly increased MAP and HR in a dose-dependent manner, whereas ADM at 0.001 pmol was ineffective. Microinjections of ADM (0.01 pmol) outside the RVLM had no effects on MAP or HR. Coinjections of a putative ADM receptor antagonist, ADM22–52 (0.01 pmol), abolished the increases in MAP and HR evoked by ADM (0.01 pmol). The vasopressor effects of ADM (0.01 pmol) in the RVLM were abolished by coinjections of either dizocilpine hydrogen maleate (a selective NMDA glutamate receptor antagonist, 500 pmol) or 6-cyano-7-nitroquinoxaline-2,3-dione (a selective non-NMDA glutamate receptor antagonist, 50 pmol). The ADM-induced vasopressor effects were also abolished by coadministration of either 7-nitroindazole sodium salt (a selective neuronal NO synthase inhibitor, 0.05 pmol) or methylene blue (a soluble guanylyl cyclase inhibitor, 100 pmol). These results suggest that ADM in the RVLM stimulates increases in MAP and HR through ADM receptor-mediated mechanisms. These effects are mediated by glutamate via both NMDA and non-NMDA receptors. NO, derived from neuronal NO synthase, also contributes to the ADM-induced vasopressor effects via a soluble guanylyl cyclase-associated signaling pathway.

N-methyl-D-aspartate receptor; non-N-methyl-D-aspartate receptor; neuronal nitric oxide synthase; soluble guanylyl cyclase

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MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (250–350 g) were purchased from the Biological Animal Center, University of Alberta, housed in a 12:12-h light-dark cycle at 22°C, and given free access to food and water. All experimental protocols were approved by the local Animal Welfare Committee.

Preparation of animals. Rats were anesthetized with intraperitoneal injections of urethane (1.75 g/kg, Sigma Chemical, St. Louis, MO). Body temperature was monitored with a rectal thermometer and maintained at 37°C with a heating pad. To measure MAP and HR, the left femoral artery was cannulated using PE-50 tubing (Becton Dickinson, Sparks, MD), which was connected to a computer-based data acquisition system DI-150 RS (DATAQ Instruments, Akron, OH) via a pressure transducer.

Rats were placed in a stereotaxic apparatus, and a guide cannula (C315G; Plastics One, Wallingford, CT) was lowered into the left RVLM according to the coordinates 3.5–3.8 mm posterior, 1.9–2.1 mm lateral, and 0.1–0.3 mm dorsal to the interaural zero (15, 31). An internal cannula (C315I; Plastics One), which was connected to a syringe filled with chemicals via PE-50 tubing, was inserted into the guide cannula. Microinjections of the chemicals into the RVLM were performed with an electronic infusion pump (Harvard Apparatus, Holliston, MA). All injections were made in a volume of 100 nl over 1 min.

Experimental protocol. At the beginning of each experiment, the functional location of RVLM neurons was identified with elicitation of a transient increase in MAP (25–30 mmHg) induced with microinjections of l-glutamic acid (2 nmol, Sigma Chemical). After stabilization for 20–30 min, saline, 0.001–0.1 pmol ADM (American Peptide, Sunnyvale, CA), or ADM (0.01 pmol) plus various pharmacological agents (dissolved in saline) were injected unilaterally into the RVLM. MAP and HR were recorded for at least 1 h after microinjections; 1% Evans blue (Sigma Chemical) (7), 0.05 pmol 7-NiNa (AG Scientific, San Diego, CA) (28), and 50 pmol CNQX (Sigma Chemical) (7), 500 pmol MK-801 (Sigma Chemical) (28), and 100 pmol methylene blue (Sigma Chemical) (28). The doses of the pharmacological agents were chosen based on separate experiments, which showed that unilateral microinjections of individual agents, at the doses used, did not affect MAP and HR.

Brain histology. At the end of the experiment, rats were killed, and brains were removed and fixed in 4% paraformaldehyde for 48–72 h. The brains were then frozen, and coronal brain stem sections (40 μm) were cut using a cryostat (20°C). The sections were thaw-mounted onto slides, stained with neutral red (Allied Chemical, New York, NY), and observed under a microscope. Accurate RVLM microinjections were confirmed when Evans blue was present within the RVLM.

Statistical analyses. For each rat, averaged MAP and HR values were calculated every 5 min. For each group, all values were expressed as means ± SE. The temporal effects of various treatments on MAP and HR and differences among groups were assessed using two-way ANOVA with repeated measures. This test was followed by the Student-Newman-Keuls test for post hoc comparison of individual means. P < 0.05 indicated statistical significance.

RESULTS

Microinjections of ADM into the RVLM induce vasopressor effects. Although microinjections of ADM (0.001 pmol) into the RVLM had no effect on MAP and HR compared with microinjections of saline (controls), higher doses of ADM (0.01 and 0.1 pmol) induced significant and long-lasting increases in MAP and HR in a dose-dependent manner. Microinjections of ADM (0.01 pmol) increased MAP by ~12 mmHg and HR by ~39 beats/min. ADM at 0.1 pmol increased MAP by ~27 mmHg and HR by ~128 beats/min. These vasopressor effects of ADM at both doses lasted at least 1 h (Fig. 1).

Figure 2A is a representative brain stem section showing an injection site that is considered to be within the RVLM. Figure 2B summarizes the location of 12 injection sites in a schematic drawing of the brain stem. Although microinjections of ADM (0.01 pmol) into the RVLM significantly increased MAP and HR, the same dose of ADM injected outside the RVLM did not change MAP or HR (Fig. 3). Vasopressor effects of ADM in the RVLM are abolished by ADM22,52. Increases in MAP and HR evoked by microinjections of ADM (0.01 pmol) were abolished by coinjections of ADM22,52 (0.01 pmol) (Fig. 4). In separate experiments, microinjections of ADM22,52 (0.01 pmol) alone into the RVLM did not significantly alter MAP and HR over 1 h (Table 1).

Vasopressor effects of ADM in the RVLM are abolished by MK-80I and CNQX. Although microinjections of ADM (0.01 pmol) alone into the RVLM induced significant vasopressor effects, coinjections of ADM with MK-801 (500 pmol) or CNQX (50 pmol) did not significantly increase MAP and HR (Fig. 5). Microinjections of only MK-80I (500 pmol) or CNQX (50 pmol) into the RVLM did not produce significant changes in MAP and HR over 1 h (Table 1). Vasopressor effects of ADM in the RVLM are abolished by 7-NiNa and methylene blue. The vasopressor effects caused by ADM (0.01 pmol) were abolished with coinjections of 7-NiNa (0.05 pmol) (Fig. 6). A similar effect was achieved when ADM was coinjected with methylene blue (100 pmol) (Fig. 6). Microinjections of neither 7-NiNa nor methylene blue alone, at
the doses used, caused significant changes in MAP and HR over 1 h (Table 1).

DISCUSSION

In the present study, we show that unilateral microinjections of ADM (0.01 and 0.1 pmol) significantly increased MAP and HR in a dose-dependent and site-specific manner. Furthermore, we demonstrate that the vasopressor effects of ADM were blocked by an ADM receptor antagonist. In addition, we showed that coinjections of NMDA or non-NMDA glutamate receptor antagonists blocked the ADM-induced responses in MAP and HR. Finally, we showed that inhibition of nNOS or sGC also abolished the vasopressor effects of ADM in the RVLM.

ADM in the RVLM exerts vasopressor effects. Premotoneurons in the RVLM project to the sympathetic preganglionic neurons in the spinal cord and play an important role in the regulation of MAP and other autonomic functions (11). Nuclei that project to RVLM neurons, including the PVN and nucleus of the solitary tract, have been shown to express ADM (30, 35, 40, 43). In addition, RAMP-2, a component of the ADM receptor system, is expressed in the RVLM (40). Taken together, these findings suggest that ADM acts on RVLM neurons to contribute to the regulation of MAP.

Our experiments showed that, although ADM at 0.001 pmol was ineffective, microinjections of ADM at 0.01 and 0.1 pmol into the RVLM induced dose-dependent and long-lasting vasopressor effects. We confirmed that these effects were site specific for the RVLM, as changes in MAP and HR were absent when ADM was injected outside the RVLM. It is interesting to note that ADM at 0.1 pmol induced a robust
MK-801, or /H11001 plus 50 pmol 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). plus 500 pmol dizocilpine hydrogen maleate (MK-801), or 0.01 pmol ADM microinjections into the RVLM of saline, 0.01 pmol ADM
A) in rats that received unilateral
B) and HR (Fig. 6. Temporal MAP (A) and HR (B) in rats that received unilateral microinjections into the RVLM of saline, 0.01 pmol ADM, 0.01 pmol ADM plus 0.05 pmol 7-nitroindazole sodium salt (7-NiNa), or 0.01 pmol ADM plus 100 pmol methylene blue. Time 0 indicates the beginning of injections. Values are means ± SE; n = 6 in each group. Significant difference (P < 0.05) vs. *saline, †0.01 pmol ADM plus 0.05 pmol 7-NiNa, or ‡0.01 pmol ADM plus 100 pmol methylene blue at corresponding time points.

Table 1. Effects of pharmacological agents on baseline MAP and HR

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Maximal Changes in MAP, mmHg</th>
<th>Maximal Changes in HR, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>2.4±1.5</td>
<td>8.7±5.1</td>
</tr>
<tr>
<td>ADM22,52 (0.01 pmol)</td>
<td>−2.0±1.3</td>
<td>−1.6±3.6</td>
</tr>
<tr>
<td>MK-801 (500 pmol)</td>
<td>−1.5±1.0</td>
<td>−3.5±3.1</td>
</tr>
<tr>
<td>CNQX (50 pmol)</td>
<td>−2.1±1.8</td>
<td>−3.3±4.3</td>
</tr>
<tr>
<td>7-NiNa (0.05 pmol)</td>
<td>−2.2±1.6</td>
<td>−1.2±3.7</td>
</tr>
<tr>
<td>Methylene blue (100 pmol)</td>
<td>−2.0±1.2</td>
<td>−1.2±5.1</td>
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Values are means ± SE; n = 6 for each group. Saline or different pharmacological agents were unilaterally microinjected into the RVLM, and the maximal changes in mean arterial pressure (MAP) and heart rate (HR) over 1 h were recorded. ADM, adenomedullin; MK-801, dizocilpine hydrogen maleate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; 7-NiNa, 7-nitroindazole sodium salt. There were no significant differences between the changes in MAP and HR after saline injections or after injections of any of the pharmacological agents.

increase in HR (128 beats/min), whereas the increase in MAP (27 mmHg) was more modest, suggesting that ADM particularly stimulates RVLM neurons, which control cardiac sympathetic activity. Consistent with our observation, a previous study (19) showed that microinjections of ADM (2 pmol in 200 nl) into the RVLM of anesthetized rats increased MAP and HR by 99 mmHg and 370 beats/min, respectively.

Our laboratory has shown previously (35) that expression of the ADM gene in the PVN and/or nucleus of the solitary tract was attenuated in response to intravenous injections of lipopolysaccharide and restraint stress, physiological stressors that both lead to increases in MAP (9, 45). We hypothesized that decreased production of ADM in these autonomic centers is part of the animal’s attempt to reestablish cardiovascular homeostasis (37). This hypothesis is further supported by our present observation that ADM injected into the RVLM increased MAP and HR.

ADM-induced vasopressor effects are mediated by ADM receptors. Because of the homology of ADM and calcitonin gene-related peptide (CGRP), ADM has appreciable affinity for the CGRP receptor, composed of calcitonin-receptor-like receptor and RAMP-1 (26, 34). To determine whether the vasopressor effects of ADM in the RVLM are mediated by ADM receptors, we coinjected ADM with ADM22,52, a putative ADM receptor antagonist (16). Our experiments showed that the ADM-induced vasopressor effects were eliminated by ADM22,52. Thus, together with the results from a previous study that showed that ADM-induced effects in the RVLM were not affected by CGRP26,37 (a CGRP receptor antagonist) (19), these results suggest that ADM in the RVLM induces vasopressor effects through ADM receptor-mediated mechanisms.

ADM in the RVLM induces vasopressor effects by potentiating both glutamatergic neurotransmission and nitrergic neurotransmission. Glutamate has been shown to be one of the neurotransmitters that provides tonic excitatory drive to the sympathetic premotoneurons in the RVLM (1, 4, 5, 17). To investigate whether the ADM-induced vasopressor effects are mediated by glutamatergic neurotransmission, we coinjected ADM with MK-801, a selective NMDA glutamate receptor antagonist, or CNQX, a selective non-NMDA glutamate receptor antagonist. Interestingly, both MK-801 and CNQX completely blocked the ADM-induced vasopressor effects, sug-
gesting that the vasopressor effects of ADM in the RVLM are mediated by both NMDA and non-NMDA glutamate receptors. A similar phenomenon has recently been reported in a study that showed that increases in MAP and HR induced by NO in the RVLM were mediated by both of the ionotropic glutamate receptor subtypes (7).

NO is also an important neurotransmitter for central cardiovascular regulation in the RVLM (7, 8, 20, 22, 28). It has been shown that, in the RVLM, both nNOS and inducible NO synthase are expressed in neurons, whereas endothelial NO synthase is associated primarily with blood vessels (8). Interestingly, NO derived only from nNOS exerts vasopressor effects (7), whereas NO synthesized from endothelial NO synthase or inducible NO synthase has depressor effects (7, 20). Therefore, we investigated whether nNOS is involved in the ADM-induced vasopressor effects by coinjecting ADM with 7-NiNa, a selective nNOS inhibitor. The blockade of the ADM-induced responses in MAP and HR by 7-NiNa indicates that the vasopressor effects of ADM in the RVLM are mediated by neuronal NO produced by nNOS.

One of the mechanisms for NO-induced actions is through stimulation of sGC, which, in turn, catalyzes production of cyclic GMP (3). This NO-sGC-cyclic GMP cascade is involved in various NO-induced effects in the brain (12), including NO-mediated cardiovascular responses in the RVLM (7, 44). In the present study, we demonstrate that coinjections of methane blue, an sGC inhibitor, into the RVLM blocked the ADM-induced increases in MAP and HR. Together with the data derived from the experiments with 7-NiNa, these results show that the vasopressor effects of ADM in the RVLM are mediated by NO through a nNOS-sGC signal transducing system.

Based on our results, therefore, we speculate that the binding of ADM to its receptors potentiates excitatory synaptic inputs to RVLM neurons from both glutamate and NO and that the activation of these RVLM neurons, in turn, leads to vasopressor effects.

**ADM in the RVLM initiates an ADM-glutamate-NO and/or ADM-NO-glutamate cascade.** Because both the inhibition of glutamatergic and nitrergic neurotransmission abolished the ADM-induced effects in the RVLM, we propose that ADM in the RVLM potentiates the actions of these two neurotransmitters (either pre- or postsynaptically) in sequence to exert vasopressor effects. However, the order by which glutamatergic and nitrergic neurotransmission are recruited by ADM is unclear. One possibility is that ADM in the RVLM directly potentiates glutamatergic neurotransmission, which, in turn, results in increased NO production (an ADM-glutamate-NO cascade). Indeed, it has been shown that NMDA microinjected into the RVLM in anesthetized cats can enhance extracellular NO levels and increase MAP and that the NMDA-induced increase in MAP can be attenuated by nNOS and sGC inhibitors (44). Similarly, the enhanced MAP caused by microinjections of l-glutamate into the RVLM in awake rats can be blocked by pretreatment with sGC inhibitors (23, 24). These results indicate the existence of a glutamate-NO cascade in the RVLM that is involved in the control of MAP. Thus ADM in the RVLM may act through this cascade to mediate its vasopressor effects, although there is, as yet, no evidence to show a direct interaction between ADM and glutamate in the RVLM.

Alternatively, but not exclusively, ADM in the RVLM may stimulate NO production first, and NO, in turn, may potentiate glutamatergic neurotransmission (an ADM-NO-glutamate cascade). Our observation that ADM can increase NO release from SK-N-SH human neuroblastoma cell line (unpublished data) supports the possibility that ADM directly increases NO production from RVLM neurons. In addition, existence of a NO-glutamate cascade in the RVLM is suggested by experiments done in anesthetized rats, which showed that the vasopressor effects of NO in the RVLM can be blocked by glutamate receptor antagonists (7, 29). Consistent with these results, NO has been demonstrated to increase glutamate release from RVLM neurons via a cyclic GMP signaling pathway in the rat brain stem slice preparation (18).

**Perspectives**

Previous studies have shown that ADM in the central nervous system plays an important role in the control of MAP and that effects in the brain are area specific. Our study shows that ADM in the RVLM stimulates increases in MAP and HR through the binding of ADM to its receptors. We further demonstrate that ADM in the RVLM exerts these effects by potentiating both glutamatergic and nitrergic neurotransmission. These data are critical to the understanding about the roles of central ADM in the control of cardiovascular activity, and they also contribute to the knowledge required for the development of therapeutic advances for the use of ADM and its analogs in the control of arterial pressure.

**GRANTS**

This work was supported by the Heart and Stroke Foundation of Alberta/Northwest Territories/Nunavut.

**REFERENCES**


ROLE OF NO AND GLUTAMATE IN CV RESPONSES TO ADM IN RVLM


