Effect of adrenomedullin on the activity of barosensitive neurons in the rostral ventrolateral medulla of rats

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Abstract: To investigate the electrophysiological effect of rat adrenomedullin (rADM) on barosensitive neurons in the rostral ventrolateral medulla (rVLM) and its potential mechanisms, the extracellular recording and multi-barrel iontophoresis methods were used. Of the 29 barosensitive neurons in the rVLM, 20 neurons demonstrated excitatory response to iontophoretically applied rADM and increased the firing rate from (10.8 ± 2.7) spikes/s to (14.6 ± 3.6), (19.8 ± 4.7) and (31.9 ± 6.4) spikes/s (P<0.05, n=20) at the current of 30, 60 and 90 nA, respectively. Application of human adrenomedullin (22-52) [hADM (22-52)], a specific antagonist of rADM receptor, distinctly attenuated the augmentation of firing rate induced by rADM—the firing rate was increased by 15.4% [(11.4 ± 2.5) spikes/s, P<0.05, n=10]. Another antagonist, human calcitonin gene-related peptide (8-37) [hCGRP (8-37)] had no significant effect on rADM-induced excitation. Other 23 barosensitive neurons were recorded to test the influence of nitric oxide synthase (NOS) inhibitors on the excitatory effect of rADM. In 10 neurons, 7-NiNa (neuronal NOS inhibitor) decreased the firing rate from (10.1 ± 3.5) spikes/s to (7.5 ± 2.5), (5.3 ± 2.1) and (3.1 ± 1.4) spikes/s (P<0.05, n=10) at the current of 10, 20 and 40 nA, respectively. The excitatory effect of rADM (60 nA, 30 s) during 7-NiNa application was nearly eliminated and the magnitude of firing rate was increased only by 17% of the basal level (6.2 ± 1.9) spikes/s (P<0.05, n=7). While aminoguanidine (AG, iNOS inhibitor) increased the firing rate at the resting level from (11.5 ± 5.1) spikes/s to (17.8 ± 5.6), (22.5 ± 6.3) and (29.1 ± 6.4) spikes/s (P<0.05, n=8) at the current of 10, 20 and 40 nA in 8 barosensitive neurons, respectively. When rADM (60 nA, 30 s) was delivered during AG iontophoresis period, the firing rate significantly increased by 60% of the basal level [(22.5 ± 6.3) spikes/s, n=5]. These results indicate that rADM activates the barosensitive neurons in the rVLM directly and acts as a cardiovascular regulator, and that this function might be mediated by its specific receptor. NO, mainly neuronal NOS-originated might be involved in the excitatory effect of rADM in the rVLM.

Key words: adrenomedullin; receptor; nitric oxide; rostral ventrolateral medulla; iontophoresis; extracellular recording

肾上腺髓质素对大鼠延髓头端腹外侧区压力反射敏感神经元的作用

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摘 要: 采用多管微电泳结合细胞外记录的方法研究了肾上腺髓质素(adrenomedullin, ADM)对大鼠延髓头端腹外侧区(rostral ventrolateral medulla, rVLM)压力反射敏感性神经元电活动的作用及其可能机制。结果显示: 在29个rVLM压力反射敏感神经元中, 20个神经元在30, 60和90 nA的电流微电泳大鼠ADM(rADM)过程中, 放电频率由(10.8 ± 2.7) spikes/s分别增加到(14.6 ± 3.6),(19.8 ± 4.7)和(31.9 ± 6.4) spikes/s (P<0.05, n=20)。微电泳rADM特异性受体阻断剂人ADM(human ADM, hADM) (22-52)可明显减小神经元放电频率的增加幅度, 比正常放电频率仅增加15.4% [(11.4 ± 2.5) spikes/s, P<0.05, n=10], 而降钙素基因相关肽1(CGRP1)受体阻断剂hCGRP (8-37)对rADM兴奋性神经元电活动影响较小。在另外23个神经元中, 10个神经元的放电频率在10, 20和40 nA电流微电泳神经型NOS(nNOS)抑制剂7-NiNa过程中放电减少, 由正常的(10.1 ± 3.5) spikes/s分别减少为(7.5 ± 2.5),(5.3 ± 2.1)和(3.1 ± 1.4) spikes/s (P<0.05, n=10)。在微电泳7-NiNa过程中同时微电泳rADM, 则rADM增加神经元放电频率的效应减弱, 增加幅度为基本水平的17% [(6.2 ± 1.9) spikes/s]。8个神经元在10, 20和40 nA电流微电泳诱导型NOS(iNOS)抑制剂aminoguanidine(AG)过程中放电频率由(11.5 ± 5.1) spikes/s增加到(17.8 ± 5.6),(22.5 ± 6.3)和(29.1 ± 6.4) spikes/s (P<0.05, n=8)。rADM与AG同时微电泳时, AG对rADM本身增加神经元放电的效应无明显影响。上述结果提示,
Adrenomedullin (ADM), a peptide with 52 amino acids encoded by the preproadrenomedullin (ppADM) gene, together with its specific receptor components are widely expressed in various tissues, such as the kidney, heart, lung, adrenal gland, hypothalamus-pituitary-adrenal (HPA) axis and the central nervous system (CNS)\[1,2,5,6\]. In the CNS, ADM is particularly present at the medulla oblongata, midbrain, hypothalamus and amygdala, all sites known to be important in the organization of autonomic tone\[3,4\]. Therefore, ADM may be an important neurotransmitter or neuromodulator in the regulation of autonomic functions\[5,6\].

Despite massive reports on its peripheral functions, ADM has been shown to be involved in the balance of body fluid and electrolyte homeostasis together with the regulation of cardiovascular function through its central mechanism. ADM in the CNS plays an important role in the regulation of mean arterial pressure (MAP) in an area specific way. Microinjection of ADM into the rostral ventrolateral medulla (rVLM) or the area postrema (AP) exerted a hypertensive effect associated with an increased sympathetic output, while microinjection into the hypothalamic paraventricular nucleus (PVN) produced a hypertensive effect\[7-11,27\].

The rVLM is a very important area for the regulation of blood pressure (BP). Neurons in the rVLM are located at a crucial site involved in the baroreflex pathway and play a key role in controlling peripheral sympathetic nerve activity (SNA) and BP through many neurotransmitters, such as amino acid, enkephalin, substance P (SP), neuropeptide Y (NPY), NO, etc\[12-15\]. According to Xu et al\[11\], microinjection of ADM into the rVLM could increase BP, heart rate (HR), and renal sympathetic nerve activity (RSNA) through specific ADM receptor and NO in a dose-dependent manner, which suggested that ADM was involved as a neuropeptide in the central regulation of sympathetic outflows and cardiovascular system activities.

However, it is not clear whether these biological effects of ADM, observed in microinjection studies, are produced by directly exciting the barosensitive premotor sympathetic neurons in rVLM, and whether there are other neurotransmitters or neuromodulators involved in it. The present study was conducted to investigate whether central administration of ADM could directly influence the activity of rVLM barosensitive neurons and its possible mechanisms.

1 MATERIALS AND METHODS

Male Sprague-Dawley rats (220–250 g) were anesthetized with a mixture of urethane and chloralose (700 mg/kg and 35 mg/kg, respectively, i.p.)

1.1 Surgical procedure

Briefly, after anesthesia, catheters were placed into the trachea, right femoral artery and vein. For the recording and iontophoresis from the ventral medulla, the trachea and esophagus were ligated and cut off, the oral part was pulled to the head, parts of muscle and occipital bone were removed to expose ventral surface of the medulla and the aortic nerve (AN) was unmasked.

1.2 Single-unit recording and microiontophoresis

The anaesthetized rats were fixed on a stereotaxic frame (Type: SN-3, Narishige, Japan) with ventral upwards, ventilated with room air. Rectal temperature was measured and maintained around 37.8 °C with a heating pad. BP and electrocardiogram were sequentially measured by a Biological Signal Recording System (BSRS, SMUP-PC, Shanghai).

Five-barrel glass pipettes were used. The central barrel for recording contained 2% pontamine sky blue dye dissolved in 0.5 mol/L sodium acetate. The balance barrel contained 0.25 mol/L NaCl and other barrels were used for iontophoresis of rat ADM (rADM) (100 mmol/L), Glu (100 mmol/L), 7-NiNa and aminoguanidine (AG) (50 mmol/L). All drugs were dissolved in 0.9% NaCl. Glu was ejected with negative current and other drugs with positive current. A retaining current of 8 nA was applied between ejection periods.

Extracellular single-unit recordings were made from spontaneously active neurons in the rVLM. Signals were pre-amplified and then fed into a signal collection system (SMUP-E, Shanghai) to be recorded and analyzed. To verify the drugs were applied to the cell body by an iontophoresis machine (JL-H2003, Shanghai), Glu was first iontophoresically applied to the neuron recorded. If the firing rate of this neuron unit increased during the Glu application period, this unit could be used for further experiment. Otherwise, this unit would be discarded.

1.3 Histology

At the end of experiment, the neuronal recording sites were marked by electrophoretic application of pontamine sky blue dye (10 µA for 30 min, negative currents). The brain
stem was removed and fixed in 10% formaldehyde for about 72 h in room temperature, then frozen and sectioned. According to Paxinos and Watson atlas[26], all recording sites were located in the rostral medulla, just ventrolateral to the nucleus ambiguous, lateral to the nucleus inferior olive (Fig.1).

1.4 The verification of barosensitive neurons in the rVLM

Three methods were used to determine if a unit recorded was a barosensitive neuron locked to the cardiac cycle: (1) The spikes were inhibited by stimulation of AN through a stimulator (SEN-7103, Nihon Kohden, Japan) with specific parameters (duration 200 μs, current 0.2–0.8 mA, interval 5 ms, frequency 1 Hz); (2) Recording the change of BP and the frequency of spikes during pentoadrenallin application (i.v., 10 μg/kg); (3) The correlation and coherence analysis of spikes to cardiac rhythm, with the R wave of ECG triggered superposition of ECG and spike histogram, respectively (Fig.2).

Fig. 1. Diagrammatic representation of the medulla oblongata at rVLM level, and the distances in millimeters of each section caudal to the interaural line were indicated. Dots show the location where iontophoresis of drugs elicited significant effects on the firing rate of barosensitive neurons and extracellular recording was performed. Amb, ambiguous nucleus; IO, inferior olive; LPGi, lateral paragigantocellular nucleus; Py, pyramidal tract.

Fig. 2. Verification of barosensitive neurons in the rVLM. A: Histogram shows a decrease of firing rate after stimulating the AN. B: Changes of BP and the frequency of spike during the application of pentoadrenallin (i.v., 10 μg/kg). C: The histogram of the R wave of ECG triggered superposition of BP and spike, respectively. D: Histogram shows the correlation and coherence of spike to cardiac rhythm.
1.5 Data analysis
Values are expressed as mean±SD. Statistical analysis was performed by Student’s t-test for paired and unpaired data. Criterion for statistical significance was \( P<0.05 \).

2 RESULTS
The average resting firing rate of a total of 63 barosensitive neurons correlating to cardiovascular activity in the rVLM was \((10.8 \pm 1.7)\) spikes/s \((4.8-35.6\) spikes/s). Among them 52 units increased their firing rate \([(29.2 \pm 6.8) \text{ spikes/s}, \ P<0.01\] when Glu (-30 nA) was iontophoretically applied, therefore they were used for further study. The overall neurons recorded and their responses to drugs were shown in Table 1.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Total number</th>
<th>Excitatory effect</th>
<th>Inhibitory effect</th>
<th>No response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu (100 mmol/L)</td>
<td>63</td>
<td>52 (87%, 29 for rADM, 23 for NOS inhibitors)</td>
<td>7 (11%)</td>
<td>4 (2%)</td>
</tr>
<tr>
<td>rADM (100 mmol/L)</td>
<td>29</td>
<td>20 (69%)</td>
<td>2 (7%)</td>
<td>7 (24%)</td>
</tr>
<tr>
<td>7-NiNa (50 mmol/L)</td>
<td>12</td>
<td>0 (0%)</td>
<td>10 (83%)</td>
<td>2 (17%)</td>
</tr>
<tr>
<td>AG (50 mmol/L)</td>
<td>11</td>
<td>8 (73%)</td>
<td>2 (18%)</td>
<td>1 (9%)</td>
</tr>
</tbody>
</table>

2.1 Excitatory effect of rADM on rVLM barosensitive neurons
Of the 29 recorded barosensitive neurons recorded, 20 neurons enhanced their firing rate \([\text{resting level: } (10.8 \pm 2.7) \text{ spikes/s}]\) in response to rADM. Iontophoretic application of rADM at 30, 60 and 90 nA resulted in an increase of the firing rate to \((14.6 \pm 3.6), (19.8 \pm 4.7)\) and \((31.9 \pm 6.4)\) spikes/s \((P<0.05 \text{ at 60 nA and } P<0.01 \text{ at 90 nA})\), respectively, namely, increased by 35%, 80% and 195% of the resting level. The firing rate restored quickly following the termination of the injection current. The barrel containing saline at the same current (30, 60, 90 nA) had no effect (Fig.3).

2.2 Effects of rADM antagonists on rADM exciting barosensitive neurons
In 12 barosensitive neurons, inhibitory response to human ADM (hADM) (22-52), an antagonist of rADM receptor, were observed in a current-dependent way. The firing rate decreased to \((8.1 \pm 1.9), (7.7 \pm 1.6)\) and \((5.4 \pm 2.1)\) spikes/s at 20, 40 and 60 nA, respectively \((P<0.05, n=12)\). During hADM (22-52) application (40 nA, 3 min), the response to rADM (60 nA, 30 s) was attenuated and the firing rate was increased by 15.4% \([(11.4 \pm 2.5) \text{ spikes/s}, P<0.05, n=10]\), significantly smaller than that evoked by rADM alone (increased by 80% of the resting firing rate).

Another 10 barosensitive neurons excited by rADM also demonstrated an inhibitory response to human calcitonin-gene related peptide (8-37)[hCGRP (8-37)], an antagonist of ADM receptor. The firing rate decreased to \((9.5 \pm 3.2), (8.2 \pm 2.6)\) and \((7.3 \pm 2.4)\) spikes/s at the current of 20, 40 and 60 nA, i.e., decreased by about 12%, 24% and 32% \((P<0.05)\), respectively. During hCGRP (8-37) (40 nA, 3 min) application, the excitatory effect of rADM (60 nA, 30 s) on these neurons were little affected, and the firing rate was increased by 93% \([(15.8 \pm 3.1) \text{ spikes/s}, P<0.05, n=10]\) (Fig.4).

2.3 Influence of NOS inhibitors on the excitatory effect of rADM on barosensitive neurons
To test the influence of NOS inhibitors on the excitatory effect of rADM, another 23 barosensitive neurons in the rVLM were recorded. Their resting firing rate was \((10.1 \pm 3.5)\) spikes/s. 7-NiNa, an neuronal NOS (nNOS) inhibitor, showed a current-dependent inhibition in 10 neurons and decreased the firing rate to \((7.5 \pm 2.6), (5.3 \pm 2.1)\) and \((3.1 \pm 1.4)\) spikes/s \((P<0.05)\) at the current of 10, 20 and
40 nA for 30 s, respectively. The firing rate restored within 2 s once the injection current stopped. Seven out of these 10 barosensitive neurons increased their firing rate from the resting level of (10.1 ± 3.5) to (17.3 ± 4.2) spikes/s (increased by 72% of the resting level, $P<0.05$) when rADM was applied alone at a current of 60 nA. The firing rate was increased only by 17% ($P<0.05$, $n=7$) when rADM (60 nA, 30 s) was applied during iontophoretical application of 7-NiNa (20 nA, 3 min), significantly lower than that of rADM alone (increased by 80% of the resting firing rate).

AG, an iNOS inhibitor, excited other 8 barosensitive neurons and increased their firing rate to (17.8 ± 5.6), (22.5 ± 6.3) and (29.1 ± 6.4) spikes/s from the resting level [(11.5 ±
5.1) spikes/s] at the current of 10, 20 and 40 nA ($P<0.05$), respectively. During the iontophoretic application of AG (20 nA, 3 min), rADM (60 nA, 30 s) still showed a significant excitatory effect in 5 neurons and increased their firing rate by 60% of the basal level ([22.5 ± 6.3] spikes/s, $P>0.05$, $n=5$) (Fig.5 and Fig.6).

Fig. 5. The effects of NOS antagonists on the spike of barosensitive neurons in the rVLM. A: The nNOS inhibitor, 7-NiNa, decreased the firing rate when iontophoretically applied. B: The iNOS inhibitor, AG, increased the firing rate when iontophoretically applied in the same neuron. Bars show the time course. C, D: Corresponding graphs illustrated the different effects of iontophoretic application of hADM (22-52) or hCGRP (8-37) on the barosensitive neurons in the rVLM. *$P<0.05$, **$P<0.01$ vs 0 nA current intensity, respectively.

Fig. 6. The influence of NOS antagonists on rADM activating barosensitive neurons. A: Histogram showing that the effect of rADM on the firing rate was attenuated markedly during the application of the nNOS inhibitor, 7-NiNa. B: The effect of rADM on the firing rate was not significantly affected by AG. Bars show the time course. C: Graph showing the change of firing rate before and after the NOS inhibitors were applied. *$P<0.01$ vs rADM application alone.
3 DISCUSSION

According to the previous studies[10,11], microinjection of ADM into the rVLM increased the BP, HR, and RSNA. These biological effects were blocked by ADM specific receptor antagonist, hADM (22-52), and nNOS inhibitor, 7-NiNa. It is logical to speculate the involvement of ADM specific receptor and nNOS-derived NO in the hypertensive effect of ADM in the rVLM. The present work showed clearly that the hypertensive effect of ADM in the rVLM might correlate to the activation of barosensitive neurons in the rVLM.

Up to now, it has been known that ADM receptors include the calcitonin gene-related peptide 1 (CGRP1) receptor and ADM specific receptor that consists of two components, receptor activity modifying proteins (RAMPs) and calcitonin-receptor-like receptor (CRLR). RAMP had three isoforms, i.e., RAMP1, RAMP2 and RAMP3, which control both the transport and functional state of the CRLR through differential glycosylation or allastereic modulation of agonist binding sites and the alteration of conformational state of CRLR[17-19]. RAMP1 was found to serve as an accessory protein involved in the transport of an orphan receptor, while co-transfecting RAMP2 or RAMP3 with CRLR into mammalian cells was found to function as an ADM receptor and then two ADM specific receptor subtypes had now been defined in molecular terms: ADM1, composed of CRLR with RAMP2, and ADM2, composed of CRLR and RAMP3[20].

To evaluate the physiological and pharmacological characteristics of central ADM, several laboratories used the CGRP1 receptor antagonist hCGRP (8-37) and the ADM specific receptor antagonist hADM (22-52), but neither of them contains disulfide bond. In this study, we used these two ADM receptor antagonists to test which receptor was involved in the excitatory effect of ADM on the barosensitive neurons in the rVLM. We observed that iontophoresis of hADM (22-52), but not hCGRP (8-37), could partially antagonize the excitatory effect of ADM, which supported the view that the action of ADM in the rVLM may be dependent on the ADM specific receptor. According to Hay et al.[21], there were pharmacological differences between CRLR/RAMP2 and CRLR/RAMP3 receptors. hADM (22-52) is a more significantly effective antagonist of ADM at the CRLR/RAMP2 receptor compared with the CRLR/RAMP3 regardless of species composition. Therefore, our present study suggests that ADM exerts a direct excitatory effect on the barosensitive neurons in the rVLM through ADM specific receptor, especially ADM1 receptor.

NO acts as a non-conventional neurotransmitter in the CNS and mediates some of the effects of central ADM[7,11]. Co-injection of nNOS eliminated ADM’s hypertensive effect in the rVLM, suggesting that NO derived from nNOS contributes to this effect[21]. In vitro experiment further showed that binding of ADM to its specific receptor could activate nNOS and then increased the synthesis and release of NO in the neuron-like cells, SK-N-SH human neuroblastoma cells through stimulating calcium-induced calcium release and cAMP-PKA pathway[22]. Although there are several studies concerning the effect of NO inhibitors on the rVLM[24,25], the direct effect of NO inhibitors on the rVLM barosensitive neurons hasn’t been reported. So we used iontophoresis and extracellular recording methods to test the responses of these neurons to 7-NiNa and AG. Our results showed that AG could significantly increase the firing rate of rVLM barosensitive neurons while 7-NiNa had the contrary effect. The results were consistent with other studies using microinjection method which showed that iNOS inhibitor could increase, while nNOS inhibitor could decrease, blood pressure, when injected into the rVLM of rats[25].

To further clarify ADM-NO cascade in the regulation of sympathetic output in the rVLM, rADM was applied during iontophoretic application of 7-NiNa and AG. Despite the effects of NO inhibitors on rVLM barosensitive neurons, they also influenced the effect of ADM when applied simultaneously. We noted that nNOS inhibitor 7-NiNa, but not iNOS inhibitor AG, could partially attenuate the excitatory effect of ADM on the barosensitive neurons in the rVLM. In this condition, both AG and rADM had excitatory effects on barosensitive neurons and the final effect observed was the simple summation of them. Therefore, they might exert their effects through different pathways and had little influence on each other. It seems that NO derived from nNOS is involved in the excitatory effect of ADM on the rVLM barosensitive neurons, but NO derived from iNOS was independent of the effect of rADM, and that their effects can be simply summated. Our results demonstrate that ADM is an important neurotransmitter or neuroregulator in the rVLM for the modulation of sympathetic output and cardiovascular function through binding with ADM1 receptor, and that this procedure is possibly associated with nNOS activity.

REFERENCES

3. Satoh F, Takahashi K, Murakami O, Totsune K, Sone M, Ohneda...


