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Role of cerebellar adrenomedullin in blood pressure regulation

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ABSTRACT

Adrenomedullin (AM) and their receptor components, calcitonin-receptor-like receptor (CRLR) and receptor activity-modifying protein (RAMP1, RMP2 and RAMP3) are widely expressed in the central nervous system, including cerebellum. We have shown that AM binding sites are altered in cerebellum during hypertension, suggesting a role for cerebellar adrenomedullinergic system in blood pressure regulation. To further evaluate the role of AM in cerebellum, we assessed the expression of AM, RAMP1, RAMP2, RAMP3 and CRLR in the cerebellar vermis of 8 and 16 week old spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats. In addition, the effect of microinjection of AM into rat cerebellar vermis on arterial blood pressure (BP) was determined. Animals were sacrificed by decapitation and cerebellar vermis was dissected for quantification of AM, CRLR, RAMP1, RAMP2 and RAMP3 expression using western blot analysis. Another group of male, 16 week old SHR and WKY rats was anesthetized, and a cannula was implanted in the cerebellar vermis. Following recovery AM (0.02 to 200 pmol/5 μL) or vehicle was injected into cerebellar vermis. BP was determined, before and after treatments, by non-invasive plethysmography. In addition, to establish the receptor subtype involved in AM action *in vivo*, animals received microinjections of AM_{22-52} (200 pmol/5 μL), an AM1 receptor antagonist, or the CGRP1 receptor antagonist, $CGRP_{8-37}$ (200 pmol/5 μL) into the cerebellar vermis, administered simultaneously with AM or vehicle microinjection. Cannulation was verified *post mortem* with the *in situ* injection of a dye solution. Our findings demonstrated that the expression of CRLR, RAMP1 and RAMP3 was higher in cerebellum of SHR rats, while AM and RAMP2 expression was lower than those of WKY rats, both in 8 and 16 week old rats. *In vivo* microinjection of AM into the cerebellar vermis caused a profound, dose dependent, hypotensive effect in SHR but not in normotensive WKY rats. Coinjections of a putative AM receptor antagonist, AM_{22-52} abolished the decreases in mean arterial pressure (MAP) evoked by AM, showing that AM acts through its AM1 receptor in the vermis to reduce MAP. These findings demonstrate a dysregulation of cerebellar AM-system during hypertension, and suggest that cerebellar AM plays an important role in the regulation of BP. Likewise; they constitute a novel mechanism of BP control which has not been described so far.

1. Introduction

Adrenomedullin (AM) is a 52- (human) or 50-amino acid (rat) peptide which shows some homology with calcitonin gene-related peptide (CGRP) (Kitamura et al., 1993) and has therefore been added to the calcitonin/CGRP/amylin peptide family (Ichiki et al., 1994; Sakata et al., 1994). AM has two specific receptors, the AM1 and AM2 receptors, formed from the obligate co-expression of a class-B, G protein coupled receptor (GPCR), the calcitonin receptor-like receptor (CRLR) and receptor activity-modifying proteins (RAMP) 2 or 3, respectively (McLatchie et al., 1998). The calcitonin gene-related peptide 1 (CGRP1) receptor is formed of a complex between CRLR and RAMP1 (McLatchie et al., 1998; Cases and Mora-Macía, 2001). AM1 receptors are highly selective for AM over CGRP and other peptides while AM2 receptors binds both AM and AM2 (intermedin) with high affinities (Hong et al., 2012). AM also has appreciable affinity for the CGRP1 receptor (Gibbons et al., 2007).

AM is found in the peripheral circulation, in the cerebrospinal fluid and in several organs including the central nervous system (CNS) (Ichiki et al., 1994; Kitamura et al., 1993; Satoh et al., 1995, 1996; Wei et al., 1998; Wang et al., 2015). In the CNS, AM and its receptors are particularly localized to the autonomic nuclei, including nucleus tractus solitarii (NTS), lateral parabrachial nucleus (LPBN) and rostral ventrolateral medulla (RVLM) and in cerebral cortex, pituitary gland, thalamus, hypothalamus, brainstem, amygdala and cerebellum (Hinson et al., 2000; Serrano et al., 2000; Juaneda et al., 2003; Macchi et al., 2006; Owji et al., 1996; Xu and Krukoff, 2004a). Also it was reported the presence of AM and its receptor components in cerebrospinal fluid-contacting nucleus (Wu et al., 2015). In cerebellum, AM immunoreactivity is located in cerebellar Purkinje cells and mossy terminal nerve fibers as well as neurons of the cerebellar nuclei (Serrano et al., 2000).

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AM exerts several effects in the CNS. Central AM administration results in various neuroendocrine responses such as the inhibition of arginine vasopressin secretion induced by hypovolemic and osmotic stimuli, and the secretion of oxytocin by activating hypothalamic oxytocin-producing cells (Serino et al., 1999; Yokoi et al., 1996). In addition, central administration of AM increases urinary excretion of water, sodium and potassium, in a dose dependent manner in conscious hydrated rats (Israel and Diaz, 2000; Díaz and Israel, 2001), and microinjection of AM into the area postrema (AP) and the RVLM causes a hypertensive effect; while in the paraventricular nucleus (PVN) of the hypothalamus produces hypotension (Allen et al., 1997; Ji and He, 2002; Xu and Krukoff, 2004b). This evidence indicates that central AM may play an important role in body fluid homeostasis and central regulation of the cardiovascular system.

Several experimental and epidemiological studies have shown that AM and its receptor components expression are altered during hypertension (Cases and Mora-Macía, 2001). In effect, it has been shown an increased expression of AM and its receptor components in rat aorta and ventricle during hypertension possibly due to a compensatory response to the cardiovascular pathophysiological process (Gibbons et al., 2007; Zhao et al., 2006; Pan et al., 2005).

AM immunoreactivity, AM binding sites and CRLR, RAMP2 and RAMP3 are expressed in rat cerebellum (Serrano et al., 2000; Chakravarty et al., 2000; Sone et al., 1997; Uezono et al., 2001), however little is known about their functional role in this structure. The fact that AM binding sites are increased in SHR rats' cerebellum when compared with WKY control (Pastorello et al., 2007) suggest a possible role for cerebellum AM in the blood pressure regulation. Thus, we were prompted to assess the expression of AM and its receptor components in the cerebellar vermis in Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR) of 8 and 16 week old. In addition, the effect on blood pressure of AM microinjection into the cerebellar vermis *in vivo* was determined.

2. Materials and methods

2.1. AM and its receptor components expression

Male, 8 and 16 week old WKY and SHR rats, provided from Venezuelan Institute of Scientific Research (IVIC) (Caracas, Venezuela), were maintained in single cages under controlled conditions of temperature and photoperiod (lights on 06.00 to 18.00 h) and provided with free access to tap water and standard laboratory chow.

Cerebellar CRLR, RAMP1, RAMP2, RAMP3 and AM expression was performed by Western blot analysis by a modification of the method described by Urrecheaga et al. (2007). Rats were killed by decapitation, the skull was rapidly removed, and brain extracted and immediately placed on ice-cold plate. Cerebellar vermis was dissected, homogenized in ice-cold lysis buffer for receptor expression assessment (Tris–HCl 200 mM, CHAPS 160 mM, Na_2EDTA 10 mM, PMSF 5 mM and protease inhibitors cocktail [pepstatin A 5 μM, aprotinin 10 μg/mL, leupeptin 10 μM pH 7.5]) or in ice-cold lysis buffer for AM expression evaluation (NaCl 50 mM, Tris–HCl 25 mM, Triton X-100 1%, PMSF 1 mM and protease inhibitors cocktail [pepstatin A 5 μM, aprotinin 10 μg/mL, leupeptin 10 μM, pH 8.1]) and centrifuged at 10.000 rpm for 10 min at 4 °C. The supernatant was collected and protein concentration was determined by Lowry et al. (1951) using bovine serum albumin as standard.

For Western blot analysis, samples of rat cerebellar vermis were subjected to 10% SDS-polyacrylamide gel electrophoresis under denaturizing and reducing conditions and transferred to a nitrocellulose membrane. Membranes were incubated for 1 h in blocking buffer (TBS-Tween 0.1% + 5% non-fat dried milk) at room temperature. Next, membranes were incubated overnight at 4 °C with the following rabbit primary antibodies: anti-CRLR, anti-RAMP1, anti-RAMP2, anti-RAMP3 and or anti-AM (Santa Cruz, Biotechnology, Inc.), diluted in 3% bovine serum albumin solution in TBS-Tween 0.1% (1:1000). Then, membranes were incubated with secondary antibody anti-rabbit IgG conjugated to horseradish peroxidase (Cell Signaling Technology), for 1 h at room temperature. After each incubation, membranes were washed 10 min 3 times with TBS-Tween 1×. The immunostaining was visualized by a chemiluminescent detection system (SuperSignal® West Pico Chemiluminescent Substrate) and densitometry analysis was performed using Quantity One 1-D®-BioRad software. CRLR, RAMP1, RAMP2, RAMP3 and AM expression were normalized with β-actin expression.

2.2. Intracerebellar cannulation

Sixteen-week-old SHR and normotensive WKY male rats were maintained in single cages under controlled conditions of temperature and photoperiod (lights on 06.00 to 18.00 h) and provided with free access to tap water and standard laboratory chow. With the aid of a stereotaxic instrument and under sodium pentobarbitone anesthesia (40 mg/kg, intraperitoneally), a cannula was implanted into the cerebellar vermis with the following stereotaxic coordinates: anteroposterior (AP) = −10.3, lateral (L) = 0 and ventral (V) = 2.4 according to Paxinos and Watson (1986) and Sacchetti et al. (2002). The cannula was secured to the skull with acrylic cement. A minimum of 3 days was allowed for recovery. Single intracerebellar injection was made with a Hamilton syringe fitted with a stop to prevent needle penetration past the cannula tip. At 09:00 h, half of the rats received AM (0.02 to 200 pmol/5 μL) or vehicle (5 μL) into the cerebellar vermis. To establish the receptor subtype involved in the hypotensive action of intravermis microinjection of AM in SHR rats, animals received microinjections of AM_{22-52} (200 pmol/5 μL), an AM1 receptor antagonist, or the CGRP1 receptor antagonist, $CGRP_{8-37}$ (200 pmol/5 μL) into the cerebellar vermis, administered simultaneously with AM or vehicle microinjection. To determine whether the effect of AM was site specific, animals received injections of AM (200 pmol/5 μL) or vehicle (5 μL) outside the cerebellar vermis. And to determine whether the effect of AM was specific, animals received injections of angiotensin II (ANG II) (200 pmol/5 μL) or vehicle (5 μL) into cerebellar vermis. Cannula placement was confirmed postmortem by examining the distribution of an intravermis injection of 5 μL of fast green dye, given before animal sacrifice. Data were used only if the dye was distributed in the cerebellar vermis.

2.3. Measurement of cardiovascular responses

Blood pressure (BP) was measured by the tail-cuff method according to Israel et al. (2000). Systolic and diastolic pressure (SP, DP) and heart rate were recorded daily using a tail-cuff digital plethysmograph (Digital Pressure Meter LE 5002 LETICA®, Panlab, S.L. Barcelona-Spain). To minimize stress, rats were trained daily with the plethysmograph one week prior to the experiment. Cardiovascular parameters were measured daily at the same time of the day during the training and experimental periods. Plethysmographic pressure values were validated in early experiments with direct measurements with intra-arterial catheter recordings (data not shown). Mean arterial pressure (MAP) was calculated as follows: MAP = DP + 1/3(SP–DP), where DP: diastolic pressure and SP: systolic pressure.

The procedures used in these experiments were reviewed and approved by the Animal Care and Use Committee of The Central University of Venezuela, School of Pharmacy, Caracas, Venezuela. All experiments were done according to good practice of laboratory animal management (NIH Guide, 1996).

2.4. Statistics

All data are presented as means ± S.E.M. Statistical differences between groups were analyzed using Kruskall–Wallis and U Mann–Whitney analysis. A value of $p < 0.05$ was considered statistically significant. Data analysis and graphs were performed using Graph Pad Prism Program, 5.1 version.