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Vasodilatory effects of homologous adrenomedullin 2 and adrenomedullin 5 on isolated blood vessels of two species of eel

Melissa S. Cameron [a,*], Shigenori Nobata [b], Yoshio Takei [b], John A. Donald [a]

[a] School of Life and Environmental Sciences, Deakin University, Geelong, Victoria 3216, Australia
[b] Atmosphere and Ocean Research Institute, University of Tokyo, Kashiwa, Chiba 277-8564, Japan

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ABSTRACT

In mammals, adrenomedullin (AM) is a potent vasodilator through signalling pathways that involve the endothelium. In teleost fishes, a family of five AMs are present (AM1/4, AM2/3 and AM5) with four homologous AMs (AM1, AM2/3 and AM5) recently cloned from the Japanese eel, *Anguilla japonica*. Both AM2 and AM5 have been shown to be strong *in vivo* vasodepressors in eel, but the mechanism of action of homologous AMs on isolated blood vessels has not been examined in teleost fish. In this study, both eel AM2 and AM5 caused a marked vasodilation of the dorsal aorta. However, only AM5 consistently dilated the small gonadal artery in contrast to AM2 that had no effect in most preparations. Neither AM2 nor AM5 had any effect when applied to the first afferent branchial artery; in contrast, eel ANP always caused a large vasodilation of the branchial artery. In the dorsal aorta, indomethacin significantly reduced the AM2 vasodilation, but had no effect on the AM5 vasodilation. In contrast, removal of the endothelium significantly enhanced the AM5 vasodilation only. In the gonadal artery, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) significantly reduced the AM5 vasodilation suggesting a role for soluble guanylyl cyclase in the dilation, but L-NNA and removal of the endothelium had no effect. The results of this study indicate that AM2 and AM5 have distinct vasodilatory effects that may be due to the peptides signalling via different receptors to regulate vascular tone in eel.

1. Introduction

Mammalian adrenomedullin (AM) was first isolated from human pheochromocytoma cells and was classified as a member of the calcitonin gene-related peptide (CGRP) family (Kitamura et al., 1993). In pufferfish (Ogoshi et al., 2003) and medaka (Ogoshi et al., 2006), comparative genomic analyses have shown that AM is highly diversified and consists of five paralogous AMs termed AM1 to 5; multiple AMs have been found in other teleost species including zebrafish and trout (Takei et al., 2004a). In medaka and pufferfish, the five AMs can be divided into three groups, AM1/4, AM2/3 and AM5, based on deduced precursor sequences, with AM1 considered the orthologue of the original mammalian AM (Ogoshi et al., 2006; Takei et al., 2010). In Japanese eel, *Anguilla japonica*, four AMs (AM1, AM2, AM3 and AM5) have been cloned and sequenced (Nobata et al., 2008). Following the discovery of five AMs in teleost fishes, genomic analysis revealed that AM2 (also known as intermedin) and AM5 are also found in mammals, reptiles and amphibians (AM2: Roh et al., 2004; Takei et al., 2004a,b; AM5: Takei et al., 2008, 2010, 2013).

It is known that members of the CGRP family, and more specifically mammalian AM, bind to calcitonin receptor-like receptors (CLRs; McLatchie et al., 1998). The CLRs on their own offer little binding affinity for mammalian AM and must associate with one of three receptor activity-modifying proteins (RAMPs) to become functional. In mammals, it has been shown that AM binds to a CLR in association with RAMP2 (called the AM_1 receptor) or RAMP3 (called the AM_2 receptor). A recent study has shown that mammalian AM2 demonstrates binding affinity to both AM_1 and AM_2 receptors, but preferentially activates the AM_2 receptor (Hong et al., 2011). In addition, mammalian AM2 has also been shown to interact with the CGRP receptor (CLR/RAMP1) but with much less affinity (Kuwasako et al., 2011). As the discovery of AM5 in mammals is relatively new, the receptor(s) are as yet unknown, but it has been suggested that mammalian AM5 may act through a receptor other than a CLR/RAMP complex (Takei et al., 2008, 2010, 2013; Rademaker et al., 2012). In teleost fish, AM receptors have been characterised in pufferfish, *Takifugu obscurus*. Three CLRs and five RAMPs were cloned and it was found that AM2 and AM5 could bind to the CLR1/RAMP3 combination or AM_2 receptor (Nag et al., 2006).

It has been well documented that mammalian AM is a key regulator of the cardiovascular system of mammals by mediating hypotension via central and peripheral mechanisms (Brain and Grant, 2004). In addition, both AM2 and AM5 have vasodepressor effects, but these are not as potent as AM (Fujisawa et al., 2004; Pan et al., 2005; Takei et al., 2010). AM is a dilator of isolated blood vessels but the signalling systems involved in vasodilation are variable and dependent on the type of blood vessel

* Corresponding author. Tel.: +61 3 5227 3449; fax: +61 3 5227 1040.
E-mail address: msca@deakin.edu.au (M.S. Cameron).

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(Brain and Grant, 2004). A considerable number of studies have provided evidence that AM acts indirectly via the calcium-dependent activation of endothelial nitric oxide synthase (NOS3) in endothelial cells (Hayakawa et al., 1999; Ross and Yallampalli, 2006) and the subsequent release of NO, as AM vasodilation is attenuated by inhibitors of soluble GC (sGC) and NOS. In contrast, other studies have shown that AM mediates endothelium-independent vasodilation involving cAMP and K channels (Roh et al., 2004; Bayram et al., 2010).

Previously, mammalian AM was found to cause hypotension in trout, *Oncorhynchus mykiss* but not in the cod, *Gadus morhua* (Aota, 1995), and a weak dilation of the trout coeliac artery (Aota, 1995). In *A. japonica*, injection of homologous AM1 (AjAM1), AM2 (AjAM2) and AM5 (AjAM5) *in vivo* caused varying degrees of hypotension of both the branchial and systemic circulations (Nobata et al., 2008), with the vasodepressor effects of both AjAM2 and AjAM5 being significantly more potent than that of AjAM1. However, the effects of homologous AMs on isolated blood vessels from the systemic and branchial vasculature of teleost fish have not been determined. Therefore, the aim of this study was to assess the effects of AjAM2 and AjAM5 on eel blood vessels to determine if the endothelium, sGC or prostaglandins are involved in their vascular effects. AM1 was not examined because of its weak hypotensive effect in *A. japonica* (Nobata et al., 2008).

2. Materials and methods

2.1. Animals

2.1.1. Australian eels, Anguilla australis

Australian short-finned eels, *A. australis*, of both sex and a body mass of 736 ± 53 g, were purchased from Western Victorian Eel Growers Group (Skipton, Australia). *A. australis* were maintained in re-circulating tanks of aerated, filtered fresh water (100 L) held at 19–21 °C. The tanks were cleaned weekly and animals were not fed during captivity (up to 2 weeks). All experiments on *A. australis* were approved by the Deakin University Animal Welfare Committee (approval A32/2008).

2.1.2. Japanese eels, A. japonica

Japanese eels, *A. japonica*, of both sex and a mass of 173 ± 15 g, were obtained from a local supplier and maintained at the Atmosphere and Ocean Research Institute at the University of Tokyo. *A. japonica* were maintained in re-circulating tanks of aerated, filtered fresh water (50 L) held at 18 °C. All experiments on *A. japonica* were approved by the Animal Experiment Committee of the University of Tokyo.

2.2. Molecular biology

Total RNA was isolated from the heart, kidney and gill of *A. australis* using TRI-Reagent (Sigma) with a Polytron homogeniser. The RNA concentration and purity were determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Three micrograms of RNA was reverse-transcribed into first strand cDNA using oligo$(dT)_{15-18}$ and SuperScript™ II Reverse Transcriptase (Life Technologies) according to manufacturer's instructions. Kidney and gill were chosen to amplify AM2 and AM5, and the heart was used for ANP based on the tissue distribution of each mRNA species in the Japanese eel (Nobata et al., 2008, 2010). Primer design for AM2 and AM5 was based on the alignment of the respective sequences from *A. japonica* and the European eel *A. anguilla*. Analysis of the *A. anguilla* genome shows that AM2 and AM5 of *A. anguilla* are identical to *A. japonica*. Given that the mitochondrial genome analysis of the *Anguilla* genus places *A. anguilla* in the same clade as *A. australis* and that *A. japonica* is in a separate clade (Minegishi et al., 2005), then it is reasonable to assume that primers designed for *A. australis* could be used to amplify AM2 and AM5 from *A. australis*. Amplification of AM2 was performed using 3′ Rapid Amplification of cDNA Ends (RACE) as a suitable gene-specific primer pair could not be identified. 3′ RACE was performed using a forward primer (Table 1) designed on the AM2 sequence from *A. japonica* and *A. anguilla* and was carried out using the 3′-Full RACE Core Set (Takara Biotechnology), according to the manufacturer's instructions. Forward and reverse primers were designed based on sequence similarity between *A. japonica* and *A. anguilla* to amplify AM5 from *A. australis* (Table 1). The ANP sequence of *A. australis* has previously been sequenced (Healy and Toop, unpublished); therefore the primers used in this study were used to confirm the sequence, which was found to be identical between *A. japonica* and *A. australis* (Healy and Toop, unpublished). After an initial denaturation at 94 °C for 2 min, 36 cycles of PCR was performed consisting of denaturation of 94 °C for 45 s, annealing at 56 °C for 30 s, and extension at 72 °C for 45 s, and a final extension was performed at 72 °C for 2 min. PCR products were resolved on 1.5% TBE agarose gels and viewed under UV light after ethidium bromide staining. Products of interest were excised and purified using a Wizard SV Gel and PCR Clean-up kit (Promega). Sequencing was performed using BigDye™ Terminator v3.1 kit (Life Technologies) and a 3130 Genetic Analyser (Applied Biosystems) at Deakin University. Sequences obtained were translated into amino acids using GeneRunner v3.05. Sequences were analysed using NCBI BLAST and aligned using ClustalW.

2.3. Homologous A. japonica peptides

The predicted mature peptides of *A. japonica* AM2 (AjAM2), AM5 (AjAM5), and ANP (AjANP) were chemically synthesised by the Peptide Institute (Osaka, Japan), as described previously (AMs Nobata et al., 2008; ANP Nobata et al., 2010). *A. japonica* AMs were used at a concentration of 10^{-8} M which is in excess of the concentration of 6.5×10^{-9} M used by Nobata et al. (2008) to achieve the profound *in vivo* hypotension in eel. Furthermore, a preliminary dose response analysis performed with AjAM5 on the gonadal artery found that concentrations less than 10^{-8} M had only a small effect on the vessel and that the majority of the dilation occurred at a concentration of 10^{-8} M.

2.4. Dual-wire myography

A. australis was anaesthetised with 0.1% benzocaine buffered with sodium bicarbonate, and *A. japonica* was anaesthetised in tricaine methane sulphonate (1:1000) due to anaesthetic availability in Japan. In our experience, neither anaesthetic affects the vascular responses of blood vessels in fish. For both species, the gonadal artery (300–400 μm in diameter) and the first afferent branchial artery (300–400 μm in diameter) were dissected free and placed in HEPES-buffered physiological saline (138 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2 \cdot 6H_2O$, 5.6 mM glucose, 10 mM Hepes, 7 mM NaOH, and 2.5 mM $CaCl_2$, pH 7.4), which was maintained at 19–21 °C. Dual-wire myography was not performed on the dorsal aorta due to the large size of the vessel; this vessel was studied with organ bath physiology (see below). The vessels were cut into individual rings of approximately 2 mm in length and were mounted horizontally between two pieces of 40 μm wire attached to separate jaws of a dual-wire myograph (Model 410A, Danish Myo Technology). The vessel rings were bathed in 5 ml of physiological saline and were maintained at 19–21 °C and bubbled with room air. Tension was placed on the arteries by increasing the distance between the internal wires until they were flush against the vessel wall; they were then left to equilibrate for at least 30 min. The myograph was linked to a Myo-Interface system, which was attached to a PowerLab (ADInstruments

Table 1
List of primers used.

AM2 forward (3′RACE)	5′-AGGGTGAGACCTTCATCGC-3′
AM5 forward	5′-ACAGCACAGAGCCCAGAGTC-3′
AM5 reverse	5′-GAGGAAGTGAGGGTCAAATGA-3′
ANP forward	5′-CATGAGGAAGATCATTCTCACC-3′
ANP reverse	5′-TCCGTAGCAAACCACACCAG-3′