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Adrenomedullin: a new target for the design of small molecule modulators with promising pharmacological activities

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Abstract

Adrenomedullin (AM) is a 52-amino acid peptide with a pluripotential activity. AM is expressed in many tissues throughout the body, and plays a critical role in several diseases such as cancer, diabetes, cardiovascular and renal disorders, among others. While AM is a protective agent against cardiovascular disorders, it behaves as a stimulating factor in other pathologies such as cancer and diabetes. Therefore, AM is a new and promising target for the development of molecules which, through their ability to regulate AM levels, could be used in the treatment of these pathologies.

Keywords: Adrenomedullin; Cancer; Diabetes; Hypertension; Positive and negative modulators

1. Introduction

Adrenomedullin (AM) is a 52-amino acid peptide identified in 1993 and isolated from a human pheochromocytoma (adrenal tumor) [1]. The authors also reported in this initial paper the first function of AM as a vasodilator, given its relaxing action on blood vessel smooth muscle cells [1]. Although the vascular actions of AM remain the main focus of AM research, in the following years many other functions have been ascribed to AM, such as bronchodilation, neurotransmission, hormone regulation, antimicrobial activity or growth regulation. An extensive review has recently been published which summarizes the state of the art in our collective knowledge on AM science [2].

2. Chemical structure

Human AM is a 52-amino acid peptide with a single disulfide bond between the cysteine residues located in positions 16 and 21 and an amidated tyrosine at the carboxy end. This structure confers some homologies between AM and the calcitonin super family of peptides based on its structural similarity with calcitonin gene-related peptide (CGRP) and amylin. The chemical similitudes among these peptides include the 6-amino acid ring, and the amidated tyrosine at the carboxy terminus. Another related peptide is calcitonin, but its similarity is not as high as the others. In any case, the four molecules can be considered members of the same super family of peptides [3]. More recently, intermedin has been identified as a new member of the calcitonin/CGRP family [4,5].

The amino acid sequence for AM is known for different vertebrate species (Table 1). Porcine, bovine and canine AM contain 52-amino acids and have only one, four and three substitutions, respectively, when compared with the human peptide. Rat and mouse peptides have only 50 amino acids, with two deletions and six and seven substitutions, respectively. Only partial sequence for the horse peptide is known. All these sequences show a high degree of similarity with human AM. Recently, the presence of five peptides of the AM family (TrAM-1–5) has been detected in Takifugu and Zebrafish and their amino acid sequences have been published [6] (TrAM-1 in Table 1).

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Table 1
Comparison of amino acid sequences of human AM with homologue peptides from other species. Amino acids with one letter symbol. The dashes (–) symbolize missing amino acids; bold symbols (**X**) represent amino acid substitutions. Sequence for equine AM is incomplete and only the aminoend is known

Species	Peptide sequence
Human	H_2N-YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY-$CONH_2$
Porcine	H_2N-YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKD**G**VAPRSKISPQGY-$CONH_2$
Bovine	H_2N-YRQS**L**NNFQGLRSFGCRFGTCTVQKLAHQIY**H**FTDKDKD**GS**APRSKISPQGY-$CONH_2$
Rat	H_2N-YRQSMN--QG**S**RS**T**GCRFGTCT**M**QKLAHQIYQFTDKDKD**GM**APR**N**KISPQGYCONH_2
Mouse	H_2N-YRQSMN--QG**S**RS**N**GCRFGTCT**F**QKLAHQIYQ**L**TDKDKD**GM**APR**N**KISPQGY-$CONH_2$
Dog	H_2N-YRQSMNNFQG**P**RSFGCRFGTCTVQKLAHQIYQFTD**N**DKD**G**VAPRSKISPQGY-$CONH_2$
Horse	H_2N-YRQSMNNFQGLRSFGCRFGTCTVQKLAH(...)-$CONH_2$
Zebrafish	H_2N----S**K**N**S**I**N**Q**S**R**RS**GC**SL**GTCT**VHD**LAH**RLHDLNNKLKIGN**AP**V**DKI**NP**Y**GY-$CONH_2$
Takifugu	H_2N-**TKRS**K**N**L**V**N**Q**S**R**K**N**GC**SL**GTCTV**HD**LA**FRLH**Q**LGFQY**K**IDI**AP**V**D**KISPQGY-$CONH_2$

A preliminary model of AM structure is shown in Fig. 1. This model was obtained using the available sequence information [1] and several servers such as PSI-BLAST, FASTA and JPRED [7–9]. The resulting model was later submitted to a 3 ns molecular dynamics simulation with explicit inclusion of water as a solvent and using the amber suite of programs [10].

3. Gene structure and regulation of gene expression

In the same year of the discovery of AM, the complementary DNA (cDNA) sequence for the human gene was published [11], followed by the cDNA sequence for the rat [12]. The structure of the complete gene is available for two species, human [13] and mouse [14]. Both genes contain four exons with three short introns among them, and have been mapped to single loci in chromosome 11 in humans and in chromosome 7 of the mouse. There are TATA, CAAT, and GC boxes in the 5′-flanking region of the gene, together with numerous binding sites for regulatory proteins and transcription factors. The boundaries between introns and exons coincide with the characteristic motifs described for mammals, although some mismatches in the sequences of the third intron have important consequences in the alternative splicing of the messenger RNA (mRNA) [15]. The complete sequences for the five different genes in Takifugu have been also reported [6].

AM mRNA can be detected by northern analysis and identified as a band of 1.6 kb. This mRNA encodes for a long precursor molecule, termed preproadrenomedullin. In both rat and human this precursor consist of 185 amino acids [11,12], while the porcine precursor has 188 residues [16]. The amino end of preproadrenomedullin contains 21 amino acids that function as a signal peptide, targeting the nascent polypeptide to the rough endoplasmic reticulum. As soon as the precursor penetrates in the lumen of the endoplasmic reticulum, the signal peptide is cleaved off, resulting in a 164-amino acid-long molecule termed proadrenomedullin. This prohormone is further modified while traveling through the endoplasmic reticulum, the Golgi complex, and the secretory granules by the sequential action of endopeptidases, exopeptidases, and finally the amidating enzymes (Fig. 2). This post-translational process generates two biologically active amidated peptides, the 52-amino acid peptide AM, which is situated toward the carboxy end of the precursor molecule, and proadrenomedullin N-terminal 20 peptide (PAMP), consisting of the 20 amino acids situated in the amino terminus of proadrenomedullin. The coding sequence for PAMP is split between exons 2 and 3, whereas the AM sequence is contained in the fourth exon. A third peptide, encompassing amino acid 153–185 of preproadrenomedullin, may have some functional attributes [17], but these results still need confirmation.

As both peptides AM and PAMP are originated from the same precursor molecule, we could expect equimolecular amounts of these peptides in the tissues and in the body fluids, but this never happened. In fact, there are different values for AM and PAMP obtained from cell and tissue extracts by radioinmunoassay and wild variations in the PAMP/AM ratio

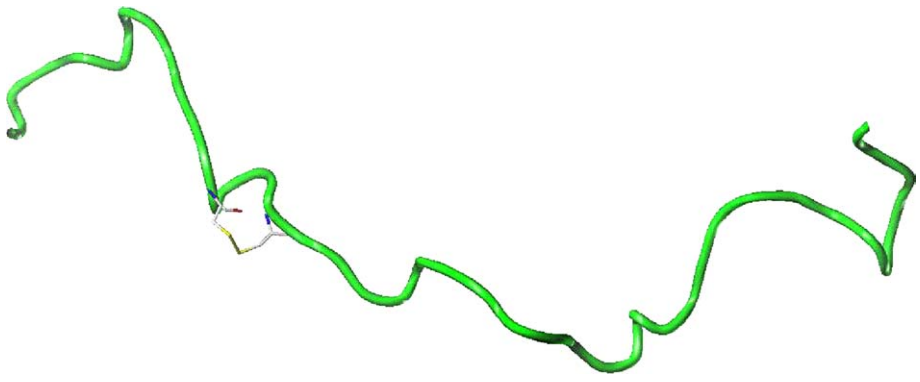

Fig. 1. Schematic representation of AM structure. The amidated carboxy-end is located at the right hand side of the figure. The disulfide bond is also depicted.

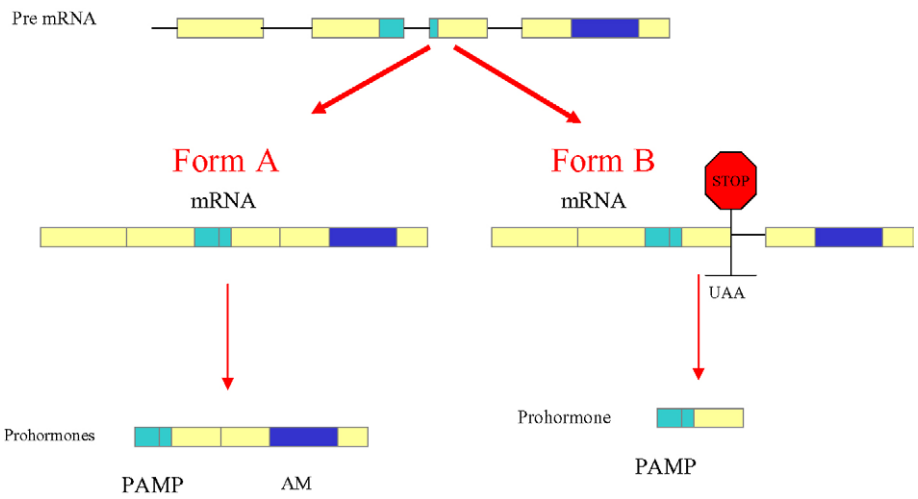

Fig. 2. Schematic drawing representing the genomic structure of the AM gene and the alternative splicing mechanism governing the differential expression of AM and PAMP. Removal of the three introns yields form A mRNA, which carries information for both peptides (left hand side). Retention of the third intron results in the introduction of a premature stop codon that prevents AM transcription. Reprinted with permission from Martínez et al. [15].

observed in many organs and tissues. These facts remained unexplained for several years, until Martínez et al. [15] proposed the existence of an alternative splicing mechanism of the AM gene. Depending on the cell type, during the maturation process of the pre-mRNA, some molecules are only partially spliced and retain their third intron, thus resulting in a longer cytoplasmatic mRNA species, named form B. When this longer message gets translated, a stop codon inside the third intron ends translation prematurely, generating a shorter preprohormone. Because the complete reading frame for AM is located in the fourth exon, the shorter preprohormone contains PAMP but no AM (Fig. 2). Different treatments that modify expression can modulate the amounts of AM mRNA that matures through one pathway or the other, therefore influencing the ratio of secreted PAMP/AM from the cell [15].

4. AM receptors and signal transduction pathways

Within the past years four putative receptors for AM have been reported: calcitonin receptor-like receptor (CL) [18], L1 [19,20], RDC-1 [21] and hrhAM [22,23]. However, the lack of experimental consistency for both AM-binding and functional response has disqualified some of these molecules as AM receptors [24]. It has now become clear that only the combination between CL and a receptor activity modifying protein (RAMP) results in true AM receptor [25,26]. CL was cloned in 1993 [27] and belongs to the seven transmembrane domain G-protein-coupled receptor (GPCR) family of receptors. It can bind two members of the CGRP family of peptides; AM, and CGRP [28–30]. Ligand specificity is controlled by a unique mechanism consisting in the specific interaction of different RAMPs to CL [31].

Three human RAMPs (RAMP1–3) have been cloned. They trigger different pharmacologies in combination with CL through the following binding pattern: CL + RAMP1 results in CGRP1 pharmacology, whereas the combination CL + RAMP2/RAMP3 results in an AM receptor [32,33].

CL interacts with heterotrimeric Gs protein and after binding to AM activates the adenylyl cyclase-PKA pathway resulting in elevation of intracellular cAMP [34,35]. Elevation of cAMP was the end point used to demonstrate AM activity in the seminal paper by Kitamura et al. [1] and since then a number of studies have demonstrated cAMP mediated actions after stimulation with AM [36,37]. However, several papers have demonstrated cAMP-independent actions for AM as well [38,39]. AM produces vasodilatation via elevation of Ca^{2+} and activation of endothelial nitric oxide (NO) synthase [40,41]. Furthermore, AM inhibits mitogen-activated protein kinase (MAPK) [42,43] and stimulates MAPK phosphatase [44] in a set of cell types. In other cell lines the situation is reversed and AM stimulates MAPK activity [38]. This stimulation of MAPK could be related to the mitogenic ability of AM [45]. A possible role of K+-ATP channels has been proposed in AM-mediated vasodilatation [46].

5. Distribution of AM in healthy organs and tissues

AM is found as a circulating peptide with a normal concentration in humans that varies between 1 and 10 pM, with most values ranging from 2 to 3.5 pM [36,47]. AM has been found in urine, in which the reported concentrations are much higher than in plasma and are independent of circulating levels [48,49]. AM is also present in the saliva [36], amniotic fluid [50,51], cerebrospinal fluid [52,53], sweat [54], and milk [55,56]. The presence of fully processed AM in these locations may be related to the antimicrobial activity of AM [57], which would protect these fluids and the surfaces they impregnate from bacterial colonization. AM is also expressed by almost every organ in the body, reflecting its many roles as a modulator of diverse physiological activities. Other consequence is the need of a tight regulatory system to control AM expression in each particular case.

In the next paragraphs, we will try to summarize the most important aspects of this widely distributed expression pattern.

The heart was one of the organs where AM expression was found, both at the mRNA and protein levels in rat and humans [11,12,58–63]. AM secretion by cardiomyocytes could be stimulated by either volume or pressure overload [64,65], mechanical stress [66,67], and hypoxia [68–70]. Expression of the putative AM receptors L1, RDC-1, and CL has been found in cardiac myocytes of both neonatal and adult rat heart [71], as well as the RAMP2-CL complex in human heart [65,72]. AM is also present in other components of the cardiovascular system, such as endothelial cells and vascular smooth muscle cells from the aorta and other blood vessels of different organs and tissues [60,73–82].

The presence of AM in the central nervous system was soon described by several authors [58,83–85]. Higher levels of the peptide were found in the thalamus and hypothalamus, with lower concentrations in cortex, medulla, pons, and cerebellum [86–90]. Neurons inmunoreactive for AM were identified in the supraoptic nucleus and in the magnocellular pars of the paraventricular nucleus [85,91–93]. A very detailed map of AM distribution in the rat brain was provided by Rodrigo's group using both light and electron microscopy immunocytochemical methods [94,95].

AM is also present in sensory organs. Expression of AM has been studied in the eye. AM was found in the outer neuroblastic layer of the developing retina [96] in the iris ciliary body [65,97,98], and in the retinal pigment epithelial cells [99,100]. AM was also found in the cochlear epithelium of mouse embryos, but there is no confirmation of this observation in the adult ear [96,101]. In addition, AM was also found in the carotid bodies of the rat [102].

Since AM was discovered in a tumor derived from the adrenal medulla [1], this peptide has been found in a number of locations of the endocrine system. AM is located in the chromaffin cells [59,103,104] and was found in zona glomerulosa and fasciculata of the adrenal cortex [61,105,106]. Interestingly, AM is not expressed by the vascular endothelium of the adrenal gland.

There are several studies reporting the presence of AM and PAMP in the pituitary [58,61,107–110]. AM concentration in pituitary extracts was higher than in the brain [65,91,111]. Immunocytochemical studies in several mammalian species, including humans, have shown a widespread expression of AM in the adenohypophysis and the neural lobe, whereas the intermediate lobe showed a lower amount [79,112].

In the endocrine pancreas, the existence of AM was shown in the islets of Langerhans [50,61,79]. Studies by RIA methods detected very low levels of AM in the submandibular glands, small intestine, colon, and liver [58,61,107,113,114]. Using immunohistochemical techniques, AM has been detected in different gastrointestinal glands and epithelia [79,115], and was specifically identified in the chief cells of the rat gastric mucosa [116]. AM mRNA expression has been reported in some non-endocrine epithelial gastric cells [117]. In the liver, AM immunostaining has been described in the biliary ductal epithelia and in the mucosal epithelium of the gallbladder, but not in hepatocytes [79].

AM immunoreactive cells have been found in the digestive diffuse endocrine system. Some studies reported a few of them in the gastric glands of the rat stomach, colocalizing with a subpopulation of serotonin-containing cells, and also in the pyloric glands, colocalizing with gastrin [118]. AM positive cells with endocrine morphology have also been described in small and large intestine [79,119–122].

Several studies found elevated amounts of AM in the kidneys. This high levels of peptide pointed to the kidney as an important source of AM [1,12,58,60,63,123–126]. Several authors have reported the distribution of AM immunoreactivity in renal structures [59,114,127,128].

Several studies revealed the presence of AM in the lung [11,12,16,59,61,63,81,107]. Detailed immunohistochemical studies have shown expression in different lung structures such as vascular and bronchial smooth muscle cells, the apical region of columnar epithelial cells of bronchi and bronchioli, endothelial cells, some glands, neurons of the intrinsic parasympathetic nervous system, chondrocytes, and alveolar macrophages [70,79,114,129].

AM peptide, AM-mRNA, and AM receptor mRNA have been localized in the normal female reproductive tract throughout all structures of the system with marked expression in the epithelial cells of the uterus, Fallopian tubes, and blood vessels [130]. In the ovary, many cell types show AM staining, including the granulose and thecal cells, cells of the corpus luteus, and the germinal epithelium [131,132]. AM expression has also been found in human and rat placenta [51,96,101,133–137].

AM is also present in the mammary glands, particularly in the epithelia of small and large ducts and in the terminal end buds of glands [55,79]. AM immunoreactivity has also been found in the milk present in the ducts, suggesting a secretion of the peptide into the milk [55,56].

AM and AM receptors are also present in the male reproductive system [61,79,101,107,138–140]. The highest AM levels in the male reproductive tract occurs in the prostate gland, and its expression is induced by androgens [79,141,142].

AM and AM-binding sites have been found in the skin. They have been described in epithelial cells of mammalian skin, including keratocytes, hair follicles and sebaceous and sweat glands [54,79,143]. The presence of AM in these locations may be related to the antimicrobial role played by AM and PAMP [57,144].

AM has been found in the spleen [12] and in the connective tissue [82]. AM also seems to be produced by mast cells [129,145,146] and by components of the blood, such as granulocytes, lymphocytes, and circulating monocytes [147]. Adult osteoblasts also produce AM protein and possess AM receptors, with CL and RAMPs having been detected [32,148–150].

6. Functions

AM has a remarkable range of actions which have been extensively reviewed [2,36]. This peptide can act as a vasodi-

lator [151], a bronchodilator [152], a regulator of hormone secretion [145], a neurotransmitter [84], an antimicrobial agent [57], and a controller of renal function [128]. In addition, several reports implicate AM in different aspects of tumor biology [153,154].

6.1. Cardiovascular actions

AM and PAMP increase heart rate and coronary blood flow [155–158]. Increases in heart rate by AM could be considered a direct effect of the vascular activity of the peptide in peripheral blood vessels. Heart rate would increase to compensate for peripheral vasodilatation and to maintain blood flow. Intracerebroventricular administration of AM or PAMP causes an increase in heart rate associated with vasoconstriction [126,159–162]. Addition of AM to cardiomyocytes resulted in a reduction in the expression levels of atrial natriuretic factor (ANF) [163,164].

The impact of AM and PAMP in peripheral vasculature elicits a well-established potent hypotensive action when applied intravenously [1,11,165]. The vasorelaxant activity of AM and PAMP reduces peripheral vascular resistance by inhibiting vascular smooth muscle contraction, therefore increasing blood flow.

AM-mediated vasorelaxation has also been reported in veins [166,167]. The vasodilatory effect of AM has also been found in microlymphatic vessels [168].

6.2. Bronchodilation and pulmonary actions

AM causes pulmonary vasodilatation and increases blood flow on the pulmonary vascular bed [169–173]. AM is also a mediator in the inhibition of pulmonary vascular remodeling [174]. The pulmonary vascular bed seems to play an important role in relation to AM metabolism, constituting the main site of AM clearance [80,175–177].

Another important function of AM on the airways is its capability to produce a long lasting bronchodilatation [178,179]. AM also induces the secretion of phosphatidylcholine, the main component of pulmonary surfactant, from type II pneumocytes, thus modulating surfactant formation [180]. The presence of AM and PAMP in the bronchial lumen may be related to the antimicrobial activity of these peptides, which would be part of the passive defense system against pathogenic invasions [144,181].

6.3. Electrolyte balance

There are many organs involved in the control of fluid and electrolyte homeostasis including the central nervous system, kidneys, pituitary and adrenal glands, in a very complex system of connected processes [182]. The presence of AM and PAMP in all these locations suggests that these peptides could have an important role in electrolyte balance. In addition, there is a close relationship between whole body fluid volume and blood pressure, and AM is able to regulate it as discussed above.

There are a number of studies that confirm this idea. For instance, when AM is directly administered into the central nervous system, it has an antidipsogenic effect, that is, it inhibits water drinking and therefore leads to a drop in total fluid content [65,90,183,184]. AM is also able to regulate salt intake and secretion in experimental animals [182].

6.4. Neurotransmission

AM and its receptors exist in the central nervous system and its cellular components. Focal brain ischemia is the most common event leading to stroke in humans and a role for AM in this condition has been suggested. The effects of intracerebroventricular injection of AM on cardiovascular regulation, electrolyte balance, and bronchodilatation clearly show the neurotransmitter or neuromodulator character of this regulatory peptide. In addition, intracerebral administration of AM acts directly on paraventricular and supraoptic nuclei [162,185–187] resulting in an elevation of oxytocin an arginine-vasopressin in plasma and decrease in food ingestion [188].

The experimental demonstration that AM is able to alter neuronal excitation patterns definitively established the idea that AM is a neurotransmitter [84,189]. In addition, increasing renal sympathetic nerve activity by AM via the central nervous system has been demonstrated [88,155,190,191] as has AM inhibition of adrenergic neuronal transmission [190,192].

6.5. Renal actions

Circulating AM can affect renal function, and evidence exists for a role for locally produced AM in tubular function. AM induces renal vasodilatation, influencing blood flow and glomerular filtration rate, thus resulting in a concomitant enhancement of diuresis, natriuresis, and kaliuresis [40,123,193–198].

AM can also regulate blood pressure through the renninangiotensin system. AM increases renin secretion by stimulating juxtaglomerular cells [155,199–201].

In mesangial cells AM inhibits proliferation and migration, and induces apoptosis [42,202–208]. It also stimulates hyaluronic acid release from mesangial cells to the extracellular matrix, indicating that AM may play a role in inducing differentiation in these cells [209].

6.6. Growth regulation

Both stimulation and inhibition of cell proliferation have been reported upon exposure to synthetic AM. This different behavior may be cell context dependent.

AM stimulates proliferation of the zona glomerulosa cells of the adrenal cortex [109,210,211], in both normal and malignant skin [54], in human oral keratinocytes [212], osteoblast [148–150], C-6 glioma cells [213], Swiss 3T3 fibroblast cell line [214,215], and in many tumor cell lines [145].

The antiproliferative action has been found in rat cardiac fibroblasts [216,217], and in mesangial cells [206,207,218]. AM is also involved in proliferation reduction of vascular smooth muscle cells [174,216,217,219,220], cultured cardiomyocytes [221], and human teratoblastoma cells [222].

6.7. Hormone regulation

One of the most important functions of AM is the ability to regulate the secretion of other hormones.

AM regulates secretion of arginine-vasopressin and oxytocin from paraventricular and supraoptic hypothalamic neurons and moderately increases growth hormone secretion in human and rat pituitary somatotrophs [223]. AM inhibits basal and stimulated ACTH secretion from corticotrope cells [224–227]. A detailed review of AM and the hypothalamus—pituitary—adrenal axis was published by Nussdorfer [109].

The effects of AM on the adrenal gland are mainly associated with regulation of zona glomerulosa function in the cortex of the chromaffin cells of the medulla. The main hormone secreted by the zona glomerulosa is aldosterone, and evidence on whether AM stimulates or inhibits its secretion is contradictory. Both decrease [201,228–232], and increase [104,233–235] have been reported. These contradictions could be explained, at least partially, by the use of different adrenal tissue preparations, which would involve increased or decreased levels of regulation, each adding new layers of complexity [36].

Corticosterone secretion seems to be elevated by AM, possibly by enhancing perfusion of the adrenal gland through AM's vasodilatatory activity [236].

6.8. Endocrine pancreas

The main function of AM in the pancreas is to maintain an inhibitory tone of insulin secretion, as demonstrated in vitro and in vivo [37,237,238]. We have recently found that the AM-binding protein, complement factor H is present in β-cells. Addition of factor H in the presence of AM increased the function of AM, thus decreasing further insulin release [239]. In exocrine pancreas function, AM has been implicated in the inhibition of amylase secretion from pancreatic acini [240].

6.9. Reproductive physiology

The fact that AM is present with high expression in female reproductive tract and the fluctuations of the amount of this peptide along the menstrual cycle point to important roles in female reproductive physiology [61,112,131,241].

Effects of AM on the uterus include vasodilation of local vessels, uterine smooth muscle relaxation, angiogenesis, antiapoptotic actions, and antimicrobial activities [130,131,242,243]. During particular periods of human pregnancy, AM contributes to the maintenance of uterine quiescence through CGRP receptors in myometrial cells [244,245]. AM could be also responsible for the reduction in stretch-induced ANF release observed during pregnancy [164].

The most important action reported for the AM in the male reproductive tract concerns penile erection. Intracavernosal injection of AM induces an increase of penile blood flow and penile erection in normal cart specimens [246,247].

6.10. Antimicrobial activity

AM is present in a number of protective tissues and secretions, suggesting a putative role in the defense against microbial colonization. AM and mRNA are present in epithelial surfaces constituting the external barrier of the organism (skin, airways, genitourinary tract, digestive tube, cornea, etc.) and protective secretions (saliva, sweat, and milk, among others). This role was experimentally demonstrated for AM and PAMP [144], and against a variety of Gram-positive and Gram-negative bacterial strains that are frequent in human skin, digestive tract, and the airways [57]. Interestingly, addition of AM-binding protein, complement factor H, results in a reduction of the antimicrobial activity of AM [248].

6.11. Apoptosis

Some reports implicate AM as a direct apoptosis survival factor. This was first observed in rat endothelial cells, where AM reduces serum deprivation-induced apoptosis via a cAMP-independent mechanism [249]. It was later reported that this action was also cGMP independent [250]. The same group demonstrated that AM abrogates serum deprivation-induced endothelial apoptosis by upregulation of the max gene in an autocrine/paracrine manner [250].

Sata et al. [251] reported that AM and NO reduce endothelial cell apoptosis via a bcl-2-cGMP-independent mechanism.

Interestingly, other laboratories have reported conflicting findings. Oehler et al. have recently reported that stably transfected Ishikawa cells overexpressing AM show resistance to hypoxia induced apoptosis via a bcl-2 mediated mechanism [243].

A recently published report by Rebuffat et al. demonstrates expression of AM and its receptor in the human adrenal gland. The authors have found evidence that AM not only enhances proliferation but decreases apoptosis in primary cultures of zona glomerulosa cells [252].

Martínez el al. have recently shown that AM-overexpressing cells show lower levels of proapoptotic factors such as Bax, Bid and caspase 8, concomitant with higher resistance to apoptosis (after serum deprivation), than cells transfected with the empty vector [253].

6.12. Other AM functions

AM has some actions on the digestive tract. It inhibits activity on gastric secretion in rat stomach [254], modulates water and ion transport and bowel movement in rat distal colon [255]

and elevates cAMP levels in hepatic lypocytes or stellate cells resulting in relaxation of these cells and facilitation of sinusoidal microcirculation [256,257].

In bone, stimulation of osteoblastic activity by AM has been found [148]. AM seems to constitute a growth factor for bone and this knowledge may eventually be useful in the understanding and treatment of osteoporosis [149,150].

AM inhibits angiotensin-II-induced tissue factor and also plasminogen activator inhibitor-1 in vascular endothelial cells. Through this mechanism AM could contribute to the regulation of blood coagulation and fibrinolysis [258].

7. Implication of AM in cardiovascular disorders, cancer and diabetes

There is strong evidence for the implication of adrenomedullin in several diseases, such as cardiovascular and renal disorders, cancer and diabetes.

7.1. Cardiovascular disorders

Elevation of AM levels in plasma has been observed for a variety of cardiovascular disorders, including hypertension [259], congestive heart failure [260] and myocardial infarction [261]. This increment is always accompanied by a fall of blood pressure and a reduction in stroke volume. Usually, these higher levels of AM correlate with elevations in atrial, brain, and C-type natriuretic peptides (ANP, BNP, and CNP) [260], which also have hypotensive properties. The plasma AM level decreases on recovery from the corresponding disorder [261]. All these observations correlate with in vivo experiments carried out in animals, and in healthy volunteers. Thus, it has been shown that an intravenous injection of AM (0.1 nmol/kg) causes a significant fall of blood pressure in normal rats [262]. Similarly, at certain doses (8 ng/kg min, during 90 min) AM provokes a remarkable decrease in blood pressure in healthy volunteers, without altering other processes also related to AM, such as secretion of catecholamines, renin, adrenal hormones and natriuretic peptides, suggesting that the latter effects need higher levels of AM, or are regulated via an endocrine rather than paracrine or autocrine mechanism [263]. All these data suggest that the increase of AM levels, found in pathological disorders affecting the vascular system, is the consequence of a compensatory mechanism to maintain a physiological blood pressure [63].

Administration of AM in rats by osmotic minipump [264], or adenovirus-mediated gene delivery [265,266], has demonstrated that AM has a protective effect against infarction, cardiac remodeling and cardiomyocyte apoptosis induced by acute ischemia/reperfusion.

7.2. Cancer [154]

AM was first identified from extracts of a human adrenal tumor (pheochromocytoma), but soon was localized in a variety of neural and epithelial cancers [1]. Ehlenz et al. [267] found elevated AM levels in plasma of patients with lung and gastrointestinal cancer. In microchip analysis comparisons between lung cancer and normal lung specimens, AM overexpression in cancers is one of the main selected markers [268]. In cancer cells, AM can be considered as a tumor survival factor. It works as a multifunctional regulatory peptide, and exerts its action by regulating several aspects of tumor cell physiology that ultimately promote malignant growth. In fact, AM has been reported to act as a growth factor in tumor cells, to be involved in survival from apoptosis, and in angiogenesis. Additionally, an elevation of AM levels is observed in hypoxic conditions. Hypoxia is a well-established feature of the microenvironment of solid tumors and, in order to survive in this environment, tumor cells alter their transcription levels of a wide range of genes whose protein products serve to stimulate blood vessel growth (pro-angiogenic factors) [269] and other survival strategies.

The proliferative activity of AM has been experimentally demonstrated in vitro [54,56,109,148–150,210–213,270]. When an anti-AM monoclonal antibody was added to a culture of breast cancer cell lines, a marked reduction of proliferation was observed, and this effect was reversed by addition of exogenous AM [145]. These results correlate with in vivo experiments carried out recently in xenograft tumors. Intratumoral administration of the anti-AM antibody resulted in a dramatic reduction in subcutaneous U87 xenograft tumor weight after 21 days of treatment, and the density of blood vessels decreased, supporting the angiogenic activity of AM [271]. In an attempt to further understand the role of AM in tumor growth, AM cDNA was transfected into two representative types of endometrial carcinoma cells (Ishikawa-estrogen receptor (ER) positive- and RL95.2-ER negative-) [272]. The RL95.2 transfectants showed a marked growth increase in vitro, whereas no effect was observed on Ishikawa transfected cells. The transfectants were xenografted into athymic mice to analyze their ability to form tumors in vivo. In agreement with in vitro results, small effect was observed on Ishikawa tumor growth. In contrast, RL95.2 transfectants not only had a marked effect on elevated tumor growth, but also in tumorigenecity, greatly facilitating the tumor take in the mice. In a recently published study, Martínez et al. [253] further characterized the effect of AM overexpression in breast tumor. Two human breast cancer cell lines (T47D and MCF-7) were stably transfected with an expression vector containing the coding region of the human AM gene. Transfected cells expressed higher levels of AM mRNA and protein as compared to the cells transfected with empty vector. AM-overexpressing cells displayed a more pleiotropic morphology, an increased angiogenic potential both in vitro and in vivo, and less apoptosis after serum deprivation. In a preliminary in vivo experiment, three of 10 nude mice injected with AM-overexpressing T47D cells developed xenograft tumors, whereas none of the 10 nude mice injected with cells carrying the empty plasmid developed tumors [253].

The implication of AM in angiogenesis was first demonstrated using the chick chorioallantoic membrane assay [273].

Since then, a large number of experiments have confirmed the angiogenic potential of AM using in vitro and in vivo models [242,253,272,274]. These studies are the starting point for the development of new and more effective antiangiogenic therapies.

7.3. Diabetes

The presence of AM in the islets of Langerhans in the endocrine pancreas, and its function as inhibitor of insulin secretion have been demonstrated by several experimental methods [237,275]. Addition of AM into isolated rat pancreatic islets resulted in a reduction of insulin secretion in a dose dependent manner and, more interestingly, the presence of a monoclonal antibody against AM induced a fivefold increase of the amount of insulin secreted to the medium, thus indicating that the AM intrinsically secreted by the islets was actively inhibiting β-cell function [237]. Moreover, when synthetic AM is injected in rats, the reduction of insulin in blood is accompanied by a concomitant increase of the levels of circulating glucose [237]. When diabetic rats were injected with the monoclonal antibody against AM, glycemia was reduced to normal levels [276,277]. In humans, the levels of circulating AM are clearly elevated in patients with type II diabetes when compared to normal controls [278,279]. As hyperglycemia is responsible for all the disorders associated to diabetes [280], a control of the levels of circulating glucose based on the manipulation of AM levels, could offer a valuable alternative to the current methods in the long-term treatment of diabetes.

Interestingly, heterozygous AM knockout mice, which are able to survive to adulthood, develop peripheral resistance to glucose at mature age [281].

8. Positive and negative modulators of AM

In summary, while AM is a protective agent against cardiovascular disorders, it behaves as a stimulating factor in other pathologies such as cancer and diabetes. Therefore, AM is a new and promising target in the development of molecules which, through their ability to regulate AM levels, could be used in the treatment of these pathologies.

The first attempt in this area has been related to the development of peptide fragments that may interfere with the AM receptor binding process, thus inhibiting AM action [17,281]. However, these compounds are rapidly metabolized when injected into the bloodstream and show a low potency. Monoclonal antibodies have been also proposed as regulators of the physiologic effects of AM, but they have significant limitations as potential drugs given the lack of humanized blocking antibodies [145].

To overcome this problem, in a recent work Martínez et al. [282] have developed a new and efficient method to detect non-peptidic modulators of AM from a large library of small molecules that the NCI has collected since 1955. The first stage of the method is based on the search of compounds that inhibit the binding between AM and its monoclonal antibody. All the compounds that gave a positive response to this assay were subjected to a secondary screening that consisted in an analysis of their ability to modify the production of cAMP, a second messenger elicited by the specific receptor system. Chemical structures of some of the compounds that presented an interesting activity as negative or positive modulators are shown in Figs. 3 and 4, respectively.

Validation of biological activity of some of these small molecules has been carried out, both as potential antiproliferative and hypotensive agents. Over the past few years, a number of reports have confirmed the proliferative capabilities of AM in a breast cancer cell line (T47D) [253]. Accordingly, negative modulators **1**–**10** showed an interesting antiproliferative activity in the MTT assay with T47D cells, which is probably related to their ability to interact with AM [283] unpublished results.

On the other hand, a central function of AM is the regulation of blood pressure [1]. Injection of selected positive modulators (**12** and **14**) in hypertensive rats induced a decrease from the basal levels in blood pressure, while selected AM negative modulators (**1**, **7** and **8**) injected in normotensive rats brought about an elevation of blood pressure [282]. The molecules that diminished blood pressure in hypertensive animals are interesting pharmacological candidates for vascular diseases, renal disorders, sepsis and brain ischemia. Negative

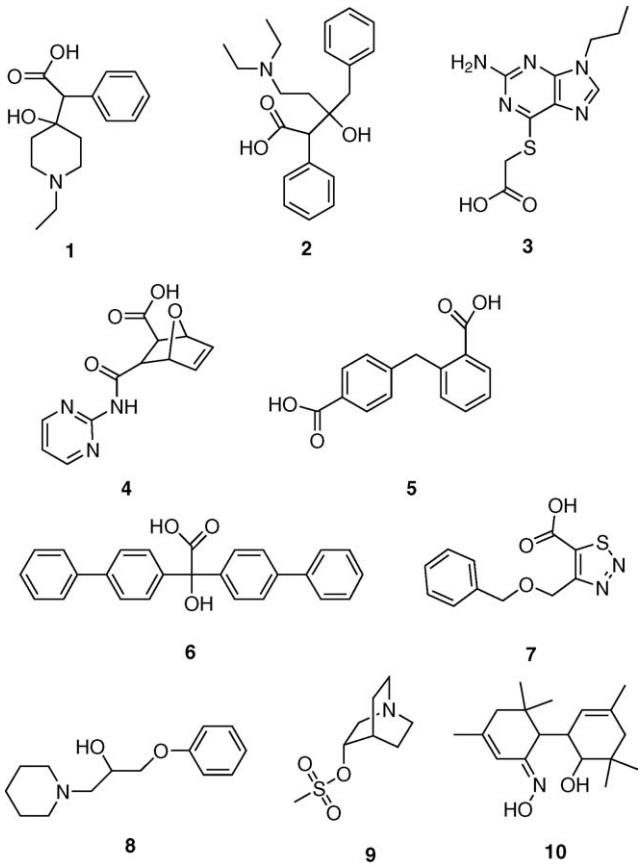

Fig. 3. Chemical structures of some AM negative modulators.

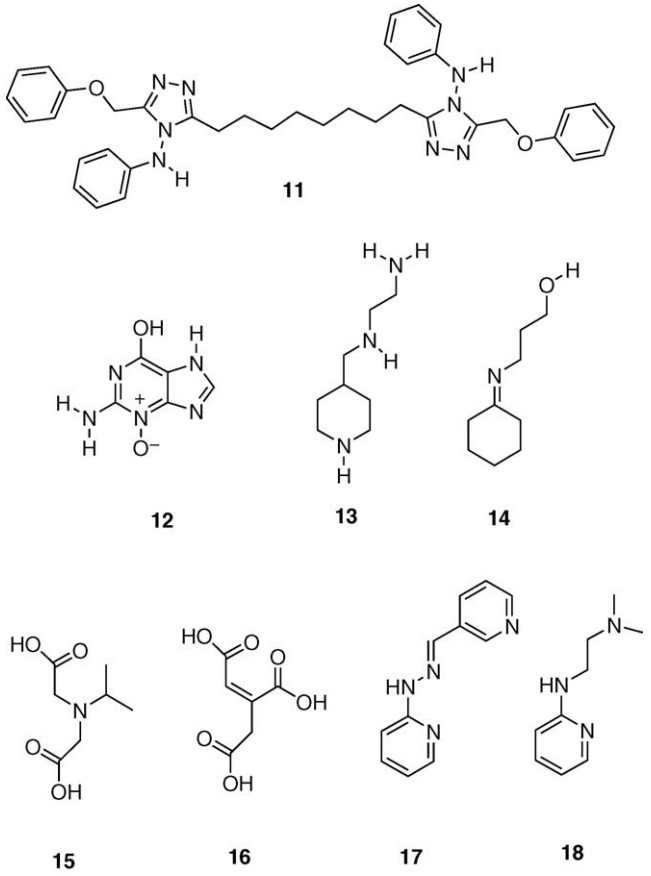

Fig. 4. Chemical structures of some AM positive modulators.

modulators could be also useful against trauma, malignant hypotension or catecholamine disorders.

To investigate the mechanism of action by which the small molecules regulate AM activity, receptor binding studies in the presence or absence of the modulators have been carried out, without seeing any difference [282]. However, surface plasmon resonance studies have clearly shown a specific binding between AM and its small molecule regulators with dissociation constants as low as 6.56 nM. Thus, the molecular mechanism by which the modulators exert its action may involve actual binding to the peptide.

The small molecules detected as modulators of the AM physiology and collected in Figs. 3 and 4, are the starting point for the design and development of new potential drugs against several diseases. Currently, we are carrying out the synthesis of a series of analogues, following a classical approach of molecular modulation. A study of the structure activity relationships using computational techniques has been carried out, based on the activities of the compounds detected in the NCI library of small molecules, and the new synthesized analogues [284]. NMR studies are also being carried out to establish the three-dimensional structure of AM, that will allow us to gain insight into the mode of binding of these small molecules to AM using molecular modeling and molecular mechanics techniques.

9. Conclusions

Adrenomedullin is a 52-amino acid peptide isolated in 1993 from a human pheochromocytoma. Initially it was reported as a vasodilator peptide and, although this relaxing action on vascular smooth muscle cells has remained the main focus on AM research, many other functions were described in the following years. It is now well-established that AM is directly involved in cardiovascular disorders, cancer and diabetes. While AM behaves as a protective agent against cardiovascular disorders, it behaves as a promoting factor in cancer and diabetes. This fact makes AM a promising new target for the rational design of small molecule modulators that could regulate these functions. Here we review the main functions described for AM as well as the first series of chemical compounds capable of regulating AM function. These molecules have been selected from the NCI small molecule collection, through a high throughput screening method recently reported [282]. All these compounds can be considered as prototypes for the design of new agents with different pharmacological applications.

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