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アドレノメデュリン

Adrenomedullin

北村和雄

アドレノメデュリンとPAMPは，共通の前駆体より生合成される降圧作用を有した生理活性ペプチドで，心血管系をはじめとする幅広い組織で生合成されており，降圧因子として循環調節や心血管病の病態に関与している．

構造・分布

アドレノメデュリン(AM)はヒト褐色細胞腫組織抽出液から発見された強力な血管拡張性ペプチドであり，特徴として分子内に6個のアミノ酸よりなるリング構造とC末端のアミド構造をもっている．生理活性に必須であるこれらの構造はカルシトニン遺伝子関連ペプチド(calcitonin gene-related peptide；CGRP)やアミリンなどと一部相同性があり，1つのスーパーファミリーを形成している．さらに，AM前駆体からは**プロアドレノメデュリンN末端20ペプチド(proadrenomedullin N-terminal 20 peptide；PAMP)**が，降圧作用を有した別の生理活性ペプチドとして生合成されている(図)．AMは副腎髄質だけでなく全身の臓器で発現・分泌されており，肺，腎臓，心臓，血管など循環調節に重要な臓器での発現量も多い．

作用・作用機序・受容体

AMの主作用は強力で持続時間の長い血管拡張作用であるが，それ以外にアルドステロンの分泌抑制作用や利尿作用ももち，全体として血圧を低下させる方向に作用する．さらに，血管平滑筋や心筋の増殖抑制作用，血管内皮細胞のアポトーシス抑制作用を有し，臓器障害に対し保護的に作用している可能性も考えられる．なお，AMのトランスジェニックマウスが開発されており，血圧の低下が確認されている．また，AMの欠損マウスは胎生致死であり，その原因としては血管形成不全によると考えられている．

AMの血管拡張作用の機序としては，血管平滑筋細胞では，cAMPを濃度依存性に増加させ，血管拡張を起こすと考えられている．一方，血管内皮細胞に対してAMは，cAMPを増加させると同時にCa^{2+}やIP_3も増加させることが判明している．血管内皮細胞のCa^{2+}の増加はcNOS(constitutive nitric oxide synthase)を活性化しNO産生を増すことが知られており，種による差や血管床による違いはあるものの，AMの血管拡張作用はNO依存性血管拡張作用が関与していると考えられている．さらにAMの血管新生作用やアポトーシス抑制作用は，PI3K-Akt，ERK-MAPKおよび局所接着キナーゼ(p125 FAK)などを介することが示されている．

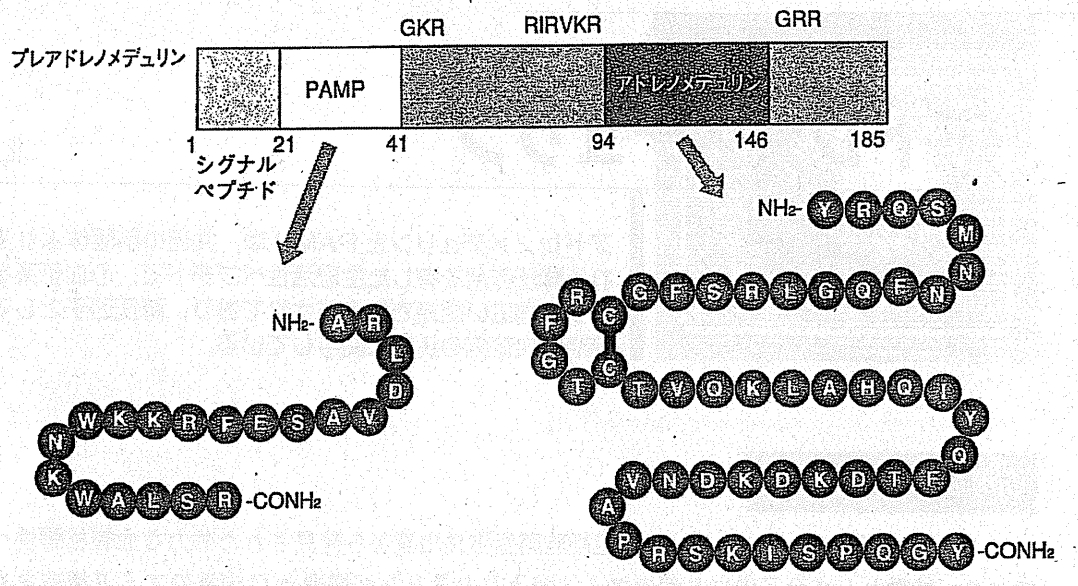
AM受容体に関しては，AMとCGRPが受容体としてCRLR(calcitonin-receptor-like receptor)という7回膜貫通型G蛋白共役型受容体を共有する．CRLRがAMとCGRPの受容体として機能を発揮するためには，1回膜貫通型の受容体活性調節蛋白(RAMP)と呼ばれる膜蛋白が必須である．RAMPがなければCRLR単独では膜表面に移行せず活性も示さず，RAMP単独でも受容体として機能しない．さらに，RAMPはAMとCGRPに対する特異性も決定する．RAMPにはRAMP1～3の相互に相同性を有する3種類の蛋白が存在し，CRLR＋RAMP1でCGRPの，CRLR＋RAMP2またはCRLR＋RAMP3でAMの受容体を形成する．CRLR＋RAMP2はAMへの親和性が高く，かつRAMP2欠

用語解説

プロアドレノメデュリンN末端20ペプチド(PAMP)
AM前駆体から生合成されるもう1つの降圧活性ペプチドで，降圧機序はAMと異なり，協調して循環調節に関与していると考えられる．

略語

AM：adrenomedullin
CRLR：calcitonin-receptor-like receptor
PAMP：proadrenomedullin N-terminal 20 peptide
cAMP：cyclic adenosine monophosphate
IP_3：inositol (1,4,5-) triphosphate
RAMP：receptor activity modifying protein



プロアドレノメデュリンN末端20ペプチド(PAMP)
降圧，交感神経抑制，アルドステロン抑制

アドレノメデュリン
降圧，利尿，抗炎症，細胞増殖の制御ほか

図　ヒトアドレノメデュリン(AM)前駆体の構造と生合成機構およびAMとPAMPの構造

AM前駆体からはAM以外に，PAMPが別の生理活性ペプチドとして生合成される．両ペプチドとも降圧作用を示すが，それらの作用機序は異なっている．

損動物ではAM欠損動物と同じく胎生致死となることから，AMの受容体としてのCRLR＋RAMP2が重要であると考えられている．

病態との関連

AMおよびPAMPの血漿中濃度は，本態性高血圧患者をはじめ腎不全や心不全患者で重症度に従い増加している．また，敗血症性ショックの患者では，AMの血中濃度が重症心不全患者の10倍以上にも増加しており，AMの敗血症性ショックでの病態生理学的意義が注目されている．AMは血管において多量に生合成分泌されており，血管内皮や平滑筋細胞からのAMの生合成・分泌はtumor necrosis factor(TNF)-αをはじめとする多くのサイトカインや血管作動物質等により調節されている可能性が示されている．血管で産生されたAMが血管に対して，エンドセリン-1やアンジオテンシンⅡに拮抗するようなかたちで，血管トーヌスの調節や動脈硬化症の病態などに深く関与していると考えられる．

トピックス：臨床応用の可能性

AMの作用の多くが心血管保護的であり，血管新生作用も有することから，心血管病治療への臨床応用に関する研究が展開されてきた．高血圧症や動脈硬化症への治療応用が期待されているが，AMペプチドそのものの単なる経口投与は不可能であり，新たな投与手段の開発が求められる．急性心筋梗塞，脳梗塞，肺高血圧症に対しては，比較的短時間の静脈内投与あるいは吸入投与にて治療効果が得られる可能性が示唆されており，今後の発展が期待されている．AMの抗炎症，抗線維化作用も強力であり，心血管病以外にも，炎症性腸疾患や各種線維症の治療薬としても検討されている．

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関連事項

アンジオテンシンⅡ ▶▶ 76頁
アルドステロン ▶▶ 88頁
NO ▶▶ 92頁
閉塞性動脈硬化症 ▶▶ 162頁

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Adrenomedullin production is increased in colorectal adenocarcinomas; its relation to matrix metalloproteinase-9

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ABSTRACT

Adrenomedullin (AM) is highly expressed in various cancer cell lines, suggesting a possible association with cancer growth. In the present study, we examined the expression and/or concentration of AM, its related peptide, adrenomedullin2/intermedin (AM2/IMD) and their receptors in human colorectal cancer and the surrounding normal tissue. In addition, we assessed the correlation between the expression of AM and AM2/IMD with that of vascular endothelial growth factor (VEGF)-A and matrix metalloproteinase (MMP)-9. Using a specific immunoradiometric assay, we found that AM concentrations were 2–11-fold higher in colorectal cancer tissues than in the surrounding normal tissues. Moreover, real-time quantitative RT-PCR showed that the expression levels of preproAM (+548%), preproAM2/IMD (+2674%), calcitonin receptor-like receptor (CLR) (+518%), receptor activity modifying protein (RAMP)2 (+281%), RAMP3 (+178%), VEGF-A (+277%) and MMP-9 (+864%) mRNAs were significantly higher in cancer tissues than in the surrounding normal tissues, and there was a positive correlation between the gene expressions of MMP-9 and preproAM ($r=0.352$; $p=0.005$), but not with preproAM2/IMD ($r=0.041$, $p=0.406$). Both AM and AM2/IMD immunoreactivity were detected mainly within cancer cells, whereas MMP-9 immunoreactivity was mostly seen in the surrounding stroma. These findings suggest that AM produced in colorectal tumors acts in concert with MMP-9 in the stroma to contribute to the pathogenesis of colorectal cancer.

1. Introduction

Colorectal cancer is the fourth most common cause of cancer death, worldwide [10]. It proliferates locally and metastasizes to liver, lung, bone and/or brain, and often recurs after surgical resection. Invasion and metastasis are multistep processes that require sequential interactions between the cancer cells and stromal components, such as the extracellular matrix [12] and micro-neovessels [35]. Matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) are key factors that facilitate invasion and metastasis, respectively by degrading extracellular matrix [35] and promoting angiogenesis [8,15] for solid tumor growth.

Adrenomedullin (AM) is a pluripotent bioactive peptide initially isolated from human pheochromocytoma [21]. Since its isolation, AM has been shown to be widely distributed among various organs and tissues, including the human digestive system [22,26], and to be involved a variety of physiological functions, such as vasodilatation, hormone secretion, neurotransmission, embryogenesis, wound healing and immunoregulation [2]. Adrenomedullin2/intermedin (AM2/IMD) was identified as an AM-related peptide by genomic searching [34,36]. AM2/IMD possesses not only a structural similarity to AM such as a ring structure and an amidated C terminus, but also a functional analogy to exert vasodilation and anti-oxidative stress [14]. The functions of AM are mediated through specific receptors comprising calcitonin receptor-like receptor (CLR) and a receptor activity-modifying protein (RAMP); when co-expressed with RAMP2 or RAMP3, CLR functions as a specific AM receptor [23]. Meanwhile, AM2/IMD binds non-selectively to three types of CLR/RAMP complexes: CLR/RAMP1, CLR/RAMP2 and CLR/RAMP3 [23]. AM and its receptors are highly expressed in some tumors and cancer cell lines, which suggests that AM may participate in tumor progression through its potent mitogenic, angiogenic and anti-apoptotic properties [1,13,29]. However, its site-specific expression profile and its concentration in human colorectal cancers and the surrounding normal tissue have not been fully explored. Therefore, our aims in the present study were to measure the expression and/or concentration of AM and its receptors in both cancerous and normal colorectal tissue in humans, and to correlate AM levels

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Table 1
Sequence of the primers and probes.

Gene	Primer/probe sequences	Product size (bp)
AM	S: 5′-TGCCCAGACCCTTATTCGG-3′ AS: 5′-CCGGAGGCCCTGGAAGT-3′ Probe: 5′-AGTCAAGCGCTACCGCCAGAGCA-3′	130
AM2/IMD	Applied Biosystems Gene Expression Assays (ID;Hs00363866_m1)	
CLR	S: 5′-CTGTACATGAAAGCTGTGAGAGCTACT-3′ AS: 5′-TGGAAGTGCATAAGGATGTGCATGATG-3′ Probe: 5′-TCCATGGCGACCTGAAGGAAAGATTGCAGA-3′	140
RAMP2	S: 5′-GCAGAGAGGATCATCTTTGAGACTC-3′ AS: 5′-CCTCCATACTACAAGAGTGATGAGGAAG-3′ Probe: 5′-TGCTCCCTGGTGCAGCCCACCTCTTCTGAC-3′	156
RAMP3	S: 5′-CCGAGTTCATCGTGTACTATGAGAG-3′ AS: 5′-CTGTGGATGCCGGTGATGAAGC-3′ Probe: 5′-AGGCCAATGTCGTGGGCTACTGGCCCA-3′	115
VEGF-A	S: 5′-GTGTGAGTGGTTGACCTTCCTC-3′ AS: 5′-CCGTATATAAAACACTTTCTCTTTTCTCTG-3′ Probe: 5′-CCTGGTCCTTCCCTTCCCTTCCCGA-3′	125
MMP-9	S: 5′-CCCTGGAGACCTGAGAACCA-3′ AS: 5′-AACCATAGCGGTACAGGTATTCCT-3′ Probe: 5′-TCTCACCGACAGGCAGCTGGCA-3′	78
β-actin	S: 5′-AGCCTCGCCTTTGCCGA-3′ AS: 5′-CTGGTGCCTGGGGCG-3′ Probe: 5′-CCGCCGCCCGTCCACACCCGCC-3′	174

S, sense strand; AS, antisense strand.

with clinical variables and the synthesis of invasive and metastatic factor, VEGF-A and MMP-9. We also discussed the expression of AM2/IMD in the disorder.

2. Materials and methods

2.1. Tissue collection

Colorectal specimens were obtained from 23 Japanese patients with colorectal cancer (8 colon cancer and 15 rectal cancer) who underwent surgical resections at Miyazaki University Hospital between June 2007 and March 2009. The histological stages were determined according to Japanese General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus, The 7th Edition. The specimens (0.2–1.0 g) included both cancerous tissue and normal-appearing colorectal tissue from the surrounding area. All specimens were immediately stored in liquid nitrogen until used for real-time quantitative PCR, immunoradiometric assays (IRMAs) and histological analysis. Written informed consent was obtained from all patients, and the study protocol was approved by the institutional review board (No. 519).

2.2. IRMA

The mature form of AM was measured using an AM mature IRMA kit (Shionogi & Co., Ltd., Osaka, Japan) [30]. Tissue samples boiled in 1.0 mol/L acetic acid were homogenized and centrifuged at 12,000 rpm for 20 min at 4 °C, after which the supernatants were stored at −30 °C until assayed.

2.3. Reverse-phase high performance liquid chromatography (RP-HPLC)

To characterize the molecular forms of immunoreactive AM in human colorectal tissues, collected samples (1.0 g) of cancer tissue and the surrounding normal tissue in one patient were immediately boiled for 10 min and then acidified by the addition of acetic acid to a final concentration of 1.0 mol/L to inactivate proteases. The samples were then homogenized and centrifuged at 12,000 rpm for 20 min, after which the supernatants were applied to Sep-Pak C18 cartridges (Nihon Waters K.K.). The resultant extracts were analyzed by RP-HPLC with a TSK ODS 120A column (4.6 × 150 mm; Tosoh) using a linear gradient from solvent A (H_2O–acetonitrile (CH_3CN)–100 mL/L trifluoroacetic acid (TFA, 90:10:1, by volume)) to solvent B (H_2O–acetonitrile–100 mL/L TFA (40:60:1, by volume)) at a flow rate 1 mL/min for 60 min. The immunoreactive AM in each fraction was measured using an IRMA [30] twice, and identical results were obtained. Synthetic human AM peptide was purchased from PEPTIDE INSTITUTE, INC. Osaka, Japan.

2.4. Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed using TaqMan probes to quantify the mRNA levels of AM, AM2/IMD, CLR, RAMP2, RAMP3, VEGF-A, MMP-9 and β-actin. The primers and probes used in this study are listed in Table 1. To quantify the mRNA, standard curves were drawn using diluted cDNA made by human colon tissue. After extracting the total RNA using TRIzol (Life Technologies, Ltd.), 2-μg aliquots were used to synthesize cDNA as previously described [28]. Quantitative RT-PCR analysis was carried out using an Applied Biosystems Prism 7300 Sequence Detector. The PCR protocol entailed a starting cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The PCR products were electrophoresed and found to be of the expected molecular sizes, and β-actin mRNA was used as an internal normalization standard.

2.5. Immunohistochemical staining

Immunohistochemical staining was conducted with samples from all 23 patients after preparation of serial sections. Tissue sections (4 μm) that had been fixed in 10% formalin and embedded in paraffin were deparaffinized, hydrated in 0.01 mol/L phosphate-buffered saline (PBS), and incubated in 3% H_2O_2 in PBS for 20 min at room temperature to block endogenous peroxidase activity. For the detection of MMP-9 and AM2/IMD, prior antigen retrieval was performed by autoclaving the specimens at 121 °C for 15 min in citrate buffer (pH 6.0). The sections were then incubated with a mouse monoclonal antibody against human $AM_{[12-25]}$ (1:500 dilu-

Table 2
Patient characteristics.

Patient characteristics	Numbers
Patients	23
Median age (years) [range]	72 [35–91]
Male/female	15/8
Primary site	
Cecum/A/T/D/S/rectum	1/3/2/0/2/15
Histological stage[a]	
I/II/IIIa/IIIb/IV	1/8/5/4/5
Histological type[a] (all adenocarcinoma, partially overlap)	
tub1/tub2/muc/pap/sig	4/16/3/1/1
Liver/lung metastasis	4/1
Median longest diameter of tumor (mm) [range]	60 [25–122]

Abbreviations: A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; tub1, well differentiated type; tub2, moderately differentiated type; muc, mucinous type; pap, papillary type; sig, signet ring cell type.

[a] Japanese General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus, The 7th edition.

tion; [26,30]) or a rabbit polyclonal antibody against human MMP-9 (1:50 dilution; #3852, Cell Signaling Technology, Inc.) and human $AM2/IMD_{[35-47]}$ (1:4000 dilution, originally made by Dr. Kitamura) for 24 h at 4 °C, followed by incubation for 30 min at room temperature with horseradish peroxidase-labeled polymer conjugated with a secondary antibody (Dako Envision+ System). The immunoreactivity was visualized using 3,3′-diaminobenzidine (Dako), and the sections were counterstained with Mayer's hematoxylin. The positive ratio of AM and MMP-9 immunostaining were analyzed by randomly selected 10 fields in 23 serial sections, and calculated as the percentage of the traced positive field, respectively.

2.6. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 (MDF Co., Ltd.). Data are expressed as means ± standard deviation. The Wilcoxon matched-pair signed rank test was used to assess the association between two categorical variables. The Spearman rank correlation test was applied for the comparison of correlations among multiple groups. Difference between variables in clinical stage or type was analyzed by Kruskal–Wallis test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Patient characteristics

Table 2 shows the characteristics of the patients enrolled in this study. They included 15 males and 8 females, ranging in age from 35 to 91 years (median, 72 years). The primary site was the rectum by 65% of cases, and the histological characteristics varied widely. Among the five patients with stage IV disease, four exhibited liver metastasis and one exhibited lung metastasis.

3.2. Concentration and molecular form of AM in colorectal tumors and surrounding normal tissues

Fig. 1A shows that the concentration of AM was significantly ($p < 0.001$) higher in cancerous tissues (2.3 ± 1.0 pmol/g wet tissue) than in macroscopically normal colorectal tissue (0.7 ± 0.4 pmol/g wet tissue). On reverse phase HPLC, the AM immunoreactivity in both the cancerous tissue and surrounding normal tissue in a patient had one major peak, the elution position of which was identical to that of authentic human $AM_{[1-52]}$ (Fig. 1B).

3.3. Expression of preproAM, preproAM2/IMD, CLR, RAMP2, RAMP3, VEGF-A and MMP-9 mRNAs in colorectal tumors and surrounding normal tissues

Fig. 2 shows that the relative expression levels of the preproAM (A; +548%; $p < 0.0001$), preproAM2/IMD (B; +2674%; $p < 0.0001$), CLR (C; +518%; $p < 0.0001$), RAMP2 (D; +281%; $p < 0.0001$), RAMP3 (E; +178%; $p < 0.005$), VEGF-A (F; +277%; $p < 0.001$) and MMP-9 (G; +864%; $p < 0.0001$) mRNAs were significantly higher in cancerous tissues (Ca) than in the surrounding normal colorectal tissues (N).

3.4. Correlation between the expression of preproAM or preproAM2/IMD and VEGF-A or MMP-9 in colorectal tumors and surrounding normal tissues

The ratios of expression levels of VEGF-A mRNA in the cancerous and surrounding normal tissues (Ca/N) correlate the Ca/N ratios neither for preproAM mRNA ($r = 0.080$; $p = 0.213$, Fig. 3A) nor for preproAM2/IMD mRNA ($r = 0.042$; $p = 0.403$, Fig. 3B). On the other hand, the Ca/N ratios for MMP-9 mRNA did correlated significantly with those for preproAM mRNA ($r = 0.352$; $p = 0.005$, Fig. 3C), but not for preproAM2/IMD mRNA ($r = 0.041$, $p = 0.406$, Fig. 3D).

3.5. Localization of AM, AM2/IMD and MMP-9 immunoreactivity in colorectal tumors and surrounding normal tissues

In normal colorectal tissue, AM immunoreactivity was detected in endothelium, surface epithelium and some stromal cells (Fig. 4A and C). MMP-9 immunoreactivity was faintly detected in some stromal cells (Fig. 4B and D). In cancer tissue, strong AM immunoreactivity was distributed mainly in the cytoplasm of cancer cells and in some fibroblast-like stromal cells (Fig. 4E). Moreover, MMP-9

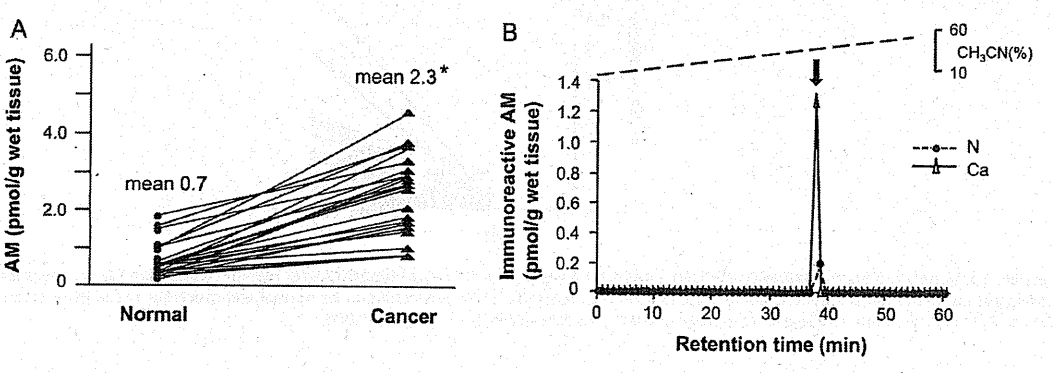

Fig. 1. (A) Concentration of immunoreactive AM in normal and cancerous colorectal tissues from 23 patients. Lines connect symbols indicating the AM concentrations in normal and cancerous tissue from each patient ($n = 23$). The absolute means are also shown in the figure. *$p < 0.001$. (B) RP-HPLC analysis of immunoreactive AM in normal and cancerous tissues from one patient. The arrow indicates the elution position of authentic human $AM_{[1-52]}$. N, Normal; Ca, Cancer tissue.

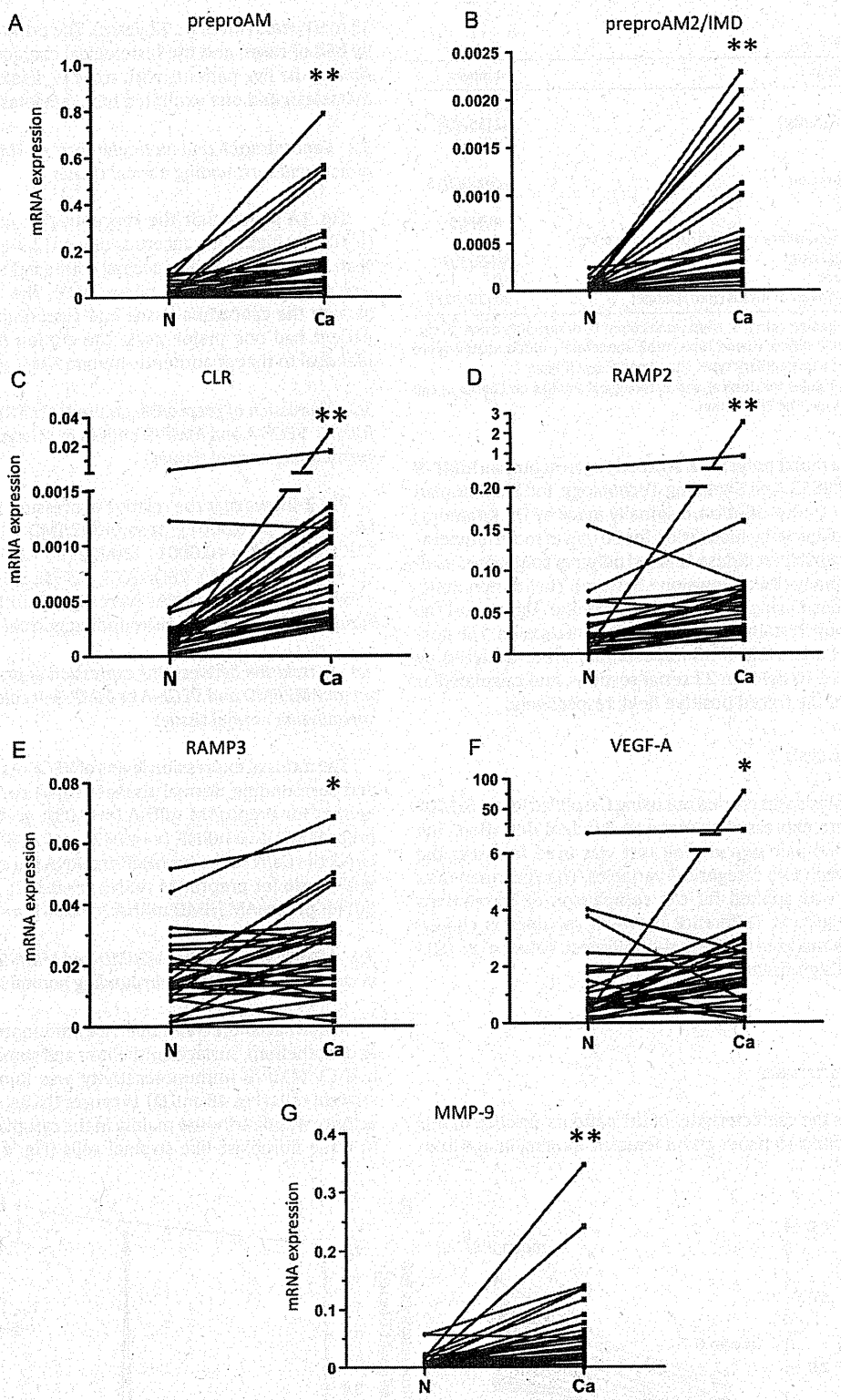

Fig. 2. Expression of preproAM (A), preproAM2/IMD (B), CLR (C), RAMP2 (D), RAMP3 (E), VEGF-A (F) and MMP-9 (G) mRNAs in normal (N) and cancerous tissues (Ca). β-actin served as an internal normalization standard. Lines connect symbols indicating the mRNA expression in the normal and cancerous tissue from each patient ($n = 23$). The *Y*-axes in C, D and F are separated into two segments to make details of the data visible. $^{*}p < 0.001$, $^{**}p < 0.0001$.

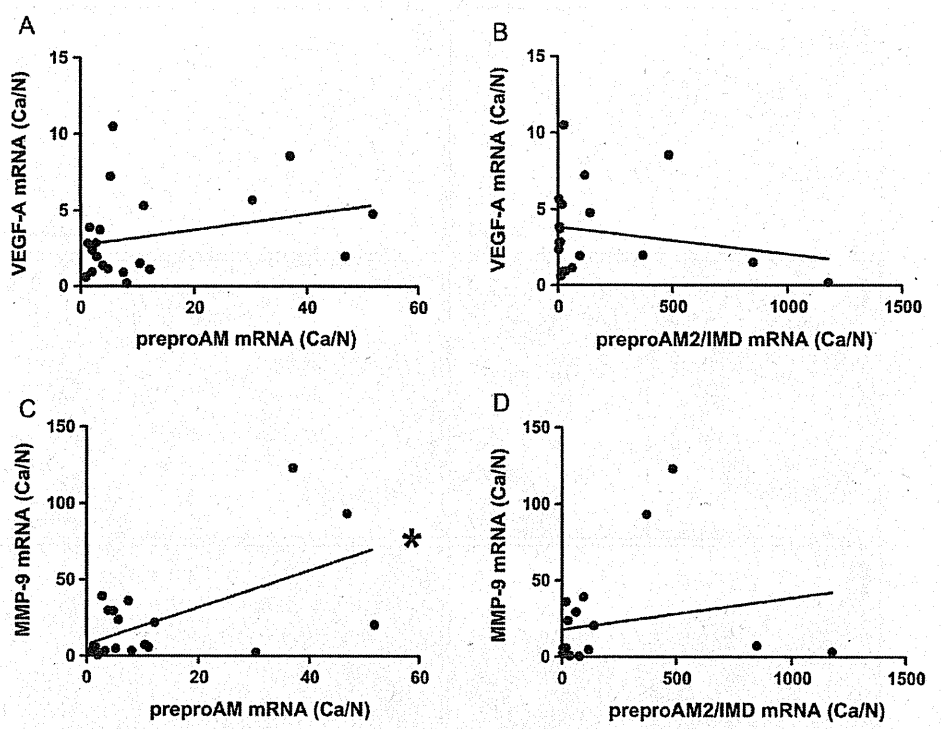

Fig. 3. (A and B) Correlation between the expression ratios of VEGF-A and preproAM mRNAs (A, $r = 0.080$, $p = 0.213$) or preproAM2/IMD mRNAs (B, $r = 0.042$, $p = 0.403$) in the cancerous and surrounding normal colorectal tissues (Ca/N) from each patient. (C and D) Correlation between the expression ratios of MMP-9 and preproAM mRNAs (C, $r = 0.352$, $p = 0.005$) or preproAM2/IMD mRNAs (D, $r = 0.041$, $p = 0.406$) in the cancerous and surrounding normal colorectal tissues (Ca/N) from each patient. *$p < 0.01$.

immunoreactivity was intensely present in the stroma of cancer tissue (Fig. 4F). MMP-9-positive cells were often found surrounding the AM-positive cells in the serial section, and the positive ratios of AM and MMP-9 in the tissue sections were 14.4% and 11.1% in the fields of cancer tissue specimens, respectively. AM2/IMD immunoreactivity was scattery found in stroma of normal colorectal tissue (Fig. 4G), while it was intensely detected in cancer cells (Fig. 4H).

4. Discussion

AM and its specific receptors, CLR/RAMP2 and CLR/RAMP3, are expressed in various cancer cell lines and in cancers of the pancreas, lung, kidney, breast, ovary and prostate [3,7,27,33]. In addition, AM has been shown to promote tumor growth by stimulating cell proliferation and angiogenesis and inhibiting apoptosis [9,13,18] under hypoxic conditions that lead to AM activation [20]. Conversely, neutralizing AM activity using a specific antibody reduces the growth in glioblastoma cells [31] and pancreatic [18] and colorectal adenocarcinoma cells [19]. However, there have been few studies examining the site-specific expression profiles of the genes encoding AM and its receptors, or the levels of the translated peptides in patients with colorectal cancer. In the present study, we found that colorectal cancers exhibited higher levels of AM synthesis than the surrounding normal tissues.

Most colonic cancer tissue contains substantial amounts of MMP-9 [6] and VEGF [5], co-activation of which is thought to facilitate invasion and metastasis [16]. Consistent with that idea, we found that cancerous colorectal tissue contains significantly higher levels of both MMP-9 and VEGF-A than the surrounding normal tissues. However, the precise mechanism of AM on angiogenesis is unclear; Iimuro et al. [17] reported that AM enhances angiogenesis mediated by VEGF, whereas Fernandes-Sauze et al. [11] found that the proangiogenic action of AM is VEGF-independent. In the present study, expression of preproAM mRNA did not correlate with VEGF mRNA expression. On the other hand, magnitude of MMP-9 mRNA significantly correlated with that of preproAM mRNA. More interestingly, AM appeared to localize specifically in cancer cells, while MMP-9 localized in surrounding stromal cells. AM modulates the production of MMP-2 in rheumatoid synovial fibroblasts [24], aortic adventitial fibroblasts [37], and hepatic stellate cells [38]. On the other hand, amino-acid sequence of AM is reported to be cleaved by MMP-2, but not by MMP-9 [25]. Our finding implicates a cross-talk between cancer cells and the microenvironment during tumor progression through activation of AM and MMP-9. We also found that AM-related peptide, AM2/IMD was highly expressed in colorectal cancer tissue as well. Importantly, the percent increase of AM2/IMD synthesis in cancer tissues to the surrounding normal tissues was greater than AM. The pathological role of AM2/IMD remains to be elucidated in cancer development. AM2/IMD mRNA is up-regulated along with AM under hypoxic conditions [4]. However, we did not find any relationship between preproAM2/IMD mRNA and VEGF-A or MMP-9 synthesis in the present study, implicating a distinct role of these peptides in the tumor progression. Further experiments will be necessary to precisely characterize the pathophysiological roles of AM and AM2/IMD in colorectal cancer development.

Although it has been suggested that plasma AM level appears to be predictive in tumor progression [32], our data are consistent with a report by Buyukberber et al. [3] that AM expression did not associate with any clinical variables, such as histological type ($\chi^2 = 1.77$, $p = 0.78$) or stage ($\chi^2 = 3.17$, $p = 0.53$). We speculate that AM does not directly contribute to tumor progression, but that they more likely have mixed biological effects supporting colon cancer survival in association with matrix degradation.

In summary, this study highlights that expressions of AM and AM2/IMD are increased in human colorectal cancer coinciding with

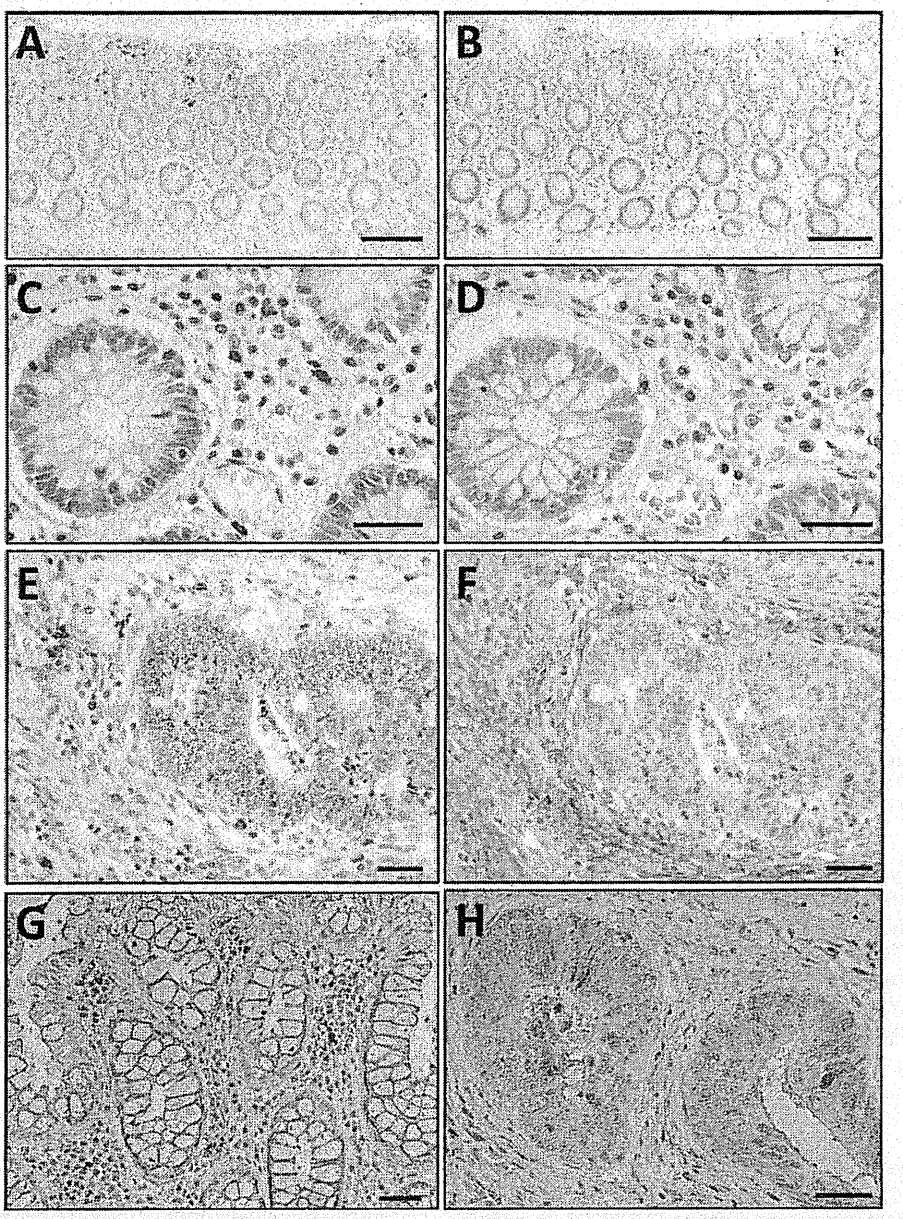

Fig. 4. Representative immunohistochemical staining of AM, MMP-9 and AM2/IMD in sections of human colorectal tissue. (A–D) Immunostaining of AM (A and C) and MMP-9 (B and D) in normal colorectal tissue collected from a patient with colorectal cancer. (E and F) Immunostaining of AM (E) and MMP-9 (F) in moderately differentiated adenocarcinoma tissue. (G and H) Immunostaining of AM2/IMD in normal colorectal tissue (G) and moderately differentiated adenocarcinoma tissue (H). Bars: A, B = 20 μm; C–H = 5 μm.

MMP-9 and VEGF-A in surrounding stroma, suggesting a possible involvement in tumor survival/progression.

Conflicts of interest

None declared.

Acknowledgments

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Structure–function analysis of helix 8 of human calcitonin receptor-like receptor within the adrenomedullin 1 receptor

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ABSTRACT

Adrenomedullin 1 (AM_1) receptor is a heterodimer composed of calcitonin receptor-like receptor (CLR) – a family B G protein-coupled receptor (GPCR) – and receptor activity-modifying protein 2 (RAMP2). Both family A and family B GPCRs possess an eighth helix (helix 8) in the proximal portion of their C-terminal tails; however, little is known about the function of helix 8 in family B GPCRs. We therefore investigated the structure–function relationship of human (h)CLR helix 8, which extends from Glu430 to Trp439, by separately transfecting nine point mutants into HEK-293 cells stably expressing hRAMP2. Glu430, Val431, Arg437 and Trp439 are all conserved among family B GPCRs. Flow cytometric analysis revealed that Arg437Ala or Trp438Ala mutation significantly reduced cell surface expression of the receptor complex, leading to a ~20% reduction in specific ^{125}I-AM binding but little change in their IC_{50} values. Both mutants showed 6–8-fold higher EC_{50} values for AM-induced cAMP production and ~50% reductions in their maximum responses. Glu430Ala mutation also reduced AM signaling by ~45%, but surface expression and ^{125}I-AM binding were nearly the same as with wild-type CLR. Surprisingly, Glu430Ala and Val431Ala mutations significantly enhanced AM-induced internalization of the mutant receptor complexes. Taken together, these findings suggest that within hCLR helix 8, Glu430 is crucial for Gs coupling, and Arg437 and Trp439 are involved in both cell surface expression of the hAM_1 receptor and Gs coupling. Moreover, the Glu430–Val431 sequence may participate in the negative regulation of hAM_1 receptor internalization, which is not dependent on Gs coupling.

1. Introduction

Adrenomedullin (AM) receptors are heterodimers composed of calcitonin receptor-like receptor (CLR) – a family B G protein-coupled receptor (GPCR) – and receptor activity-modifying protein 2 (RAMP2) or RAMP3, both of which are single membrane-spanning domain accessory proteins [19]. CLR/RAMP2 (AM_1 receptor) has proven to be the most highly specific AM receptor and is particularly sensitive to the AM receptor antagonist AM-(22-52) in all species tested [20]. By contrast, CLR/RAMP3 (AM_2 receptor) binds calcitonin gene-related peptide (CGRP) at lower concentrations than it binds AM and is more sensitive to the CGRP receptor antagonist CGRP-(8-37) than is the AM_1 receptor, particularly in rodents [20]. Although intermedin (AM2), like AM, belongs to the calcitonin/CGRP family and is more closely related to AM than to CGRP [23,29], intermedin elicits cAMP production in cells expressing CLR/RAMP3, but not CLR/RAMP2 [23,29].

AM, the endogenous agonist, is a novel vasodilator also shown to be necessary for development of the fetal cardiovascular system and able to powerfully act against various vascular diseases including hypertension, atherosclerosis and secondary lymph edema [7,11,16,18,30]. All of these effects are mediated via the AM_1 receptor [7,11,18,30]; the in vivo function of the AM_2 receptor remains unclear.

When acting as chaperones, RAMP2 and -3 transport CLR molecules from the endoplasmic reticulum to the cell surface, where the CLR/RAMP complex mediates AM-induced intracellular cAMP production and Ca^{2+} mobilization [17]. Upon AM binding, both AM receptors undergo rapid internalization via a clathrin- and β-arrestin-dependent pathway without dissociation of the CLR and RAMP molecules [8,17].

At present the crystal structures of four family A GPCRs are available: rhodopsin, β_1-adrenergic receptor, β_2-adrenergic receptor and adenosine A_{2A} receptor [21,24]. The structural findings revealed the presence of an eighth helix (helix 8), oriented per-

Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; C-tail, cytoplasmic C-terminal tail; CLR, calcitonin receptor-like receptor; GPCR, G protein-coupled receptor; GRK, GPCR kinase; h, human; HEK, human embryonic kidney; Hyg, hygromycin; RAMP, receptor activity-modifying protein; RCP, CGRP-receptor component protein; WT, wild-type.

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pendicular to the seven transmembrane helixes, in the proximal portion of the C-terminal tail (C-tail). To date, helix 8 of family A GPCRs has been shown to play key roles in the cell surface expression of GPCRs, stabilization of GPCRs at the cell-surface, the conformational switch involved in GPCR activation, Gs coupling, Gq coupling and activation of GPCR kinases [4,25,28,31,34]. On the other hand, no crystal structures of family B GPCRs are currently available, and little is known about the function of helix 8 within the receptors. Recently, Conner et al. [2] used mutagenesis and various synthetic peptides to characterize helix 8 in human (h)CLR. They showed that putative hCLR helix 8 extends from Glu430 to Trp439 and contains four residues, Glu430, Val431, Arg437 and Trp439, which are strictly conserved among family B GPCRs. In addition, putative hCLR helix 8 possesses an Arg436–Arg437 sequence, which an earlier study suggests may be part of a consensus motif involved in Gs coupling [32]. But to our knowledge, there is still no detailed information concerning the structure–function relationship of putative hCLR helix 8 complexed within AM receptors. To address that issue, we examined the effects of nine hCLR helix 8 point mutants separately expressed in human embryonic kidney (HEK)-293 cells stably expressing hRAMP2 [13], which enables hCLR to function as a hAM_1 receptor.

2. Materials and methods

2.1. Reagents and antibodies

Human AM was kindly donated by Shionogi & Co. (Osaka, Japan). FITC-conjugated mouse anti-V5 monoclonal antibody (anti-V5-FITC antibody) was from Invitrogen. All other reagents were of analytical grade and obtained from various commercial suppliers.

2.2. Expression constructs

Double V5-tagged hCLR (V5-hCLR) [14] was cloned into pIRES1/Hyg, yielding pIRES1-V5-hCLR [13]. Nine point mutations within putative hCLR helix 8 were introduced using a QuikChange® kit (Stratagene) according to the manufacturer's instructions, with pIRES1-V5-hCLR serving as the template. For each mutation, two complementary 30- to 40-mer oligonucleotides (sense and antisense) were designed with the mutation in the middle. The resultant mutants were all sequenced using an Applied Biosystems 310 Genetic Analyzer.

2.3. Cell culture and DNA transfection

Several hygromycin (Hyg)-resistant HEK 293 cell clones stably expressing hRAMP2 were isolated previously [15]. Among them, the clone that most efficiently promoted AM-mediated V5-hCLR internalization [15] was selected and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 100 μg/ml Hyg at 37 °C under a humidified atmosphere of 95% air/5% CO_2.

Transient transfection of HEK-293 cells stably expressing hRAMP2 was performed using LipofectAMINE as previously described [13]. Briefly, the cells were seeded into 12-well plates (for flow cytometric analysis) or 24-well plates (for binding and cAMP assays) and, upon reaching 70–80% confluence, were transfected with empty vector (pIRES1/Hyg) (*Mock*) or V5-tagged wild-type (WT) or mutant constructs; at all times, V5-hCLR was included in each transfection set. This was accomplished by incubating the cells for 4 h in OptiMEM 1 medium containing plasmid DNAs (0.2 μg/well for 24-well plates; 0.4 μg/well for 12-well plates), Plus regent (2 μl/well for 24-well plates; 2.5 μl/well for 12-well plates) and LipofectAMINE (2 μl/well for 24-well plates; 2.5 μl/well for 12-well plates), and all experiments were performed 48 h after transfection. Separate transfections were carried out with different passage number (1–5) hRAMP2-expressing cells; the intra- and inter-assay coefficients of variance were less than ~10%.

2.4. Flow cytometric analysis

Following transfection of the indicated V5-tagged cDNAs into hRAMP2-expressing HEK-293 cells in 12-well plates, the cells were exposed to selected concentrations of hAM in prewarmed serum-free DMEM containing 20 mM Hepes and 0.5% bovine serum albumin for 60 min at 37 °C. Receptor internalization was stopped by adding ice-cold PBS, after which the cells were harvested, resuspended in ice-cold buffer for flow cytometric analysis [17] and labeled with anti-V5 FITC antibody (1:1000 dilution) for 60 min at 4 °C in the dark. Following two successive washes, the cells were subjected to flow cytometry in an EPICS XL flow cytometer (Beckman Coulter), and cell surface expression of each V5-tagged receptor, before and after exposing cells to AM, was analyzed using EXPO 2 software (Beckman Coulter) [17].

2.5. Radioiodination and radioligand binding

^{125}I-hAM (specific activity 5 μCi/pmol) was produced in our laboratory, as previously described [12]. Briefly, hAM (10 μg) in 25 μl 0.4 M sodium acetate buffer (pH 5.6) was introduced into a tube followed by the addition of $Na^{125}I$ (0.5 mCi/5 μl, MP Biomedicals). Lactoperoxidase (Calbiochem: 600 ng/10 μl of 0.1 M sodium acetate, pH 5.6) and H_2O_2 (70 ng/5 μl of water) were then added. After letting the tube stand for 10 min at 33 °C, additional H_2O_2 (100 ng/5 μl of water) was added, and the tube was left for another 10 min at 33 °C. Immediately thereafter, the mixture was submitted to reverse-phase high-performance liquid chromatography, and mono-iodinated hAM was purified and used for the following binding assay.

To assess whole-cell radioligand binding, transfected HEK-293 cells in 24-well plates were washed twice with prewarmed PBS and then incubated with 20 pM ^{125}I-hAM for 5 h at 4 °C in the presence (for nonspecific binding) or absence (for total binding) of 1 μM unlabeled hAM in modified Krebs–Ringers–Hepes medium [17]. After washing the cells twice with ice-cold PBS, they were harvested in 0.5 M NaOH, and the associated cellular radioactivity was measured in a γ-counter. Specific binding was defined as the difference between the total binding (Bo, in the absence of hAM) and nonspecific binding (N, measured in the presence of 1 μM AM). The *Y*-axis for calculating IC_{50} values for hAM in competition with ^{125}I-hAM for binding to transfected HEK-293 cells is $(B - N)/(Bo - N) \times 100$ (%), where B is the ^{125}I-hAM bound.

2.6. Measurement of intracellular cAMP

Transfectants in 24-well plates were incubated for 15 min at 37 °C in Hanks' buffer containing 20 mM Hepes, 0.2% bovine serum albumin, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) and the indicated concentrations of hAM. The reactions were terminated by addition of lysis buffer (GE Healthcare), after which the cAMP content was determined using a commercial enzyme immunoassay kit according to the manufacturer's instructions (GE Healthcare) for a non-acetylation protocol.

2.7. Data analysis and statistics

Competitive binding data (IC_{50} values) and cAMP concentration–response data (EC_{50} values and maximum responses) were analyzed using a four-parameter logistic equation with MasterPlex ReaderFit software (Hitachi Software Engineering America Ltd., USA). Results are expressed as means ± SEM of

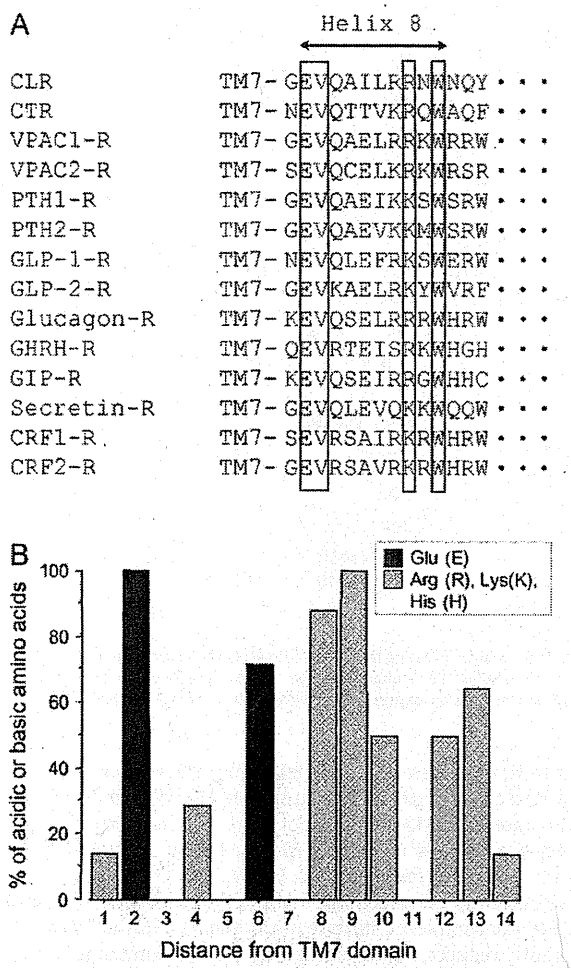

Fig. 1. (A) Alignment of the first 14 residues of the C-terminal tails of 14 family B hGPCRs. Four conserved residues, E (Glu), V (Val), R (Arg) and W (Trp), are *boxed*. CTR, calcitonin receptor; VPAC-R, vasoactive intestinal polypeptide, pituitary adenylyl cyclase activating peptide receptor; PTH, parathyroid hormone; GLP, glucagon-like peptide; GHRH, growth hormone releasing hormone. (B) Distribution of charged amino acid residues within the first residues of 14 family B hGPCR C-tails. Putative helix 8 of family B GPCRs is situated between positions 2 and 11. Acidic residues (E (Glu)) occur most frequently at positions 2 and 6, while dibasic residues (R (Arg) and K (Lys)) occur most frequently at positions 8 and 9.

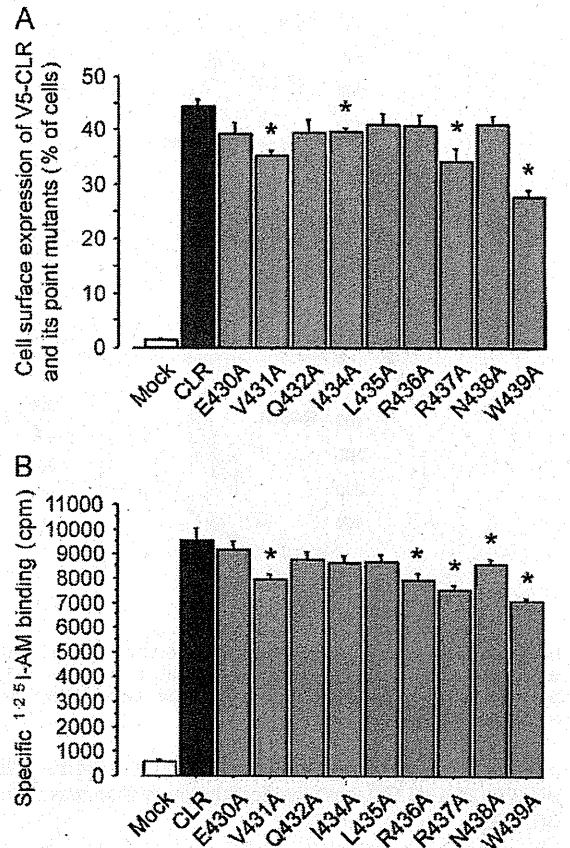

Fig. 2. Characterization of hCLR helix 8 point mutants in HEK-293 cells stably expressing hRAMP2. (A) Flow cytometric analysis of the cell surface expression of the indicated V5-tagged proteins. Transfected cells were incubated for 1 h at 4 °C with anti-V5-FITC antibody; mock incubation with the antibody served as the control. Surface V5-FITC-labeled proteins are expressed as % of cells. Cell surface expression of WT-hCLR and all hCLR helix 8 point mutants was significantly higher than that seen with mock ($p < 0.05$). Bars represent means ± SEM of six independent experiments. *$p < 0.05$ vs. hCLR. (B) Specific binding of ^{125}I-hAM. Transfected cells were incubated for 5 h at 4 °C with ^{125}I-AM (20 pM) in the presence or absence of 1 μM unlabeled hAM. Specific ^{125}I-AM binding to WT-hCLR and all hCLR helix 8 point mutants was significantly higher than that seen with mock ($p < 0.05$). Bars represent means ± SEM of six experiments. *$p < 0.05$ vs. hCLR.

at least five independent experiments; only bars in the cAMP dose–response curves (Fig. 3) are expressed as means ± SD due to limitations of the software. Differences between two groups were evaluated using Student's *t*-tests; differences among multiple groups were evaluated using one-way analysis of variance followed by Scheffe's tests. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Amino acid sequence alignment of putative helix 8 in family B GPCRs

Fig. 1A is based on results from Conner et al. [2] and shows the alignment of the first 14 residues of the C-terminal tails of 14 family B hGPCRs. Putative helix 8 is situated between positions 2–11 [2]. Among the 14 residues shown, the four boxed residues, E (Glu), V (Val), R (Arg) and W (Trp), all of which are within helix 8, are strictly conserved. It is also noteworthy that acidic residues (E (Glu)) occur most frequently at positions 2 and 6, while dibasic residues (R (Arg) and K (Lys)) occur most frequently at positions 8 and 9 (Fig. 1B).

3.2. Cell surface expression of point mutants of putative hCLR helix 8

We recently established a line of HEK-293 cells stably expressing hRAMP2 [13], which, prior to transfection with hCLR, lack AM receptors (Fig. 3). This enabled us to use these cells to examine the role of helix 8 in cellular trafficking and receptor signaling mediated via hCLR within the AM_1 receptor complex (hCLR/RAMP2). We accomplished this by testing the effects of nine helix 8 point mutations on receptor function.

Fig. 2A shows the cell surface expression of V5-tagged WT protein and the nine point mutants following their separate transfection into hRAMP2-expressing HEK-293 cells. When the cells were transfected with empty vector (*Mock*), surface binding of anti-V5-FITC antibody was within the 2% limit of resolution characteristic of flow cytometric analysis. Similar to WT-hCLR (44.2 ± 1.5%), the E430A, Q432A, I434A, L435A, R436A and N438A mutants appeared at the surface of 39–42% of cells. By contrast, cell

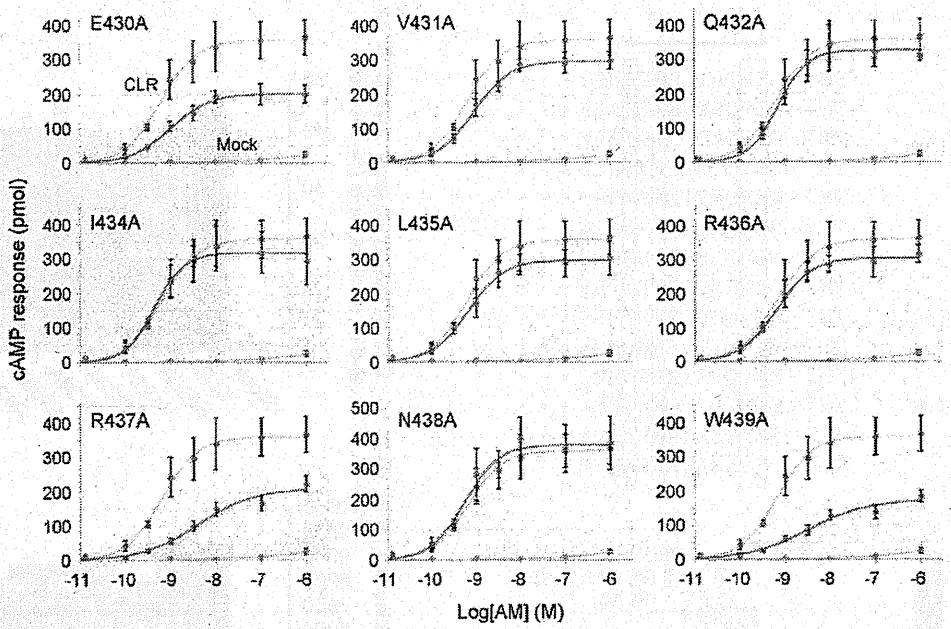

Fig. 3. AM-evoked cAMP production mediated via hCLR helix 8 point mutants in HEK-293 cells stably expressing hCLR. All cells transfected with wild-type or mutant hCLR were simultaneously exposed to the indicated concentrations of hAM for 15 min at 37 °C and then lysed. The resultant lysates were analyzed for cAMP content. Note that the same cAMP responses mediated via wild-type hCLR are shown in every mutant-mediated response. Bars are expressed as means ± SD of five experiments due to limitations of the MasterPlex ReaderFit software used.

surface expression of V431A (35.3 ± 1.0%), R437A (34.1 ± 2.4%) and W439A (27.4 ± 1.0%) was significantly lower than was seen with WT-hCLR.

3.3. Effect of helix 8 point mutation on ^{125}I-AM binding to hCLR

Fig. 2B shows that in cells expressing WT-hCLR (IC_{50} = 22.4 ± 3.0 nM), specific ^{125}I-AM binding was about 20-fold higher than in cells expressing empty vector (*Mock*), and similar levels of ^{125}I-AM binding were seen in cells expressing E430A, Q432A, I434A, L435A or N438A. On the other hand, the IC_{50} values for V431A (13.1 ± 2.0 nM), Q432A (15.0 ± 1.3 nM), R436A (14.4 ± 2.7 nM) and R437A (15.7 ± 1.4 nM) were somewhat lower, and the IC_{50} value for W439A (8.2 ± 1.2 nM) was significantly lower, than was seen with WT-hCLR ($p < 0.05$). The remaining mutants' IC_{50} values ranged from 18.3 to 19.4 nM.

3.4. Functionality of hCLR helix 8 point mutants

The functionality of the mutant receptors was assessed by measuring AM-induced intracellular cAMP production (Fig. 3 and Table 1). AM elicited little or no cAMP production in HEK-293 cells expressing hRAMP2 alone. Following transfection with WT-hCLR, however, AM elicited concentration-dependent increases in cAMP, and comparable responses were seen when cells were transfected with Q432A, I434A, L435A or N438A. The maximum cAMP levels obtained with the remaining five mutants were significantly lower than those obtained with WT-hCLR. In addition, the R437A and W439A mutants exhibited significant increases in their EC_{50} values.

3.5. Internalization of hCLR helix 8 point mutants

Our earlier flow cytometric analysis revealed that AM dose-dependently induced hRAMP2-mediated internalization of hCLR, with the peak of the receptor's internalization occurring after about 60 min [15]. In the same study, we also showed that there was no significant difference in the efficiency of receptor internalization induced by 0.1 μM and 1.0 μM AM [15]. We therefore evaluated the internalization of each point mutant after exposing hRAMP2-expressing cells to 0.1 μM AM for 60 min (Fig. 4). Surprisingly, we found that four mutants, E430A, V431A, Q432A and W439A, significantly enhanced AM-induced internalization of the mutant receptor complexes, despite the fact that E430A and W439A significantly reduced AM signaling (Fig. 3). The remaining mutants had little effect on receptor internalization.

4. Discussion

All family B GPCRs are preferentially coupled to Gs proteins responsible for stimulating cAMP production, and many undergo rapid internalization in response to an agonist. However, the roles of their C-tails in Gs coupling and in internalization have not been fully explored. We recently showed that AM elicits little or no cAMP production or internalization of receptor complexes composed of hRAMP2 and a hCLR truncation mutant totally lacking its

Table 1
AM-induced cAMP production in RAMP2-expressing HEK-293 cells co-transfected with wild-type CLR and its point mutants.

Construct	cAMP production	
	EC_{50} (nM)	Maximum response (pmol)
CLR	0.68 ± 0.10	372.4 ± 21.6
E430A	1.10 ± 0.11*	206.0 ± 9.5*
V431A	0.92 ± 0.05	300.4 ± 10.6*
Q432A	0.82 ± 0.11	328.2 ± 12.6
I434A	0.47 ± 0.04	319.3 ± 24.1
L435A	1.05 ± 0.44	306.8 ± 19.6
R436A	0.68 ± 0.09	305.6 ± 13.6*
R437A	5.15 ± 1.86*	215.7 ± 13.0*
N438A	0.59 ± 0.08	386.8 ± 29.4
W439A	3.99 ± 0.97*	182.8 ± 7.6*

The results represent the mean ± SEM of five independent experiments.
* $p < 0.05$ vs. CLR.

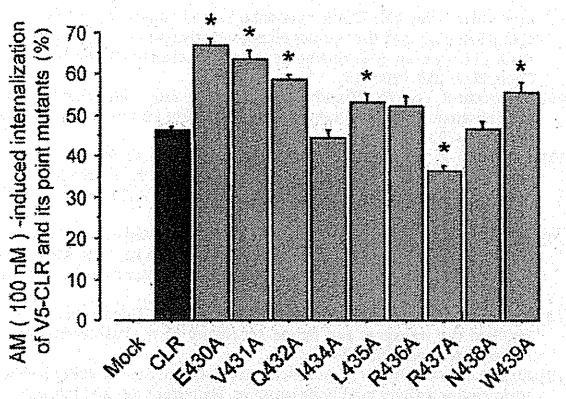

Fig. 4. Flow cytometric analysis of internalization of hCLR helix 8 point mutants in HEK-293 cells stably expressing hRAMP2. Surface expression of each construct was estimated by flow cytometry before and after exposing the cells to 0.1 μM hAM for 60 min. Internalization was estimated as the percentage of receptors lost from the cell surface after AM exposure. Internalization of WT-hCLR and all hCLR helix 8 point mutants was significantly higher than that seen with mock ($p < 0.05$). The results represent the means ± SEM of six independent experiments. *$p < 0.05$ vs. hCLR.

C-tail, despite their full cell surface expression and high-affinity ^{125}I-AM binding [13]. This means the hCLR C-tail is crucial for both AM-induced cAMP production and hAM_1 receptor internalization, which is in contrast to the earlier report that the hCLR C-tail is involved in agonist-mediated receptor internalization, but not cAMP production [2]. That finding was obtained using COS-7 cells transiently co-expressing hRAMP1 and an hCLR mutant in which the C-tail was completely removed. The discrepancies between that earlier study and our present one may reflect differences in the cell backgrounds, transfection methods, cell-surface expression levels of the target receptor complex, cAMP assays and/or the hRAMP isoform.

We observed that substituting Glu430 in putative hCLR helix 8 with Ala (E430A) reduced maximum cAMP levels by ~45% without altering the cell surface expression of the receptor or its affinity for ^{125}I-AM in HEK-293 cells stably expressing hRAMP2. This suggests Glu430 is required for the coupling of hCLR helix 8 to Gs. Notably, this negatively charged residue is strictly conserved among B GPCRs (Fig. 1), and a conserved Glu residue is also reportedly involved in Gs coupling to putative helix 8 in the hVPAC1 receptor in CHO cells [3]. In that case, substituting Glu394 with Ala (E394A) reduced cAMP responses by ~45%, although cell surface expression of the mutant receptor and agonist binding were identical to those in the wild-type receptor [3]. The C-tail of porcine calcitonin receptor is also required for evoked cAMP production [6], but the role of the conserved Glu residue in that receptor remains unknown. On the other hand, evidence suggests that the C-tails of the rat glucagon [33], parathyroid [10] and secretin receptors [9] may not be involved in coupling to Gs proteins. However, all of those studies made use of a truncation approach, which left some or all of helix 8 intact. It is therefore possible that the strictly conserved Glu residue in helix 8 is generally involved in coupling family B GPCRs to Gs.

Putative hCLR helix 8 also possesses two positively charged dibasic residues (Arg436–Arg437), both of which are highly conserved among family B GPCRs; in particular, Arg437 is strictly conserved (Fig. 1). In the present study, R437A mutation of hCLR reduced cell surface expression of the mutant receptor by ~25%, thereby reducing specific ^{125}I-AM binding by ~20%. Moreover, this mutant reduced maximum cAMP levels by ~40%, with an ~8-fold increase in the EC_{50} value. Thus, Arg437 also appears to participate in both the coupling to Gs proteins and the proper surface delivery of hCLR. Although Arg436 is next to Arg437, R436A mutation had little effect on the cell surface expression or the cAMP responses of the mutant receptor complex. By contrast, double mutation of Arg400–Arg401 to Ala400–Ala401 did not affect cell surface expression, radioligand binding or cAMP responses of the mutant hVPAC1 receptor [3]. It remains to be seen whether the highly conserved Arg residues in hCLR helix 8 are also important for other family B GPCR functions.

Unlike helix 8 in rhodopsin-like receptors (family A GPCRs), helix 8 in family B GPCRs, including hCLR, contains no cysteine residues, the palmitoylation of which has been shown to anchor the helix to the plasma membrane. Nonetheless, a recent analysis revealed that a synthetic hCLR helix 8 peptide readily anchors to liposomes in a membrane-parallel orientation via Trp439, which is strictly conserved among family B GPCRs [2]. In the intact receptor, therefore, a tethered Trp439 may partially fulfill the role of the lipid anchor seen at the equivalent position in many family A GPCRs. This function would be unaffected by the hRAMP isoform, as the hCLR C-tail does not interact with any of the three hRAMP isoforms [14]. In the present study, W439A mutation of hCLR reduced cell surface expression of the mutant receptor by ~40%, and there was a corresponding ~25% reduction in specific ^{125}I-AM binding, with a ~3-fold decline in the IC_{50} value, as compared to WT-hCLR. In addition, maximum cAMP levels were reduced by ~50%, with a ~6-fold increase in the EC_{50} value. Thus Trp439 appears to be important for both cell surface expression of the hAM_1 receptor and AM-mediated cAMP responses mediated via the receptor. Analysis of the crystal structure will be necessary to determine whether this hydrophobic residue serves as a lipid anchor in the intact form of hCLR.

It has been shown that CGRP-receptor component protein (RCP), an intracellular peripheral membrane protein, is specifically required for CLR/RAMP signal transduction [5,22]. In mouse NIH3T3 cells endogenously expressing RCP, CLR and RAMP1 or -2, RCP co-immunoprecipitates with CLR and appears to assist CGRP and AM receptor coupling to Gs [5,22]. However, nothing is known about the CLR domains responsible for the interaction with RCP, the mechanism by which RCP couples CLR to the cellular signal transduction pathway, or the role of RCP in vivo. In future experiments, it will be important to clarify whether RCP interacts with the three hCLR point mutants, E430A, R437A and W439A, all of which significantly reduced AM signaling when co-expressed with hRAMP2.

We also found that the E430A and V431A mutations significantly enhanced AM-induced internalization of the mutant receptor complexes, whereas the other mutations had little effect on internalization. As mentioned above, E430A mutation markedly reduced cAMP responses, and V431A also reduced the responses by ~20%. Taken together, these results suggest that internalization of hAM_1 receptors does not depend on Gs coupling, which is consistent with our earlier findings obtained using various C-tail deletion mutants [13]. Our present results are also supported by a recent report showing that introduction of a dileucine into helix 8 of the formyl peptide receptor (a family A GPCR) significantly enhances receptor internalization [27].

We previously showed that four GPCR kinases (GRK-2, -3, -4 and -5) bind to the Ser/Th-rich regions distal to putative hCLR helix 8, and that overexpression of these GRKs significantly enhanced internalization of the hAM_1 receptor in HEK-293 cells expressing endogenous GRKs 2–6 [13]. The helix 8 region of GPCRs moves significantly upon receptor activation [1,26,34], making it a candidate for recognition by GRKs. Perhaps the conformation of the E430A and V431A hCLR mutants favors interaction with intracellular binding partners such as GRKs.

In conclusion, we have shown that within putative hCLR helix 8, the strictly conserved Glu430 residue is crucial for Gs coupling, and that the strictly conserved Arg437 and Trp439 residues are involved in both cell surface expression of the hAM_1 receptor and Gs

coupling. We also suggest that the Glu430–Val431 sequence participates in the negative regulation of hAM_1 receptor internalization, which is not dependent on Gs coupling.

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Shared and separate functions of the RAMP-based adrenomedullin receptors

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ABSTRACT

Adrenomedullin (AM) is a novel hypotensive peptide that exerts a variety of strongly protective effects against multiorgan damage. AM-specific receptors were first identified as heterodimers composed of calcitonin-receptor-like receptor (CLR), a G protein coupled receptor, and one of two receptor activity-modifying proteins (RAMP2 or RAMP3), which are accessory proteins containing a single transmembrane domain. RAMPs are required for the surface delivery of CLR and the determination of its phenotype. CLR/RAMP2 (AM_1 receptor) is more highly AM-specific than CLR/RAMP3 (AM_2 receptor). Although there have been no reports showing differences in intracellular signaling via the two AM receptors, *in vitro* studies have shed light on their distinct trafficking and functionality. In addition, the tissue distributions of RAMP2 and RAMP3 differ, and their gene expression is differentially altered under pathophysiological conditions, which is suggestive of the separate roles played by AM_1 and AM_2 receptors *in vivo*. Both AM and the AM_1 receptor, but not the AM_2 receptor, are crucial for the development of the fetal cardiovascular system and are able to effectively protect against various vascular diseases. However, AM_2 receptors reportedly play an important role in maintaining a normal body weight in old age and may be involved in immune function. In this review article, we focus on the shared and separate functions of the AM receptor subtypes and also discuss the potential for related drug discovery. In addition, we mention their possible function as receptors for AM2 (or intermedin), an AM-related peptide whose biological functions are similar to those of AM.

Contents

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Table 1
Representative biological actions of AM.

Main actions	Reference
Stimulation of intracellular cAMP	[64,80]
Intracellular Ca^{2+} mobilization	[64,80]
Stimulation of nitric oxide (NO) production	[50,64]
Reduction of blood pressure (Vasodilation)	[20,68,132]
Reduction of vascular and cardiac oxidative stress	[90,117]
Protection from vascular hypertrophy and inflammation	[64,82]
Inhibition of vascular apoptosis	[64]
Inhibition of left ventricular hypertrophy	[20,139]
Inhibition of left ventricular remodeling	[90]
Promotion of diuresis and natriuresis	[33,93]
Inhibition of aldosterone secretion	[143,144]
Induction of angiogenesis	[57,88]
Induction of lymphangiogenesis	[37,61]

1. Introduction

Adrenomedullin (AM) and AM2 (also known as intermedin; IMD) belong to the calcitonin (CT)/CT gene-related peptide (CGRP) family [108,114,131,141]. The members of this family share very little sequence homology; only 18 amino acids are conserved between AM and AM2 (Fig. 1). However, each member contains two common structures necessary for their biological activity: a ring structure comprising six amino acids linked by a disulfide bridge and an amidated C-terminus (Fig. 1) [108,141]. AM is a 52 amino acid peptide first identified in human pheochromocytoma tissue extracts as a highly potent vasodilator comparable to CGRP [68]. Its gene expression and binding sites are widely distributed in peripheral tissues and in the central nervous system, which enables AM to exert a variety of biological effects (Table 1) [13,33,59,64,68,81,93,117,141]. Most of these effects are strongly protective against multiorgan damage induced by hypertension, oxidative stress, atherosclerosis, ischemia and sepsis. Notably, the angiogenic properties of AM support blood flow to ischemic tissue, including tumor tissue [58]. Therefore, high-affinity AM antagonists or antibodies could become effective therapeutic tools for the prevention of tumor angiogenesis and progression [28,47,92].

Genomic screens that were conducted by two independent research groups identified a novel AM-related peptide that was named AM2 (or IMD) [114,130]. Although this peptide has not yet been isolated from mammalian tissue extracts and there have been no reports on the effects of an AM2 gene knockout, the biological actions of exogenous AM2 are similar to those of AM [129,130]. Interestingly, several studies suggest that the involvement of AM2 in some pathophysiological processes differs from that of AM [51,129].

The discovery of three receptor activity-modifying proteins (RAMP1, RAMP2 and RAMP3) led to the identification of the human CGRP and AM receptors [84]. These accessory proteins enabled the then-orphan CT receptor-like receptor (CLR), which is a family B G protein coupled receptor (GPCR), to function as the CGRP receptor (CLR/RAMP1) or as the AM_1 or AM_2 receptor (CLR/RAMP2 and CLR/RAMP3, respectively) [84]. The three RAMPs are each composed of about 160 amino acids, and all exhibit a common structure that includes a large extracellular N-terminal domain (ECD), a single transmembrane domain (TM), and a very short cytoplasmic C-terminal tail (C-tail); however, they share less than 30% sequence homology [46,84,115]. Although RAMPs are ubiquitous throughout the body, there are differences in their tissue distributions, and the abundance of each isoform depends on the tissue type [100,107,115,142]. In addition, RAMP gene expression is differentially regulated under various disease conditions in animal models [46,73]. Along with AM, the AM_1 receptor facilitates the development of the fetal cardiovascular system and exerts protective effects against various vascular diseases [37,39,57,82,132]. The AM_2 receptor evidently does not mediate the major effects of AM, although there have been no reports showing the differences in intracellular signaling pathways between the two AM receptors [8].

AM2 can also interact with the CGRP, AM_1 and AM_2 receptors and, as with AM, elicit cAMP production, but the responses appear to be smaller than are seen with AM [19,114,130]. It is therefore still controversial whether the three CLR/RAMP complexes are native receptors for AM2. Interestingly, AM2, but not AM, inhibits growth hormone release from rat anterior pituitary cells [133], which suggests there is a unique AM2 receptor that is unrelated to the CLR/RAMP heterodimers.

2. Structure and function of AM, its related peptides and their peptide antagonists

The members of the CGRP family are peptides composed of 32–52 amino acids, and their receptors are all GPCRs [108] that mediate agonist-induced cAMP production, which is indicative of their G_S coupling [76]. AM is composed of 52 highly conserved amino acids, the sequence of which contains only 1–3 substitutions in humans, pigs, dogs and cows [67]. As compared to the human peptide (hAM), rat (r)AM and mouse (m)AM lack 2 resides at their N-termini, and there are 6 amino acid substitutions in each [67]. However, all of these AM peptides share a ring structure comprising 6 amino acids linked by a disulfide bridge and an amidated C-terminal Tyr residue, both of which are necessary for receptor binding and subsequent signaling [31]. In fact, these 2 structural features are common to all CT/CGRP family peptides, despite their low homology at the level of the primary sequence (Fig. 1) [67,108,141]. The 15 N-terminal residues of hAM are not essential for interaction with its receptor (compared to the hCGRP N-terminus) [31]. In addition, amidation of the C-terminal residue of hAM is more important than the amidation of Tyr52 *per se* [31]. When hAM point mutations in which the 5 residues between Cys16 and Cys21 were separately substituted with Ala were intravenously injected into rats, only Phe18Ala and Thr20Ala significantly impaired the peptide's hypotensive effects, as compared to wild-type hAM. Moreover, both of these residues are completely conserved among the aforementioned 6 species, and

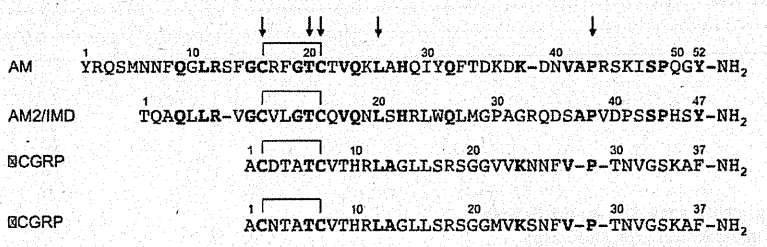

Fig. 1. Amino acid sequence alignment of human adrenomedullins (AM and AM2/IMD) with CGRPs (αCGRP and βCGRP). The human sequences are aligned for maximum homology [113]. *Arrows* indicate conserved amino acids among the AMs and CGRPs. *Bold* residues are conserved in AM.

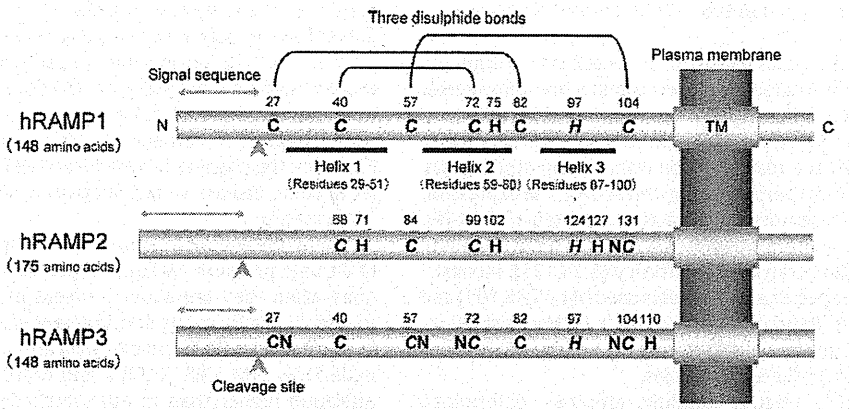

Fig. 2. Schematic representation of the structure of the three human RAMPs. Each human (h)RAMP possesses a large extracellular N-terminal domain (ECD) with a signaling sequence and cleavage site, a single transmembrane (TM) domain and a very short cytoplasmic C-terminal tail. The three hRAMPs share ~30% sequence homology, and RAMP2 is 27 amino acids longer than RAMP1 and RAMP3. Recent crystal structural analysis of the hRAMP1 ECD revealed the presence of three α-helices (helix 1, 2 and 3) and three disulfide bonds (Cys27–Cys82, Cys40–Cys72, and Cys57–Cys104) [71]. The four Cys(C) residues and one His (H) residue are conserved among all three RAMPs (shown in *bold italic*); the N-terminal glycosylation sites (N; Asn) are also shown. Note that the number of His residues is the smallest among the amino acids making up each RAMP.

Thr20 in hAM is also strictly conserved among the CT/CGRP family peptides (Fig. 1) [67,108,130]. Interestingly, Phe18 in hAM is less well conserved; it is substituted by Leu in hAM2 and hCT and by Thr in human αCGRP, βCGRP and amylin, suggesting that these residues may contribute to the determination of agonist specificity.

A C-terminal hAM fragment (residues 22–52; hAM_{22-52}) lacking the disulfide bond has been shown to act as a selective AM receptor antagonist [31]. This first report showed that the binding affinity of hAM_{22-52} was ~70-fold lower than that of hAM in cultured rat vascular smooth muscle cells (VSMCs), which express AM-specific receptors [31,32]. Similarly, the N-terminally truncated peptide $\alpha CGRP_{8-37}$ has been used as a selective CGRP receptor antagonist since 1989 [23,25]. Among CGRP peptides, αCGRP was first cloned from the CT gene [4], while βCGRP was discovered to be a second CGRP analog derived from its own unique gene [123]. Nevertheless, hβCGRP differs from hαCGRP by only three amino acids (Fig. 1), and the two display similar biological activities. Both hAM_{22-52} and $h\alpha CGRP_{8-37}$ have also been used to investigate the function of AM2. So far, there have been no reports of an AM2 peptide antagonist (e.g., $AM2/IMD_{16-47}$).

Circular dichroismspectroscopic analysis showed that the secondary structures of AM, AM2 and αCGRP differ considerably [113]. The α-helical contents of AM, AM2 and αCGRP are 31%, 51% and 80%, respectively. AM_{22-52} contains 31% α-helix and 12% β-sheet, which is identical to AM. By contrast, $\alpha CGRP_{8-37}$contains 61% less α-helical content than αCGRP, which leads to a compensatory increase in β-sheets (from 0% to 32%). Thus, the absence of the N-terminus, including the ring structure, produces a large change in the secondary structure of αCGRP but not AM.

3. Discovery of the three RAMPs and their structural characterization

Since its discovery, AM has been shown to share a number of pharmacological features with α- and βCGRP. Indeed, many effects of AM are blocked by $\alpha CGRP_{8-37}$ as well as by AM_{22-52} [41,73,89,94,137]. For these reasons, AM is believed to bind to its specific receptors, as well to receptors that bind both CGRP and AM.

The three hRAMPs were initially identified as chaperones promoting the forward trafficking of CLR from the endoplasmic reticulum to the cell surface [84]. Once at the cell surface, each RAMP governs the expression of the CLR phenotype: CLR/RAMP1, CLR/RAMP2 and CLR/RAMP3 form functional CGRP, AM_1 and AM_2 receptors, respectively [44,73,84,86,108]. The differences in the pharmacology of these three receptors are described in the next section.

Schematic diagrams showing the basic structures of the three hRAMPs are presented in Fig. 2. As mentioned earlier, all three possess a large ECD (91 amino acids in hRAMP1 and hRAMP3, 102 amino acids in hRAMP2), a single TM domain (22 amino acids), and a very short C-terminal tail (9 amino acids) [46,84,110]. There is also a predicted signal peptide (26 amino acids in hRAMP1, 44 amino acids in hRAMP2, and 27 amino acids in hRAMP3) [46,110]. Overall, the three hRAMPs share only ~30% sequence identity overall and~32% identity in their TM regions.

In 2008, Kusano et al. [71] reported the first crystal structure of the hRAMP1 ECD (residues 22–112) in the absence of hCLR. The hRAMP1 ECD is composed of three α-helices (α-helix 1, 2 and 3) that are stabilized by 3 disulfide bonds (Cys27–Cys82, Cys40–Cys72 and Cys57–Cys104). As shown in Fig. 2, the four Cys residues at positions 40, 57, 72 and 104 are strictly conserved among hRAMPs [46]. In hRAMP1 and hRAMP2, these conserved residues are required for the efficient transport of hCLR to the cell surface [79,119,120,124]. The remaining two Cys residues within the hRAMP1 ECD (Cys27 and Cys82), which are conserved in hRAMP3 but not hRAMP2, are not involved in the cell surface expression of hCLR or in CGRP potency [120]. There have been no studies examining the effects of substituting any or all of these 6 Cys residues in the hRAMP3 ECD. Interestingly, the hRAMP1 ECD possesses no putative N-terminal glycosylation sites (Asn residues), while the hRAMP2 ECD contains 1 Asn residue, and the RAMP3 ECD contains four Asn residues. Note that all of these Asn residues are located next to highly conserved Cys residues [46], although the function in the AM_1 and AM_2 receptors remains unclear.

Recently, ter Haar et al. [134] reported the crystal structure of an unbound heterodimer composed of the CLR ECD and the RAMP1 ECD. Within this structure, α-helices 2 and 3 of RAMP1 interact with the N-terminal α-helix of CLR. Surprisingly, this heterodimerization induces no significant conformational rearrangement of the RAMP1ECD structure. But this report contains no information on the crystal structures of other regions of RAMP1 and CLR or the effect of ligand binding. Consequently, the full extent of RAMP1-CLR interactions remains unknown, as does whether the formation of the CLR/RAMP1 dimer causes a conformational change in the subunits.

4. The pharmacology of recombinant CLR/RAMP complexes

The pharmacological characteristics of CLR/RAMP complexes have been extensively studied after their transfection into several cultured cell lines, including human embryonic (HEK)-293 cells, African monkey kidney (COS-7) cells and Chinese hamster ovary (CHO) cells. Although the results of the transfection studies may have been affected by factors such as the cell source, the expression levels of endogenous receptors and intracellular proteins [135,140], passage number (e.g., continuous cell passage may reduce levels of the target protein) [24], transfection efficacy [5,74,135], inconsistencies in the animal species and materials used [44,45,86,108] and the position of ligand iodination [74], there is little transfection-dependent variation in the selectivity of recombinant CLR/RAMP complexes derived from the same species.

For hCLR/hRAMP1, relative binding affinities determined based on the competitive inhibition of ^{125}I-CGRP binding are as follows: $\beta CGRP \geq \alpha CGRP > \alpha CGRP_{8-37} > AM \gg AM_{22-52}$ [2,3,84]. Similar results are obtained with cells expressing rCLR/rRAMP1 [100]. However, with a combination of rCLR and mRAMP1, the affinity $r\alpha CGRP_{8-37}$ binding is ~3-fold greater than rαCGRP binding [56]. In cells expressing hCLR/hRAMP2, the rank order of inhibition of ^{125}I-AM binding is as follows: $AM > AM_{22-52} > h\alpha CGRP_{8-37} \geq h\beta CGRP > h\alpha CGRP$ [2,36,84]. This relation is also seen in cells expressing the exogenous rodent AM_1 receptor [56,100]. The relative binding affinity for recombinant hCLR/hRAMP3 is $AM > AM_{22-52} = h\alpha CGRP_{8-37} = h\beta CGRP > h\alpha CGRP$ [2,36]. Notably, in cells transfected with rCLR/mRAMP3, the binding affinity of $r\alpha CGRP_{8-37}$ is more than or equal to rAM [56].

Only CLR/RAMP2 (AM_1 receptor) acts as a highly AM-specific receptor and is particularly sensitive to hAM_{22-52} or rAM_{20-50}, irrespective of animal species. In contrast, CLR/RAMP3 (AM_2 receptor) cross-reacts with CGRP peptides at lower concentrations and is more sensitive to $\alpha CGRP_{8-37}$ than the AM_1 receptor. With the rodent AM_2 receptor, in particular, AM binding is more effectively blocked by $\alpha CGRP_{8-37}$ than by hAM_{22-52} or rAM_{20-50}, which is not the case with the hAM_2 receptor. At higher concentrations, AM, but not hAM_{22-52} or rAM_{20-50}, can also interact with CLR/RAMP1 (CGRP receptor); indeed, there have been several reports on the effect of $\alpha CGRP_{8-37}$ on AM-mediated responses via the recombinant CLR/RAMP1 complex [1,24,55,85,89]. However, there is no evidence that the CGRP receptor functions as a $\alpha CGRP_{8-37}$-sensitive AM receptor *in vivo*.

AM2 also reportedly activates the three CLR/RAMP complexes, but its affinity and selectivity remain unclear [42,109,114,130]. In contrast to AM, the pharmacology of AM2 has not yet been well explored. We therefore examined the effect of hAM2 on cAMP responses using recombinant human AM_1 and AM_2 receptors expressed in HEK-293 cells. As shown in Fig. 3, this peptide exerted potent effects via both AM receptors: AM > AM2/IMD > αCGRP for AM_1 receptors and AM = AM2/IMD > αCGRP for AM_2 receptors. Hay et al. obtained similar results with COS-7 cells and also showed that AM2 can activate the CGRP receptor (CLR/RAMP1) with the same potency as AM [47]. These findings support the idea that many effects of AM2 are similar to those of AM, although there is evidence of a unique, unidentified AM2 receptor in rat anterior pituitary cells [133].

5. Pathophysiological functions of endogenous AM receptors in experimental rat models

There is now extensive evidence that AM and AM2 are both potent cardiorenoprotective mediators [8,13,19,51,59,64,93]. Upon their release, these peptides are well known to act locally as autocrine/paracrine regulators. Changes in the tissue expression of mRNAs encoding AMs and their receptors have been examined under various pathophysiological conditions using experimental rat models. As shown in Table 2, tissue expression of AMs and their receptor components (CLR and RAMP2 and RAMP3) is upregulated in various cardiorenal disease states, suggesting that AMs act via their receptors to compensate for pathophysiological conditions. However, the changes in RAMP2 and RAMP3 mRNA expression differ in some disease states, suggesting separate roles for AM_1 and AM_2 receptors.

The spontaneously hypertensive rat (SHR) is a useful model of chronic pressure loading. Hypertension develops in these animals when they are about 7 weeks of age (young adult) and is followed initially by the development of compensated left ventricular hypertrophy (LVH). In the myocardia and aortas of 11-week-old male SHRs, AM, CLR, RAMP2 and RAMP3 gene expression is significantly higher than in age-matched Wistar-Kyoto (WKY) rats (control) [104]. Moreover, AM gene delivery significantly reduces blood pressure and the extent of LVH in 11-week-old SHRs [20], and AM attenuates angiotens in II-induced cardiomyocyte hypertrophy *in vitro* [139]. By 20 weeks, however, left ventricular levels of AM and RAMP3 mRNAs are no longer elevated, despite the presence of myocardial concentric hypertrophy and oxidative stress. By contrast, robust induction of AM2 and RAMP3 gene expression is observed in the hypertrophied left ventricle [9]. Expression of neutral endopeptidase is known to be diminished in left ventricular tissue from 10-week-old SHRs. Downregulation of this enzyme may therefore have resulted in the augmented expression of AM2.

Chronic inhibition of nitric oxide (NO) synthesis through treatment with N^G-nitro-L-arginine-methyl-ester (L-NAME) also leads to hypertension and myocardial hypertrophy, ischemia and oxidative stress [14]. In the hypertrophied left ventricle of L-NAME-treated rats, expression of mRNAs for the two AMs and their two receptors is significantly augmented, and notably, the levels of AM2 mRNA are much higher than those of AM mRNA [10–12,145]. This suggests AM2 may play a more prominent role than AM as a local endogenous negative feedback regulator of cardiac hypertrophy. Interestingly, two antioxidants (Vitamin C and Tempol) normalized the augmented expression of myocardial AM2 and CLR (along with RAMP1) without affecting blood pressure in this model, but the expression of AM, RAMP2 and RAMP3 were unaffected. This suggests that induction of AM2 and CGRP receptor expression is influenced to a much greater extent by cardiac oxidative stress than by pressure loading [10]. Hypertension induced by L-NAME also elevates levels of mRNAs for AM and its receptor components in the aorta [105].

In the Dahl salt-sensitive (DS) rat model, systemic hypertension causes LVH by the age of 11 weeks and then heart failure by17 weeks [96]. The levels of mRNAs for AM and its two receptors in the left ventricle of DS rats were significantly increased at both the LVH stage and heart failure stage, as compared to age-matched Dahl salt-resistant (DR) rats [93]. Similar increases in the mRNA expression of AM and its receptors were also observed in the hypertrophic left ventricle in the deoxycorticosterone acetate (DOCA)-salt model, another rat model of malignant hypertension [127]. Thus, AM and both AM_1 and AM_2 receptors are upregulated in the hypertrophied left ventricle in various hypertensive rat models, but it remains unclear whether the behavior of the receptors differ depending the pathophysiological state.

In rats with congestive heart failure induced by myocardial infarction (MI; resulting from left coronary artery ligation), mRNA expression of the two AMs (AM2 > AM) and the two AM receptors was increased in both the non-infarcted and infarcted regions of the left ventricle, although the induction was significantly greater in the infarcted areas [52]. Induction of AM signaling may be beneficial because early infusion of AM (for a week) markedly inhibits left ventricular remodeling after MI in rats [90]. In this MI model, atrial