placenta was also highly immunoreactive to our anti-Ang II N-terminal antibody, and gel filtration showed the molecular weight of the antigen to be approximately 5000, just as in human urine (Fig. 3I). Moreover, although gel filtration produced a clear Ang II peak, there was little Ang I peak, indicating Ang II was produced via a renin-independent pathway.

The abundance of Bang-25 over such a wide range of tissues suggests it is important in the production of Ang II in tissue. Particularly interesting to us was the strong Bang-25 staining in endocrine tissues, including pancreatic cells and the adrenal gland medulla. It has been reported that Ang II contributes to oxidative stress, inflammation and apoptosis in pancreatic cells [21], and that local Ang II may stimulate catecholamine release from the adrenal medulla [22]. Within that context, the possible conversion of Bang-25 to Ang II in tissue suggests Bang-25 may indirectly contribute to the regulation of endocrine cell function through Ang II production. Alternatively, the observation that it is highly localized in endocrine cells suggests Bang-25 may itself function as an endocrine hormone without conversion to Ang II. In the kidney, Bang-25 is localized predominantly to podocytes (Fig. 3G and H). This is noteworthy, as it is known that pathological production of Ang II by podocytes causes injury [23] related to such disease states as primary glomerulopathy, hypertension and diabetes mellitus [24], and reduced podocyte loss can entirely account for the renoprotective effect of Ang II blockade [25]. Although it is not presently clear whether podocytes are the origin of Bang-25 in urine, it could be that Bang-25 in urine is marker of podocyte injury.

Chymase, the enzyme that cleaves Bang-25 to produce Ang II, is released into interstitial tissues from mast cell granules following tissue injury and during inflammation, and chymase expression is upregulated after kidney injury and in heart disease [26]. In human vascular extracts, chymase inhibition reduces Ang II synthesis by more than 90% [27], suggesting chymase-dependent Ang II synthesis is stimulated by tissue injury or inflammation. One possibility is that, in tissue, Aogen is subject to cleavage by a different enzyme to form Bang-25, which is in turn cleaved by chymase to form Ang II.

Table 1
Result of the immunohistochemistory.

Kidney	Podocyte	++
Heart	Left ventricle, cardiocytes	++
Lung	Pulmonary artery, SMCs	+
	Bronchiole, epithelium	+
Esophagus	Stratified squamous epithelium (basal layer)	+
Stomach	Glands (endocrine cells)	+
	Nerve (submucosal and Auerbach)	+
Large intestine	Crypts (endocrine cells)	+
Liver	Hepatocytes	+
Spleen	Histiocytes	+
Pancreas	Islets	++
	Exocrine (acinus/duct)	–
Adrenal gland	Medulla	++
	Cortex	–
Lymph nodes	Histiocytes	+
	Dendritic cells	+
Testis	Sertoli cells	++
Salpinx	SMCs	++
	Tubal epithelium	+
Placenta	Extravilous trophoblasts	++
	Villi	–

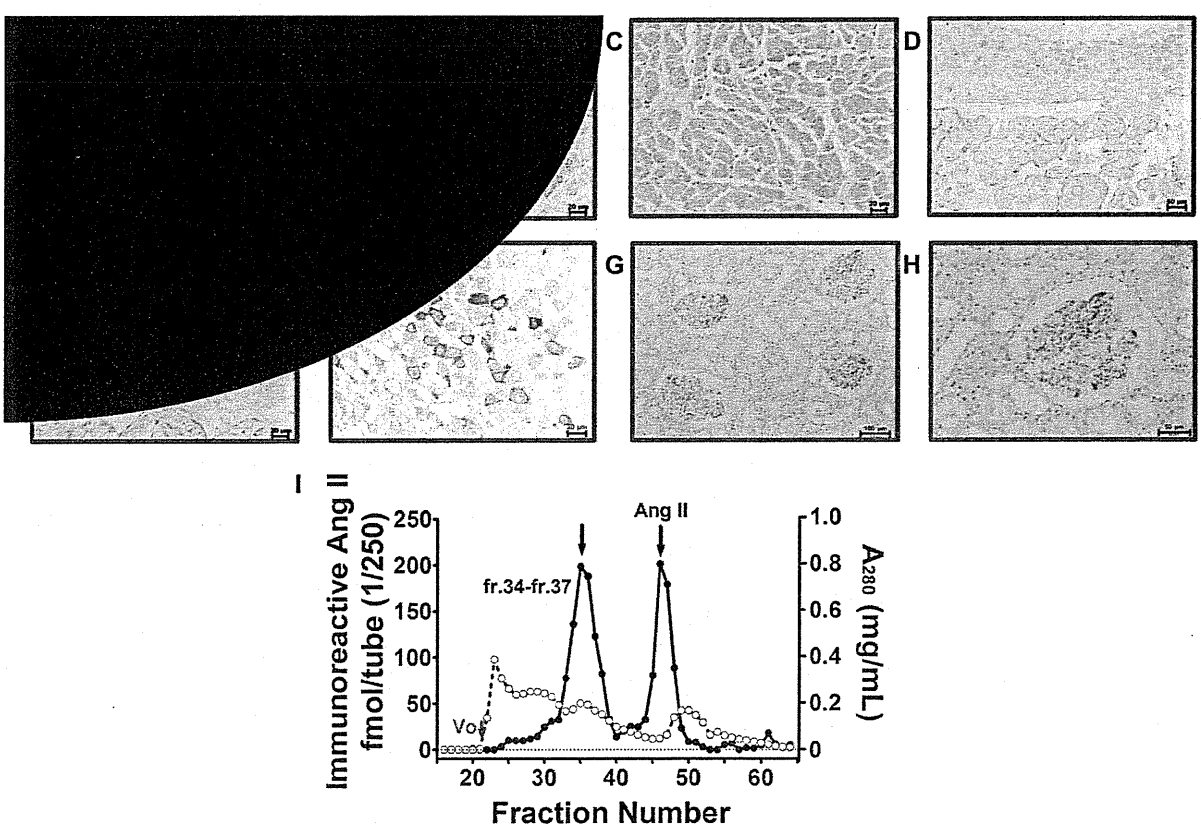

Fig. 3. (A–H) Immunohistochemical staining of Bang-25 in representative sections of human tissue. Bang-25 immunostaining in adrenal gland (A), pancreas (islet cells; B), heart (C), placenta (D–F) and kidney (G and H). The staining in kidney was localized to podocytes (G and H). (I) Immunoreactive Ang II N-terminal in placenta. An extract of human placenta was subjected gel filtration (Sephadex G50), and the immunoreactive Ang II N-terminal in each fraction was assayed using a specific RIA: closed circles, Ang II N-terminal (fmol/tube); open circles, absorbance at 280 nm (mg/ml).

In the present study, we isolated and identified a novel Aogen-derived peptide, Bang-25, which is composed of 25 amino acids, is N-glycosylated on its 14th amino acid (Asn), and has a cysteine linked to its 18th amino acid (Cys). The identification of Bang-25 suggests the existence of a RAS processing cascade different from the renin-catalyzed cleavage of Aogen to Ang I, and provides a potential target for assessing Ang II in tissue and for the development of new therapeutic approaches to related diseases.

Acknowledgments

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Adrenomedullin as a Potential Therapeutic Agent for Inflammatory Bowel Disease

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Abstract: Adrenomedullin (AM) was originally isolated from human pheochromocytoma as a biologically active peptide with potent vasodilating action but is now known to exert a wide range of physiological effects, including cardiovascular protection, neovascularization, and apoptosis suppression. A variety of tissues, including the gastrointestinal tract, have been shown to constitutively produce AM. Pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1, and lipopolysaccharides, induce the production and secretion of AM. Conversely, AM induces the downregulation of inflammatory cytokines in cultured cells. Furthermore, AM downregulates inflammatory processes in a variety of different colitis models, including acetic acid-induced colitis and dextran sulfate sodium-induced colitis. AM exerts anti-inflammatory and antibacterial effects and stimulates mucosal regeneration for the maintenance of the colonic epithelial barrier. Here, we describe the first use of AM to treat patients with refractory ulcerative colitis. The results strongly suggest that AM has potential as a new therapeutic agent for the treatment of refractory ulcerative colitis.

Keywords: Adrenomedullin, anti-inflammatory action, inflammatory bowel disease, inflammatory cytokines, translational research, ulcerative colitis.

INTRODUCTION

Inflammatory bowel disease (IBD) is a refractory ailment, involving an autoimmune response to one or more as yet unknown triggers in the intestinal tract. The two major types of IBD are ulcerative colitis (UC) and Crohn's disease (CD), and an estimated 1.4 million persons in the United States are afflicted with these diseases [1]. In Japan, the number of IBD patients has recently increased, and it has been estimated that more than a hundred thousand patients currently suffer from UC. The etiology of IBD is unknown, but it has been proposed that genetic, immunological and environmental factors are involved.

The four major classes of medication currently used to treat IBD act through the induction and maintenance of remission [2]. These medications include aminosalicylates (5-ASA), steroids (prednisolone), immune modifiers (azathioprine, 6-MP and methotrexate) and biologics (infliximab). However, the results obtained with these medications are not completely satisfactory in approximately one-quarter of UC patients, as their effects are either insufficient or lead to complications. In these cases, surgical treatment (colectomy) might be considered.

In addition to conventional steroid therapy, steroid-resistant or steroid-dependent immune modifiers or biologics are commonly used to treat refractory UC. Although these therapies are often effective, the risk of contracting potentially serious infections, such as fungal diseases and tuberculosis, particularly in patients with diabetes mellitus, is a major concern. Moreover, immune modifiers might cause malignant lymphoma in young patients. Therefore, new and effective drugs that lack these adverse side effects are highly desirable.

An estimated 1-2 million people in the United States have ulcerative colitis or Crohn disease, with an incidence rate of 70-150 cases per 100,000 individuals. An estimated 1-2 million people in the United States have ulcerative colitis or Crohn disease, with an incidence rate of 70-150 cases per 100,000 individuals. An estimated 1-2 million people in the United States have ulcerative colitis or Crohn disease, with an incidence rate of 70-150 cases per 100,000 individuals. An estimated 1-2 million people in the United States have ulcerative colitis or Crohn disease, with an incidence rate of 70-150 cases per 100,000 individuals.

Adrenomedullin (AM) is a potent vasodilator peptide originally isolated from human pheochromocytoma tissue based on its ability to elevate platelet cAMP levels [3]. In addition to hypotensive activity, AM has been implicated in a remarkable range of actions, including the regulation of cellular growth and differentiation, stimulation of angiogenesis and modulation of hormone secretion, among others [4-6]. AM also inhibits inflammation and supports tissue homeostasis through the suppression of pro-inflammatory cytokine synthesis and the induction of wound healing [6-8]. In fact, AM administration ameliorates the induction of colitis in animal models [9-13], and because AM is an endogenous

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biologically active peptide, this drug represents a potentially effective and safe treatment for IBD.

In this review article, we present general information on AM, describing the physiological and pathophysiological roles of this peptide within gastrointestinal organs and its therapeutic effects in animal models of colitis and patients with refractory UC.

GENERAL INFORMATION ON AM

AM is a biologically active peptide initially isolated from human pheochromocytoma derived from the adrenal medulla [3]. The human AM gene is located at the distal end of the short arm of chromosome 11 (p15.1-3) [14]. In humans, the AM protein is composed of 52 amino acids and belongs to the calcitonin peptide superfamily, which also includes calcitonin gene-related peptide (CGRP), amylin and AM2/intermedin [15]. The functional AM or CGRP receptor comprises a calcitonin receptor-like receptor (CLR) and a receptor activity-modifying protein (RAMP) [16]. Three types of RAMPs are expressed in mammalian tissue, and CLR functions as either a CGRP or an AM receptor, depending on the RAMP subtype coexpressed.

AM is nearly ubiquitously distributed in human tissues, including immune cells and gastrointestinal organs (Table **1**). Immunoreactive AM has been identified in cardiovascular, renal, respiratory, gastrointestinal, reproductive, neurological, endocrine and immune tissues [17]. AM is produced in stromal and parenchymal cells, and the synthesis of AM is influenced through numerous factors. For example, cardiovascular hormones, such as angiotensin II and endothelin, increase the production of AM in vascular smooth muscle cells (VSMCs). AM production is also regulated through physical factors, such as shear stress, ventricular wall stress and hypoxia, and inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 [18]. Lipopolysaccharide (LPS) also stimulates the synthesis and secretion of AM in endothelial cells, VSMCs and various other cell types [18].

AM has generally been characterized as a cardiovascular hormone. Consistently, AM is also an essential cardiovascular peptide involved in regulating circulation and body fluid control. In addition, AM reportedly exerts angiogenic, lymphangiogenic, antioxidant, tissue protective and reparative, and anti-inflammatory effects. The multiple biological activities of AM have been described in three recent reviews [4, 5, 6].

PATHOPHYSIOLOGICAL FUNCTION OF AM IN INFLAMMATION

Plasma AM concentrations are elevated in patients with hypertension, congestive heart failure, myocardial infarction, renal disease and the acute phase of stroke [19-21]. Given that AM is a circulation control factor with potent hypotensive and natriuretic activity, the elevation of this peptide in cardiovascular disease might inhibit disease progression. Plasma AM concentrations are also markedly increased in patients with septic shock [22-23], and patients with UC also show high plasma AM levels. As previously discussed, AM synthesis is induced through pro-inflammatory cytokines, such as TNF-α and IL-1, and LPS. Conversely, several groups have demonstrated that AM induces the downregulation of inflammatory cytokines in Swiss 3T3 fibroblasts [24] and rat alveolar macrophages [25]. In addition, AM possesses immunomodulatory activities, which downregulate inflammatory processes in a variety of different models, including arthritis [26] and pancreatitis [27]. These observations suggest that, beyond its vasorelaxant activity, AM might act as a negative regulator of the inflammatory response. Indeed, AM secretion and plasma AM levels are both markedly increased in response to endotoxic shock [28]. Furthermore, heterozygous AM knockout mice are significantly more sensitive to endotoxic shock than wild-type mice [29], while transgenic mice overexpressing AM are resistant to endotoxic shock [30]. Taken together, these findings suggest AM is an anti-inflammatory peptide that exerts protective effects in the context of endotoxic shock and inflammatory disorders.

AM IN THE GASTROINTESTINAL TRACT

AM is widely distributed throughout the gastrointestinal tract and markedly expressed in the stomach and colon, as summarized in Table **1**. Immunohistochemical analysis revealed the presence of AM-immunoreactive (IR) cells in the pyloric glands, where they also stained for chromogranin A and gastrin [31]. AM-IR cells are also found in the mucosal and glandular epithelia of the digestive tract, as well as in the endocrine and neuroendocrine systems [17]. Interestingly, strong staining for AM was observed in the tissue surrounding gastric ulcers [32], and the AM expression in those regions increased during the healing and scarring stages of gastric ulcers, thereby implicating AM in gastric mucosal healing. Indeed, AM exhibited significant ulcer-healing activity in an animal model of gastric ulcers [33-34]. It has been suggested that AM stimulates ulcer healing through the preservation of gastric blood flow, inhibition of gastric acid secretion, stimulation of angiogenesis and the proliferation of mucosal epithelial cells. However, it was recently reported that AM expression is increased in human colon cancer cells [35-36]. Thus, AM might contribute to the pathogenesis of colon cancer through its ability to stimulate angiogenesis and cell proliferation.

EFFECTIVENESS OF AM IN EXPERIMENTAL COLITIS

As summarized above, AM reportedly exerts anti-inflammatory effects through the inhibition of TNF-α and IL-1β expression and protects against experimentally induced ulcers of the gastric mucosa; AM immunoreactivity has also been detected throughout the gastrointestinal tract, with comparably high concentrations in the stomach and colon [31]. Although little is known about the physiological and pathophysiological functions of AM in the gastrointestinal tract, these findings suggest that the endogenous expression of AM in the colon might provide protection against inflammatory bowel diseases. To examine this idea, we assessed the effect of synthetic AM on acetic acid-induced colitis and the production of cytokines [9]. Colitis was induced using the method of Kojima *et al.* [37], after which AM or saline was administered once a day for 3-10 days. We observed that treatment with synthetic AM significantly reduced edema and the size and severity of the ulcerative

Table 1. Location and Production of Adrenomedullin (AM) in the Immune System and Bowel Mucosa

Tissue or cell	Origin	Methods	References
Thymus	Rat	mRNA	[56]
Macrophages	Mouse	RIA/mRNA	[57, 58]
Macrophages	Human	RIA	[59]
Macrophages	Human	RIA/ICC/mRNA	[60, 61]
Macrophages	Human	ICC/mRNA	[62]
Follicular Fluid Macrophages	Human	RIA	[63, 64]
Cell line RAW264-7	Mouse	RIA/mRNA	[58]
Cell line THP-1, HL60	Human	RIA	[59]
Granulocyte	Human	RIA	[59]
Lymphocyte	Human	RIA	[59]
Lymphoblastoid cell	Human	mRNA	[65]
Monocyte	Human	RIA	[59]
Monocyte	Human	mRNA/microarray	[66]
Monocyte	Human	RIA/ICC/mRNA	[60, 61]
Mast cell	Human	RIA/ICC	[67]
Mast cell	Human, Rat	ICC	[68]
Bowel mucosa	Human	ICC	[69]
Bowel mucosa	Rat, Mouse	mRNA	[70]

lesions (Fig. **1**). The microscopic examination of colonic mucosa samples from AM-treated rats showed less severe ulceration, less edema and milder inflammatory cell infiltration of the lamina propria (Fig. **1**). Furthermore, the IL-6 levels in the affected tissues were significantly lower in AM-treated rats than in control rats. However, AM had no significant effect on the interferon (IFN)-γ levels. The ability of AM to reduce the ulcerative area was dose-dependent, with the beneficial effects of AM waning somewhat at higher doses (Fig. **2**).

We further evaluated the effects and mechanisms of AM action in dextran sulfate sodium (DSS)-induced colitis, a commonly used experimental model of UC, focusing on epithelial barrier function [10]. Control mice administered DSS showed profound and sustained weight loss, with diarrhea and hematochezia, a major symptom of colitis (Fig. **3a**); AM-treated mice maintained normal body weights and other clinical symptoms were suppressed. The histological analysis of tissue samples collected from control mice on day 10 after DSS administration revealed a remarkable thickening of the colonic wall, with pronounced crypt abscesses, endothelial erosions and destruction of epithelial integrity (Fig. **3b**). In contrast, the AM-treated mice exhibited much less severe histological manifestations, although a slight thickening of the colon wall was observed (Fig. **3c**). Because intestinal intraepithelial T lymphocytes (IELs) are essential for maintaining epithelial function and preventing inflammatory responses, we examined IELs isolated from the large intestine. We observed that IELs isolated from AM-treated mice consistently produced less IFN-γ, TNF-α and IL-6 than control mice, while transforming growth factor (TGF)-β production was induced through AM treatment (Fig. **4a**).

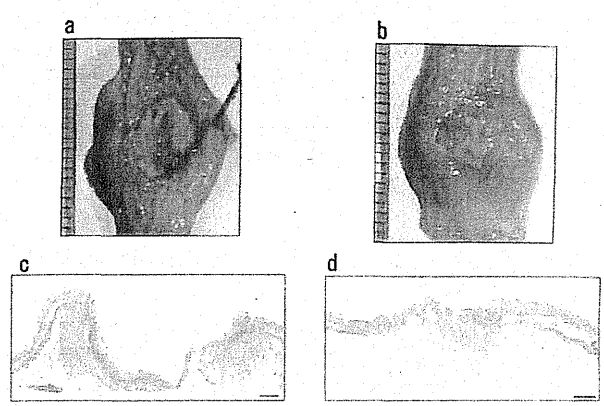

Fig. (1). Macroscopic and histological appearance of colonic ulcers induced through the subserosal injection of acetic acid. Rats ware treated with AM (b, d) or saline alone (a, c) for 5 days. The AM-treated rats showed milder ulceration, less edema and less inflammatory cell infiltration of the lamina propria (modified and redrawn from ref. 9).

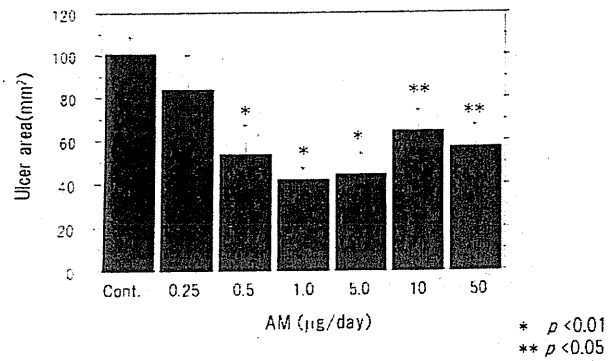

Fig. (2). Effects of AM on the size of acetic acid-induced colonic ulcers observed 5 days after induction (n = 5 for each group). AM (0.25-50.0 μg/day) dose-dependently reduced the ulcer area, but the beneficial effect diminished at higher AM doses. The bars represent the means ± S.E.; $*P < 0.01$, $**P < 0.05$ vs. control (modified and redrawn from ref. 9).

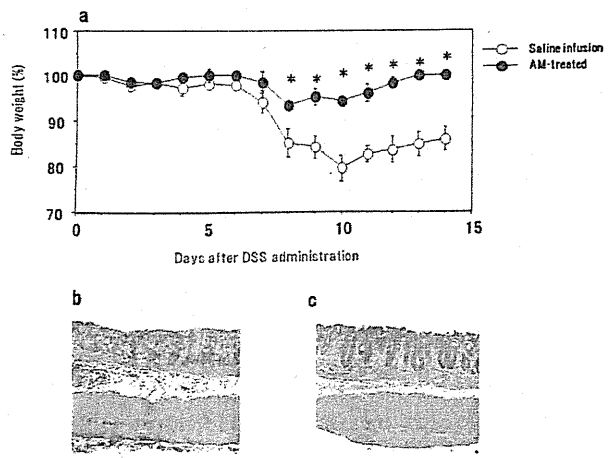

Fig. (3). Preventive effects of AM in DSS-induced colitis-associated weight loss and mucosal injury. (a) Body weight changes in mice treated with or without AM after DSS administration. The values represent the means ± SD. $*P < 0.05$ vs. control. (b, c) HE staining of colon sections prepared from control (b) and AM-treated (c) mice on day 10 after DSS administration (modified and redrawn from ref. 10).

The induction of DSS colitis leads to changes in the IEL profile, with a relative decrease in the numbers of T-cell receptor (TCR)γδ+ cells, which express TGF-β and might act as regulatory T cells to suppress inflammation. Treating mice with AM prevents the reduction in the TCRγδ IEL population, thereby inhibiting the production of inflammatory cytokines, increasing the production of TGF-β, and suppressing signal transducer and activator of transcription (STAT)3 and STAT1 activation in epithelial cells. In DSS colitis, the mRNA expression of junctional molecules, such as ZO-1 and occludin, which contribute to the structure and function of tight junctions, are drastically reduced. However, these reductions are much smaller in AM-treated mice (Fig. 4b), which might contribute to the suppression of the disease trajectory.

AM also exerts potent antimicrobial activity against a variety of bacteria [38-39]. For example, AM shows strong antibacterial activity against *Escherichia coli*, and the distribution of AM within the mucosa is similar to that of defensin family proteins [35], suggesting that AM might also contribute to defense against infection. In addition, the numbers of bacterial anaerobes present in the gastrointestinal tracts of AM-treated mice are significantly lower than those in control mice. Notably, the numbers of facultative anaerobes reportedly correlate with IBD activity in humans [40]. Thus, AM might protect against mucosal epithelium disruption through the suppression and translocation of anaerobes in the intestinal mucosa. However, the anti-bacterial effects of AM are exerted at higher concentrations (10-1000 nM) compared with the receptor mediated effects, such as anti-inflammation or mucosal regeneration [38, 41]. Because AM is typically administered at concentrations that are not high enough to exhibit anti-bacterial effects, the reduced numbers of bacterial anaerobes observed might reflect a secondary phenomenon of mucosal healing.

Reports from other laboratories also suggest that AM exerts beneficial effects against experimental models of colitis. For example, the administration of AM suppressed the inflammatory response and mediated the partial regeneration of mucosal immune tolerance in a 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model, which is a model of severe IBD [11]. In addition, AM acts as an efficient counter-regulatory molecule that protects and improves microcirculation adversely affected through cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS)/NO abnormalities during inflammation [12,42]. Hayashi *et al.* recently reported that AM ameliorates DSS-induced colitis through the suppression of systemic and local production of cytokines, such as TNF-α and IL-6, which accelerated ulcer healing and colonic mucosal regeneration [13]. AM also reportedly reduces inflammatory indices and histological inflammation in DSS-induced murine colitis, and these beneficial effects were associated with protection of the mucosa through the fine-tuning of hypoxia-induced factor (HIF) activity [43].

Given its effectiveness in experimental colitis models, we suggest that AM is potentially a new therapeutic agent for the treatment of IBD, exerting anti-inflammatory and antibacterial effects through the stimulation of mucosal regeneration and maintenance of the colonic epithelial barrier, as summarized in Table 2.

TRANSLATIONAL RESEARCH ON AM AS A THERAPEUTIC AGENT FOR REFRACTORY ULCERATIVE COLITIS

AM exerts beneficial effects in human patients with cardiovascular diseases, such as myocardial infarction, congestive heart failure, hypertension, pulmonary hypertension and limb ischemia [4,6]. Kataoka *et al.* [44] conducted the first clinical pilot study of the use of intravenous AM in patients with acute myocardial infarction. In this study, AM was infused at 12.5-25 ng/kg/min for 12 hours, during which the hemodynamic parameters in patients remained nearly unchanged. The AM infusion significantly improved the wall motion index in the infarct area at 3 months post-infarction compared with the baseline. Intravenously infused AM

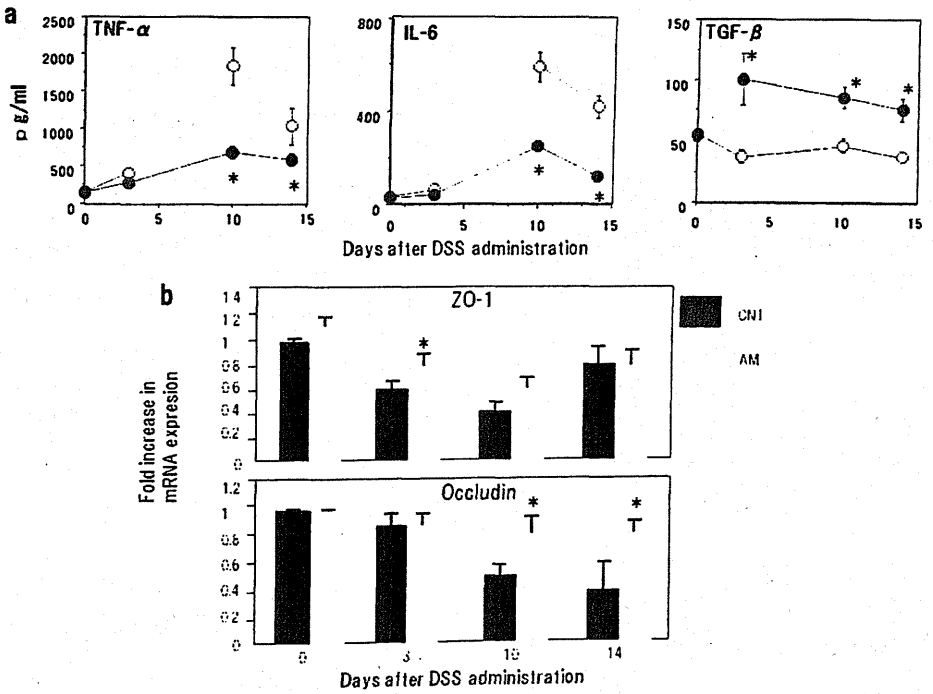

Fig. (4). AM administration suppresses the production of inflammatory cytokines in the large intestine and enhances the recovery of the expression of junctional molecules (ZO-1 and occludin) on epithelial cells. (a) Changes in the cytokine production in IELs. The values represent the means ± SD of three individual experiments. *$P < 0.05$ vs. control. (b) The mRNA expression of ZO-1 and occludin was analyzed quantitatively using real-time PCR. The data are presented as the ratio of the degree of expression at each time point to the data on day 0 in the control mice. *$P < 0.05$ vs. control (modified and redrawn from ref. 10).

potentially exerts protective cardiovascular effects, without severe adverse effects, as a potential adjunct to percutaneous coronary intervention.

Table 2. Anti-Inflammatory Activities of Adrenomedullin (AM) in the Context of Inflammatory Bowel Diseases

Anti-inflammatory activities	References
Regulation of inflammatory responses	
↓TNF-alpha	[24, 71]
↑IL-6, IL-10	[71-73]
↓Th1 response: IL-2 and INF-γ	[11, 74]
↑Regulatory T (Treg) cells	[10, 75]
Tissue repairing and protection	
Mucosal healing	[9, 76-78]
Angiogenesis, improvement of blood flow	[79, 80]
Inhibition of apoptosis	[81, 82]
↑Junctional molecules	[10, 83]
↓Permeability	[10, 84-86]
Anti-bacterial effects	[41, 86, 87]

In patients with essential hypertension [45-46], prolonged AM administration elicited a strong and steady reduction in blood pressure (Fig. 5); however, AM (15 ng/kg/min) also induced an increase in circulating C-reactive protein (CRP). This effect was confirmed in every participant, without exception, and suggests that AM might inhibit or stimulate inflammation, depending upon the setting. Consistently, AM downregulates TNF-α, an important mediator of cytokine production, in macrophages and Swiss 3T3 cells [24-25], but stimulates IL-6 production in NR8383 and Swiss 3T3 cells [47-48]. Furthermore, the bell-shaped dose-response curves in Fig. 2 suggest that the beneficial effect of AM on the ulcerative area is dose-dependent, and this effect tends to wane at higher doses. The bell-shaped dose-response curves are defined as hermetic or biphasic dose-responses, where higher doses are less effective than lower doses. It has also been reported that AM showed a bell-shaped inhibition curve for reserpine-induced gastric lesions [7]. This effect of AM has been previously observed for CGRP [49], amylin [50], and other gastrointestinal peptides [51] and might reflect the interaction of two or more receptors mediating opposite actions [52]. Alternatively, AM receptor-dependent multi-state cell-signaling pathways have been proposed [53-54].

The bell-shaped dose-response curves might suggest that an AM dose of 15 ng/kg/min is too high for the treatment of IBD. Thus, we selected a dose of 9 ng/kg/min of AM for the clinical treatment of UC, which does not evoke severe hypotension or stimulate CRP production. In addition, we confined the administration of AM to eight hours a day to ensure

Table 3. Merit and Demerit of the Therapies for Inflammatory Bowel Diseases

Agents and mechanisms	Merit	Demerit
Anti-inflammatory agent (SASP / 5-ASA) Inhibition of LTB4 production Removal of reactive oxygen	Safeness	Insufficient for severe UC
Hormonal agent (Prednisolone) Inhibition of inflammatory cytokines Inhibition of cell proliferation and differentiation Inhibition of cell infiltration	Potent anti-Inflammatory effect	Cumulative toxicity, Metabolic abnormality, Susceptibility to infection
Immune suppressor (Azathioprine/6MP) Inhibition of DNA synthesis Immune suppressor	Maintenance of remission	Myelosuppression, Susceptibility to infection, Malignant lymphoma?
Immune suppressor (cyclosporine, tacrolimus) Inhibition of IL-2	Potent anti-Inflammatory effect	Monitoring the concentration in blood, Susceptibility to infection, Finger tremor, Renal damage
Anti-cytokine agent (Infliximab, Adalimumab) Anti-TNF-α	Potent anti-inflammatory effect	Tachyphylaxis, Allergy susceptibility to infection(tuberculosis), Malignant lymphoma?
Blood component removal (LCAP/GMA) Removal of activated leukocytes	Safeness	Delayed effective (2 weeks~), Troublesome
Endogenous peptide (Adrenomedullin) Mucosal healing Inhibition of inflammatory cytokines and angiogenesis Amelioration of ischemia	Safeness New mechanism	Hypotensive effect

the safety of this treatment. Herein, we describe the first use of AM to treat UC. The results strongly suggest that AM administration is an effective treatment for refractory UC [55].

The subject was a 68-year-old woman, undergoing treatment for diabetes, who presented with a 3-year history of refractory UC. During previous UC flare-ups, clinical remission was obtained using high-dose steroid infusion and leukocytapheresis; however, because the mucosal repair was insufficient, a regimen of continuous prednisolone (PSL) and azathioprine (AZA) was also prescribed. Despite this therapy, the patient's condition worsened, and she experienced severe abdominal pain and bloody stool. A colonoscopic examination revealed deep ulcerations and erosions throughout the large intestine (Fig. **5**). Higher doses of PSL in combination with leukocytapheresis failed to induce remission (Ulcerative Colitis Disease Activity Index (UCDAI) score: 7). After ruling out ischemic heart disease, cerebrovascular disease and malignancy, AM (9 ng/kg/min) was intravenously administered 8 hours per day for 12 days. After treatment with AM for a few days, the patient noticed that her abdominal pain and bloody stool were dramatically alleviated. No adverse effects were observed, except a slight reduction in blood pressure. The colonoscopic examination performed after 2 weeks revealed significant mucosal regeneration (Fig. **6**), which gastroenterologists had never encountered, and the patient's UCDAI score was reduced to 2. By 3 months, the colonoscopic examination revealed that all of the colonic lesions had healed, with scarring, and the patient's UCDAI score reached 0, prompting the withdrawal of the PSL. Thus, AM administration might be an effective treatment for both animal models of colitis and patients with refractory UC. However, further clinical studies are recommended to confirm reproducibility and determine the optimal dose of AM for the treatment of refractory UC.

CONCLUSIONS

In this review, we provided general information concerning the physiological and pathophysiological roles of AM in the gastrointestinal tract and translational research demonstrating the safety and efficacy of AM for the treatment of refractory UC. AM is a multifunctional cardiovascular hormone that exerts curative effects on cardiocirculatory dynamics. Thus, AM has been primarily investigated in the context of cardiovascular disease. However, AM also exhibits organ-protective and tissue-regenerative properties, exerts

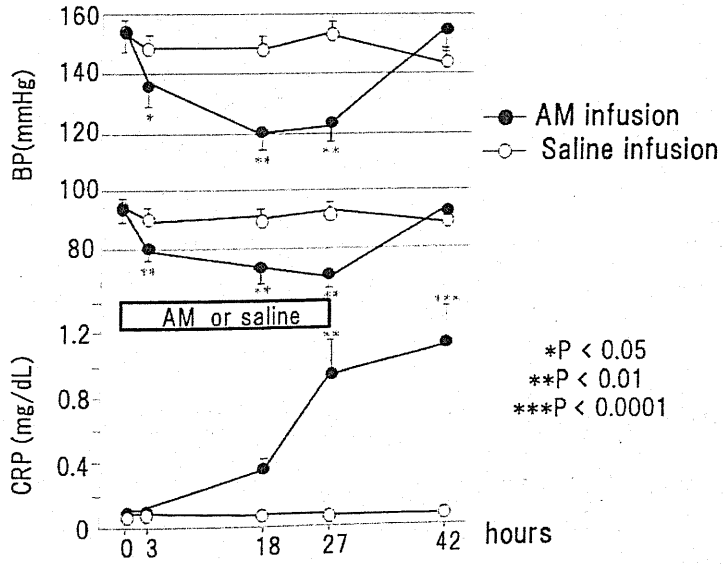

Fig. (5). Changes in the blood pressure and serum CRP levels during the infusion of AM or vehicle. The data are presented as the means ± s.e.m. **P*< 0.05, ***P*< 0.01, or ****P*< 0.0001 vs. baseline (modified and redrawn from ref. 46).

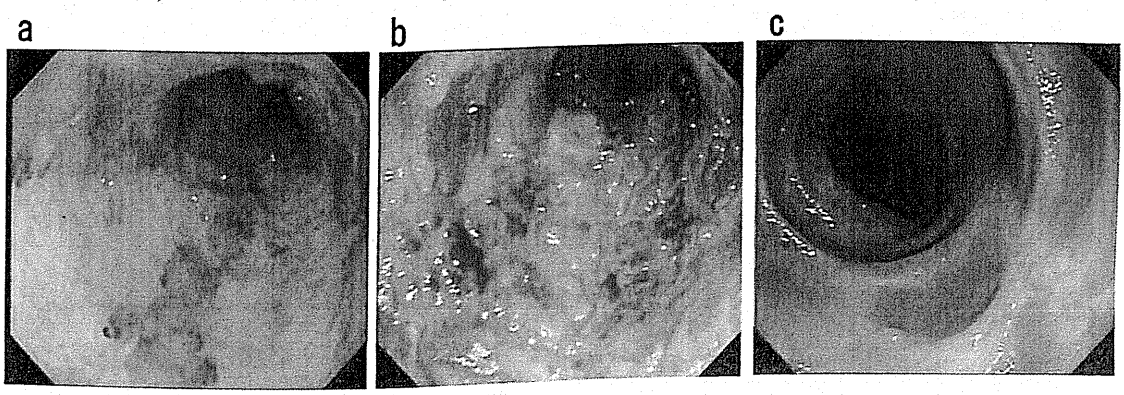

Fig. (6). Colonoscopic findings: (a) before treatment with AM, (b) 2 weeks after treatment with AM, and (c) 3 months after treatment with AM (modified and cited from ref. 55).

anti-inflammatory and angiogenic effects and stimulates epithelial and mucosal cell proliferation. These findings suggest that AM could serve as an effective therapeutic agent for the treatment of a variety of diseases. With respect to its use in the treatment of refractory UC, because AM is an endogenous peptide, no antigenicity is observed. Moreover, the functions of AM differ from those of existing immunomodulators, such as steroids, immune modifiers and biologics. Although additional study is required, AM might potentially serve as a new therapeutic agent for the treatment of refractory UC.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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LIST OF ABBREVIATIONS:

AM	=	Adrenomedullin
AZA	=	Azathioprine
CD	=	Crohn's disease
CGRP	=	Calcitonin gene-related peptide
CLR	=	Calcitonin receptor-like receptor
DSS	=	Dextran sulfate sodium
HIF	=	Hypoxia-induced factor
IBD	=	Inflammatory bowel disease
IELs	=	Intraepithelial T lymphocytes
IFN	=	Interferon

IL = Interleukin

iNOS = Inducible nitric oxide synthase

IR = Immunoreactive

LPS = Lipopolysaccharide

PSL = Prednisolone

RAMP = Receptor activity-modifying protein

STAT = Signal transducer and activator of transcription

TCR = T-cell receptor

TGF = Transforming growth factor

TNBS = 2, 4, 6-trinitrobenzene sulfonic acid

TNF = Tumor necrosis factor

UC = Ulcerative colitis

UCDAI = Ulcerative Colitis Disease Activity Index

VSMCs = Vascular smooth muscle cells

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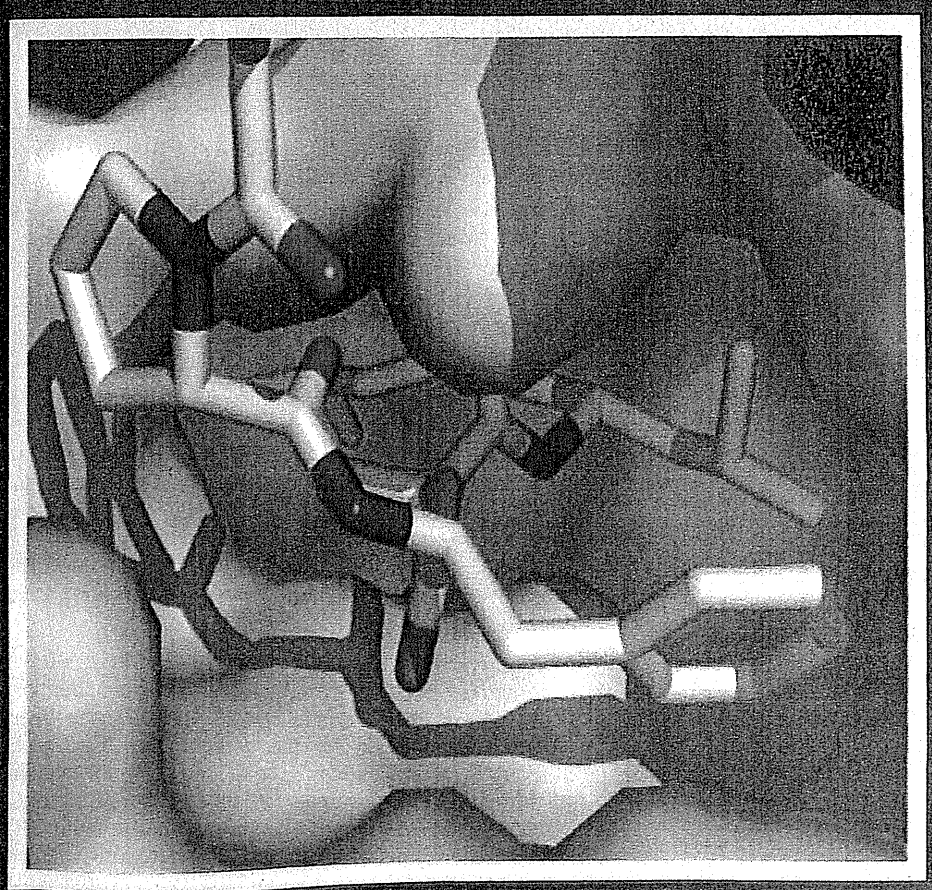

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Functions of Third Extracellular Loop and Helix 8 of Family B GPCRs Complexed with RAMPs and Characteristics of their Receptor Trafficking

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Abstract: At least one of three receptor activity-modifying proteins (RAMP1, RAMP2 and RAMP3) can interact with 10 G protein-coupled receptors (GPCRs; nine Family B GPCRs and a Family C GPCR). All three RAMPs interact with the calcitonin (CT) receptor (CTR), the CTR-like receptor (CLR), the vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating polypeptide (PACAP) 1 ($VPAC_1$) and the $VPAC_2$ receptor, which are all Family B GPCRs. Three RAMPs enable CTR to function as three heterodimeric receptors for amylin, which is a feeding suppression peptide. These RAMPs also transport the CLR to the cell surface, where they function as a CT gene-related peptide (CGRP) receptor (CLR/RAMP1 heterodimer) and two adrenomedullin (AM) receptors (CLR/RAMP2 and CLR/RAMP3 heterodimers). CGRP and AM are potent hypotensive peptides that exert powerful protective effects against multi-organ damage. We recently reported that the third extracellular loop (ECL3) of CLR governs the activation of AM, but not CGRP, signaling in the three CLR/RAMP heterodimers. Furthermore, we showed that in the presence of RAMP2, the eighth helix (helix 8) in the proximal portion of the cytoplasmic C-terminal tail of the CLR, which is thought to be present in all family B GPCRs, participates in receptor signaling. In addition, we demonstrated that overexpression of GPCR kinase (GRK) 2, GRK3 and GRK4 enhances the AM-induced internalization of the CLR/RAMP2 heterodimer. In this review, we describe these studies and consider their implications for other Family B GPCRs that can interact with RAMPs.

Keywords: Receptor activity-modifying protein, calcitonin receptor-like receptor, calcitonin gene-related peptide, adrenomedullin, receptor activation, receptor internalization, G protein-coupled receptor kinase.

1. INTRODUCTION

Among the 15 Family B (or secretin-like) G protein-coupled receptors (GPCRs) [1-2], nine can interact with at least one of three receptor activity-modifying proteins (RAMP1, RAMP2 and RAMP3) (Table **1**), and, except for the calcitonin (CT) receptor (CTR)-like receptor (CLR), the remaining eight are all able to translocate to the cell surface with no help of RAMPs [3-10]. RAMP1 and RAMP2 or RAMP3 enable CLR to function as CT gene-related peptide (CGRP) and adrenomedullin (AM) receptors, respectively [4]. The CLR/RAMP2 heterodimer (AM_1 receptor) is more specific for AM than the CLR/RAMP3 heterodimer (AM_2 receptor) [11-13]. The CLR/RAMP1 heterodimer can also induce a strong response to increased concentrations of AM; the potency of AM is approximately 10-fold lower than that of CGRP [14-15]. Both CGRP and AM, which are potent hypotensive peptides, have been shown to exert strong protective effects against multi-organ damage [16-19]. All three RAMPs interact strongly with CTR to form three high-affinity amylin (AMY) receptors and with the vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating polypeptide (PACAP) 1 ($VPAC_1$) and the $VPAC_2$ receptor, with no apparent effect on the receptor pharmacology [3, 5-10]. The parathyroid hormone type 1 receptor (PTH1), the glucagon receptor and the corticotropin-releasing factor 1 receptor (CRF_1) selectively interact with RAMP2, whereas the PTH2 and the secretin receptors selectively interact with RAMP3[4-10].

Unlike Family B GPCRs, the crystal structures of several Family A (or rhodopsin-like) GPCRs have been revealed; movements among the transmembrane (TM) domains accompany their receptor activation [20-23] and there is an eighth α-helix (helix 8) in the proximal portion of the cytoplasmic C-terminal tail (C-tail) [24]. A similar receptor activation mechanism is also thought to occur in Family B GPCRs [25-28] and the structure and functions of the putative helix 8 of Family B GPCRs have recently been reported [29-31].

All Family B GPCRs, unlike Family A GPCRs, have only endogenous peptide ligands. The 'two-step binding model' [32-34] or 'two-domain model' [35-37] has been proposed as a mechanism for the activation of Family B GPCRs. That is, agonist-specificity is primarily associated with the extracellular N-terminal domain (for the C-terminus of the peptide) and secondary recognition through the TM helix (for the N-terminus of the peptide), although there have

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Table 1. Interactions Between Family B GPCRs and RAMPs.

Family B GPCRs	RAMP1	RAMP2	RAMP3	References
CLR	Yes	Yes	Yes	[4, 10, 67]
CTR	Yes	Yes	Yes	[3, 6, 10]
$VPAC_1$	Yes	Yes	Yes	[10]
$VPAC_2$	Yes	Yes	Yes	[3]
CRF_1	-	Yes	-	[3]
PTH1	-	Yes	-	[10]
PTH2	-	-	Yes	[10]
Glucagon receptor	-	Yes	-	[10]
Secretin receptor	-	-	Yes	[7]
$VPAC_2$	-	-	-	[10, 67]
GLP-1 receptor	-	-	-	[3, 10]
GLP-2 receptor	-	-	-	[10]
GHRH receptor	-	-	-	[10]
GIP receptor	ND	ND	ND	
CRF_2	ND	ND	ND	
PAC_1	ND	ND	ND	

See "LIST OF ABBREVIATIONS" in the text. ND: not determined.

been no reports of crystal structures for TM helices and their interactions with peptide ligands in Family B GPCRs. The receptor extracellular loops (ECLs) are also thought to be involved in ligand recognition and receptor activation. Recent crystal structure analyses revealed the association of the ECDs of the PTH1 [38], the GLP-1 receptor [39], the GIP receptor [40], the CRF_1 [41], the CRF_2 [33] and the PAC_1 receptors [42] with their corresponding peptides. Also, the CLR structure was recently published which has strong similarity to the other ECD structures [43].

Upon agonist stimulation, like most Family A GPCRs, Family B GPCRs are rapidly internalized into intracellular vesicular compartments (endosomes) [7, 31, 44-51]. GPCR kinases (GRKs) phosphorylate agonist-activated GPCRs and promote high-affinity binding to arrestins, which prevent the receptor from activating additional G proteins, leading to rapid homologous desensitization [52-54]. As a result of β-arrestin binding, phosphorylated receptors are targeted for clathrin-dependent internalization [52]. As seen with Family A GPCRs, GRKs are also involved in the internalization of Family B GPCRs (Table 2). In general, Family A GPCRs are dephosphorylated in the endosomal compartment after internalization and rapidly recycled back to the cell surface for resensitization. On the other hand, Family B GPCRs tend to be retained in the endosomal compartment and slowly recycled to the plasma membrane or targeted to lysosomes for degradation.

Among three ECLs of Family B GPCRs, little is known about the function of ECL3, and no review details the role of helix 8 of Family B GPCRs. Moreover, there have been few review articles concerning the roles of GRKs in Family B GPCR internalization. Therefore, this review primarily illuminates our current view of the functions of ECL3 and helix 8 in activation of Family B GPCRs associated with RAMPs and the roles of GRKs in trafficking, notably internalization, of this family receptor complexes.

2. CHARACTERISTICS OF RAMPS AND THEIR INTERACTIONS WITH CLR

In mammals, all three RAMPs are composed of ~160 amino acids and exhibit a common structure consisting of a large extracellular N-terminal domain (or ectodomain; ECD), a single TMD and a short C-tail [4, 55-56]. Nevertheless, the three isoforms share less than 30% sequence identity [4, 55-56]. Recent studies revealed the crystal structures of the ECDs of hRAMP1 [57-58] and hRAMP2 [43]. Both hRAMP ECDs are composed of three α-helices (α-helix 1, 2 and 3) that are stabilized through disulfide bonds (three for hRAMP1 and two for hRAMP2) [43, 57-58]. Among these disulfide bonds, two are conserved between both RAMPs [43, 57-58] and their four conserved cysteine residues are crucial for the efficient transport of the hCLR to the cell surface [59-62]. Although the crystal structure of the RAMP3 ECD has not yet been determined, it is expected to possess three α-helices and three disulfide bonds [63].

Cross-linking experiments [64] and crystal structure analyses [57-58] showed a 1:1 stoichiometric association of hCLR and hRAMP1. Likewise, hRAMP2 forms a 1:1 heterodimer with the hCLR in their crystal structure [43]. To be more specific, α-helices 2 and 3 in hRAMP1 and hRAMP2 ECDs interact with N-terminal α-helix 1 of the hCLR [43, 57]. However, both CLR and RAMP1 can form homodimers when expressed alone. [4, 49, 55, 65]. In addition, bioluminescence resonance energy transfer assays showed that a single RAMP1 interacts with a CLR homo-dimer [66]; there is no information about whether two RAMPs can associate with a CLR homo-dimer. Note that the aforementioned findings are all obtained by using recombinant CLR and RAMPs. Therefore, further study will be necessary to clarify the interactions between CLR and RAMP molecules *in vivo*.

3. FAMILY B GPCRS THAT INTERACT WITH RAMPS

Among 15 Family B GPCRs, nine receptors interact with at least one RAMP [4, 6-7, 10] (Table 1). Most GPCRs that

Table 2. Trafficking of Family B GPCRs that Interact with RAMPs.

Receptors	Internalization		Post-internalization	
	Clathrin-dependency	Involvement of GRKs	Recycling	Lysosomal sorting
CLR + RAMP1	Yes [49, 50]	GRK6 [124]	Yes [66]	Yes [49, 50, 66]
CLR + RAMP2	Yes [50]	GRKs 2, 3 and 4 [30]	-	Yes [50]
CLR + RAMP3	Yes [50]	ND	Yes (+ NSF) [109]	Yes [50]
CTR	ND	GRK2 [125]	Yes [48]	Yes (+ filamin) [48]
+ RAMPs	ND	ND	ND	ND
$VPAC_1$	Yes [122]	GRKs 2, 3 and 5 [112]	Yes [46]	-
+RAMPs	ND	ND	ND	ND
PTH1	Yes [113]	GRKs 2, 3 and 5 [123]	Yes [108]	-
+ RAMP2	ND	ND	ND	ND
PTH2	ND	ND	ND	ND
+ RAMP3	ND	ND	ND	ND
Glucagon receptor	Yes [97]	GRKs 2, 3 and 5 [97]	Yes [107]	-
+ RAMP2	ND	ND	ND	ND
Secretin receptor	Yes [114]	GRKs 2, 3 and 5 [121] GRK6 [118]	- [106]	ND
+ RAMP3	ND	ND	ND	ND

Note that the co-expression of RAMP3 did not alter the rate or extent of internalization of the secretin receptor [7].
NSF, *N*-ethylmaleimide-sensitive factor; ND, not determined.
[ ]: appropriate references

interact with RAMPs were identified on the basis of an indicator of N-terminally tagged RAMP translocation to the cell surface, using fluorescence confocal microscopy [10], flow cytometry [67] or an enzyme-linked immunosorbent assay [3, 68]. As shown in Fig. (**1**), similar to the hCLR, the $hCT_{(a)}$ lacking 16 amino acids in the first intracellular loop, which is the major isoform of CTR *in vivo* [13], strongly associates with the three hRAMPs. However, the insert-positive isoform of the CTR, $CT_{(b)}$, also interacts with the three RAMPs [13, 69]. Interestingly, the $VPAC_1$ and $VPAC_2$ receptors also associate with the three RAMPs [3, 10], but there have also been several reports showing that the $VPAC_2$ does not interact any RAMPs [10, 67]; the discrepancy in reports on $VPAC_2$ may be mainly due to cell type specificity (or cell background).

It was shown that the CRF_1, the PTH1 and the glucagon receptor selectively interact with RAMP2 [3, 10], whereas the PTH2 and the secretin receptor selectively interact with RAMP3 [7, 10]. Only the RAMP3-secretin receptor interaction was determined using bioluminescence resonance energy transfer [7].

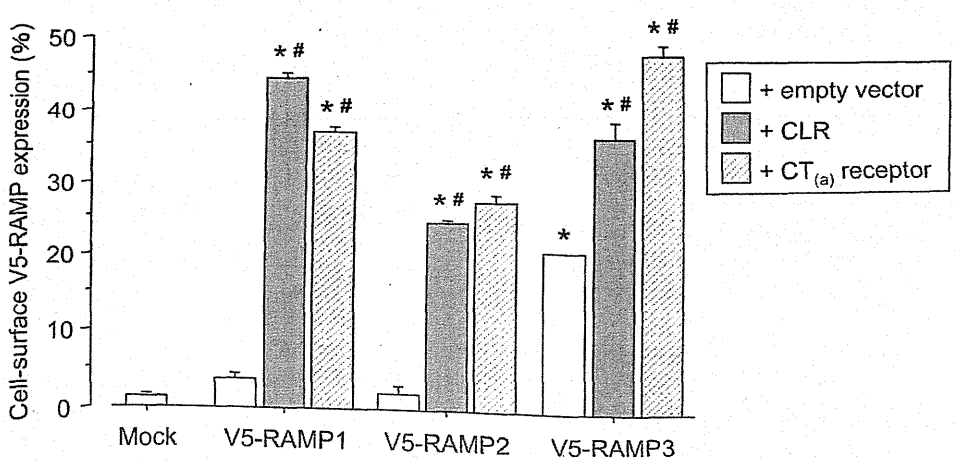

Fig. (1). Analysis of the cell-surface expression of V5-hRAMP1, -2 or -3 following transfection into HEK-293 cells, with or without wild-type hCLR or $hCT_{(a)}$ using flow cytometry. Forty-eight hours after transfection, the cells were incubated for 1 h at 4°C with a monoclonal anti-V5-FITC antibody; mock incubation with the antibody served as a control. FITC-labeled V5-hRAMP expressed at the cell surface was estimated using flow cytometry. Data are shown as the means ± SEM of four separate experiments. $^*p < 0.05$ vs. Mock; $^{\#}p < 0.05$ vs. the corresponding V5-RAMP alone.

Although the three RAMPs are required for the cell-surface translocation of CLR, the remaining eight Family B GPCRs that can interact with each RAMP are expressed at the cell surface in the absence of RAMPs.

4. ROLE OF RAMPS IN FAMILY B GPCR SIGNALING

All Family B GPCRs are preferentially coupled to Gs proteins (for stimulating cAMP signaling). The co-expression of the three RAMPs alters the phenotype of CTR. That is, the three $CT_{(a)}$/RAMP and three $CT_{(b)}$/RAMP heterodimers possess high affinities for amylin (AMY) [6, 13, 69-70], although the $CT_{(b)}$/RAMP2 heterodimer displays a greater capacity to generate AMY signaling than the $CT_{(a)}$/RAMP2 heterodimer [13, 69]. However, the exact phenotype can depend on cellular background.

In contrast, the three RAMPs do not alter the pharmacology of the $VPAC_1$ [10] and the $VPAC_2$ receptors [3]. However, the $VPAC_1$/RAMP2 heterodimer significantly enhances agonist-mediated phosphoinositide hydrolysis with no change in the cAMP response, as compared with $VPAC_1$ receptor alone [10], although the mechanism remains unclear. Similarly, overexpression of RAMP2 enables greater elevation of intracellular calcium, but not cAMP production, through enhancement of Gq coupling to the CRF_1 receptor [3]. RAMPs can also differentially modulate the G protein-coupling efficiency of amylin receptors (AMY_1, AMY_2 and AMY_3) [71]. Apart from these findings, there have been no further reports showing the modification of Family B GPCR signaling through RAMPs.

5. ROLES OF THIRD EXTRACELLULAR LOOP AND HELIX 8 IN RAMP-INTERACTING FAMILY B GPCR ACTIVATION

5.1. Overview of GPCR Activation

Agonist binding to Family A GPCRs induces the rearrangement of the transmembrane (TM) helices [20, 72-75]. That is, a rotation of TM6 and outward movement of the cytoplasmic end of TM6, leading to opening of the binding crevice for the C-terminal α5 helix of the Gα subunit and the activation of G proteins. A similar receptor activation mechanism potentially occurs in Family B GPCRs [25-28], although no crystal structure of a whole receptor is currently available. In Family B GPCRs, the entire receptor from the ECD through to the C-terminus is likely to be involved in the ligand binding and activation process.

5.2. The Role of Third Extracellular Loop of GPCRs

The role of the ECLs of both Family A and B GPCRs was well summarized by Wheatley *et al.* [76]. In addition, the role of the Family B GPCR ECLs was shown in Table 2 by Barwell *et al.* [1]. The ECLs of Family A GPCRs interact with each other and with TM helices. In addition to serving as linkers between TM helices, the ECLs of Family A GPCRs are required for ligand binding, ligand specificity, receptor activation and cell-surface expression [76-79]. The Family B GPCR ECLs are also important for peptide ligand binding and receptor activation [1, 76]. The orientation of ECL2 in the majority of Family A GPCRs is restrained through a conserved disulfide bond between ECL2 and the top of TM3 [80-81]. A previous study suggested that the two cysteine (Cys) residues in the first (Cys212) and second ECL (Cys282) form a disulfide bond for stabilizing the human CLR/RAMP2 heterodimer in the correct conformation for ligand binding and activation [62]. As both cysteine residues are strictly conserved among Family B GPCRs [1, 76], the formation of a disulfide bond between ECL1 and ECL2 is expected.

The ECL3 of Family B GPCRs, similar to ECL2, is predicted to be relatively short compared with ECL1 [1, 76]. There is some conservation of the sequence surrounding the tops (Pro-Asp/Glu) and ends (Ala/Val/Ile-X-Leu/Ile) of ECL3 [1, 76] (Fig. (2)); however, there was little information regarding the role of this loop compared with other ECLs. A recent study showed the effect of single point mutations in hCLR ECL3 on CGRP receptor function in the presence of hRAMP1 [82]. Among the mutant receptors, only the I400A/RAMP1 mutant showed a small but significant reduction in CGRP potency [82]. We recently constructed an hCLR ECL3 chimera, in which the ECL3 of hCLR was substituted with that of $hVPAC_2$ [83] because $hVPAC_2$ was unable to interact with any hRAMPs in human embryonic kidney (HEK)-293 cells used [10, 67]. The co-expression of the hCLR ECL3 chimera with hRAMP2 or hRAMP3 led to marked reductions in AM potency in cAMP signaling, but CGRP potency was barely affected [83]. Interestingly, the chimera significantly decreased AM, but not CGRP, potency in the presence of hRAMP1 [83]. Thus, the hCLR ECL3 is crucial for AM-induced cAMP responses via the three human CLR/RAMP heterodimers, but is less involved in their CGRP responses. ECL3 potentially contributes to AM-mediated conformational changes of these heterodimers, thereby inducing their activation. There have been few reports indicating that RAMPs possess critical sites for the binding of CGRP and AM [18, 63]. Although the results of a recent crystal structure analysis showed that Glu101 in the hRAMP2 ECD is critically involved in AM binding [43], an earlier study showed that the mutation of this acidic residue failed to transport the hCLR to the cell surface, leading to a lack of AM signaling [84]. Therefore, rather than being directly involved in the binding site(s) of CGRP and AM, the three RAMPs might indirectly alter the binding affinity of both peptides to the ECD and TM of the CLR. Taken together, it is likely that CGRP and AM might change the conformation of the CLR differently within the same receptor complex. It would be valuable to clarify the entire crystal structure of the three CLR/RAMP heterodimers in the presence and absence of agonist binding. The possibility that RAMPs can change receptor conformation has implications for other RAMP-complexed Family B GPCRs, particularly CTR and $VPAC_1$, both of which are associated with the three RAMPs.

5.3. The Role of Helix 8 of GPCRs

The results of the crystal structure analyses of Family A GPCRs revealed the presence of an eighth α-helix, called 'Helix 8', oriented perpendicular to the seven transmembrane (TM) helices (TM1 to TM7) in the proximal portion of their C-tails [24]. Helix 8 of Family A GPCRs has previously been reported to play important roles in the surface delivery of GPCRs, the stabilization of GPCRs at the

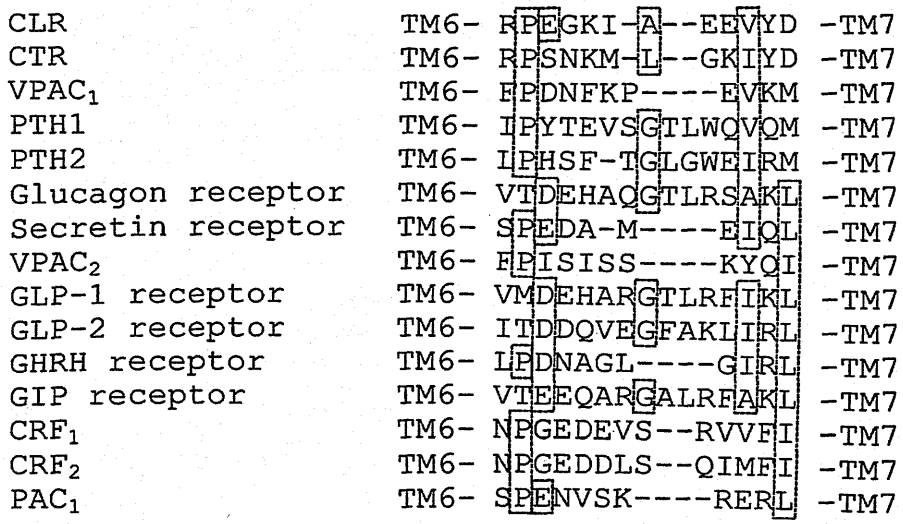

Fig. (2). Alignment of the third extracellular loop (ECL3) of 15 human Family B GPCRs. The *dashed box* represents sequence conservation of more than 50%. See "LIST OF ABBREVIATIONS" at the end of the text. Note that this figure is a modified version of Refs [1, 76].

cell-surface, the coupling of Gs and/or Gq and the activation of GRKs [85-90]. Similarly, all Family B GPCRs potentially possess the putative Helix 8 [29-31] (Fig. (3)). With respect to helix 8, there are no conserved residues among Family A GPCRs [88], while human Family B GPCRs all contain four conserved residues [1, 30-31] (Fig. (3)).

Within the putative helix 8 of the hCLR, Glu430, Val431, Arg437 and Trp439 are strictly conserved among Family B GPCRs. In HEK-293 cells stably expressing hRAMP2, the substitution of each residue within the hCLR helix 8 with alanine (Ala) did not decrease AM binding affinity relative to the wild-type hCLR [29]. Nevertheless, three mutants, Glu430Ala, Arg437Ala or Trp439Ala, showed significant reductions in the potency of AM [29]. It is well known that the receptor/G protein complex has a higher affinity for agonists than the free or uncoupled receptor [91]. These results suggest that hCLR helix 8 may participate in controlling the activation of Gs, rather than Gs coupling. In contrast, there has been one report showing hCLR helix 8 is not involved in CGRP-mediated cAMP accumulation in the presence of hRAMP1 [31]. This finding was obtained using COS-7 cells, derived from the monkey kidney, which transiently co-expressed hRAMP1 and an hCLR mutant lacking helix 8 [31]. The discrepancies between these two studies might reflect differences in the cell backgrounds, transfection methods, cell surface expression levels of the target receptor complex, cAMP assays, the hRAMP isoform and other differences. More work is needed to confirm the role of helix 8 in CLR.

Similar to the function of Glu430 in association with hRAMP2, the corresponding Glu residue (Glu394) in the $hVPAC_1$ receptor is also required for the activation of adenylyl cyclase (AC) [92]. The C-tail of the porcine CTR is also necessary for AC activation [93], but the role of conserved Glu residues in the CTR is still unknown. However, it was reported that the C-tails of the glucagon [94], PTH [95] and secretin receptors [96] in the rat might not be involved in the activation of Gs and AC. However, all of these three studies utilized a truncation approach, which left some or all of helix 8 intact [94-96]. It is therefore possible that the strictly conserved Glu residue in helix 8 of Family G GPCRs plays a general role in activation of Gs proteins.

Putative hCLR helix 8 also contains two dibasic residues (Arg436-Arg437), which are conserved among Family B GPCRs; in particular, Arg437 is highly conserved (Fig. (3)). Arg437, but not Arg436, is involved in the activation of Gs [29]. This Arg437 residue is important for the cell-surface expression of the CLR/RAMP2 heterodimer [29]. Similarly, helix 8 of the $hVPAC_1$ receptor possesses the corresponding dibasic residues (Arg400-Arg401). However, a double mutation of these two Arg residues to Ala residues did not affect the surface delivery, agonist-binding affinity or cAMP responses of the mutant $hVPAC_1$ receptor [92]. It has not been determined whether the highly conserved Arg437 in the hCLR helix 8 is also important for other Family B GPCRs.

The role of helix 8 in other RAMP-interacting Family B GPCRs in the presence of each RAMP is currently unknown. However, the co-expression of each hRAMP did not affect agonist-mediated cAMP stimulation via the $hVPAC_1$ [10] and the $VPAC_2$ receptors [3]. It is therefore unlikely that the three RAMPs modulate the function of helix 8 in either receptor. To date, there have been no reports indicating the effect of RAMP association on cAMP signaling of other RAMP-complexed GPCRs, the PTH1, the PTH2, the glucagon receptor and the secretin receptor.

Helix 8 of Family A GPCRs contains cysteine residues, the palmitoylation of which has been shown to anchor this helix to the plasma membrane [85, 88]. However, Family B GPCRs possess no cysteine residues within helix 8 (Fig. (3)). It has been reported that Trp439 in the hCLR helix 8 might partially fulfill the role of the lipid anchor in the equivalent

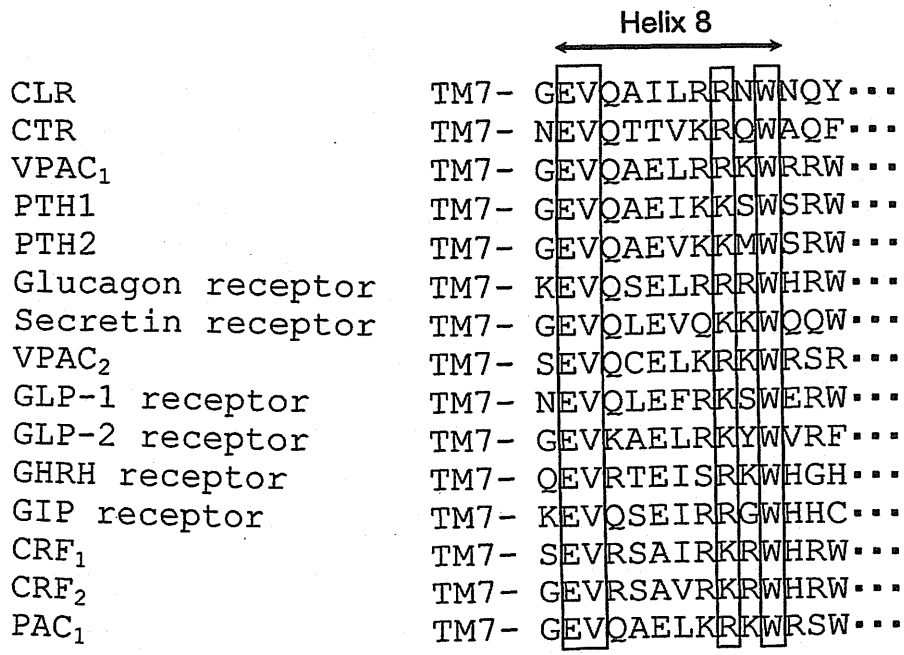

Fig. (3). Alignment of the first 14 residues of the cytoplasmic C-terminal tails of 15 human Family B GPCRs. The four conserved residues, E (Glu), V (Val), R (Arg) and W (Trp), are *boxed.* See "LIST OF ABBREVIATIONS" at the end of the text, and compare this figure with Table 1. Note that this figure is a modified version of Refs [1, 29].

position in many Family A GPCRs [31]. In addition to the activation of Gs and stabilization of the hCLR C-tail, Trp439, which is strictly conserved among Family B GPCRs, is also involved in the surface delivery of the CLR/RAMP2 heterodimer [29]. Whether the multi-function of this Trp439 residue will be applicable to other Family B GPCRs remains unknown.

6. TRAFFICKING OF FAMILY B GPCRS IN THE ABSENCE AND PRESENCE OF RAMPS

6.1. Overview of GPCR Trafficking

Following agonist stimulation, most Family B GPCRs, like most Family A GPCRs, undergo rapid internalization for desensitization (e.g., the hCLR [31, 49-51], the rabbit CTR [48], the hVPAC$_1$ [46-47], the hPTH1 [45], the hPTH2 [44], the rat glucagon receptor [97] and the secretin receptor [7]). In many cases, the agonist-induced activation of Family A GPCRs leads to receptor phosphorylation through second messenger-dependent kinases (e.g., protein kinase A (PKA), PKC), specific GRKs or both [53, 98-99]. In turn, cytoplasmic accessory proteins, called β-arrestins, readily bind to GRK-mediated phosphorylation sites in the third intracellular loop and the C-tail of the receptor, leading to the uncoupling of G proteins, so-called 'receptor desensitization' [52-54]. The complexes consisting of the phosphorylated GPCRs and β-arrestins are subsequently targeted to clathrin-coated pits [100-102]. After the vesicles are pinched off the plasma membrane through dynamin [103], the receptor is internalized into intracellular vesicular compartments (endosomes) [100-102]. There have been reports of differences in the endosomal ability to bind β-arrestins between Family A and B GPCRs [104-105]. In general, internalized Family A GPCRs are dephosphorylated in the endosomes and rapidly recycled to the plasma membrane for resensitization. While Family B GPCRs are retained in the endosomes and slowly recycled back to the cell surface [46, 48, 66, 106-109] or targeted to lysosomes for degradation [48-50, 66] (Table 2).

6.2. Trafficking of GPCRs Able to Interact With RAMPs

As shown in Table 2, the surface hCLR overexpressed in HEK-293 cells undergo agonist-induced internalization (a 30-minute exposure to 100 nM CGRP or AM), together with each hRAMP, through a clathrin-dependent pathway, and after removal of the agonists, these three recombinant CLR/RAMP heterodimers are targeted to lysosomes [50] (Fig. (4)). Hilairet *et al.* [49] clearly demonstrated that the CGRP-induced activation of the recombinant human CLR/RAMP1 heterodimer expressed in HEK-293T cells results in the phosphorylation of the hCLR, but not hRAMP1, and the CGRP receptor is internalized as a stable complex. The receptor internalization was β-arrestin-, clathrin- and dynamin-dependent, and the internalized receptor was also targeted to a degradation pathway [49]. The internalization (a 30-minute exposure to 100 nM AM), without recycling, of endogenous AM receptors has also been shown using anti-hCLR antibody against the C-terminal end of the hCLR protein (15 amino acid residues) in human dermal microvascular endothelial cells, which express CLR, RAMP2 and RAMP3 [110]. On the other hand, in HEK-293 FLP cells expressing rat CLR/hRAMP1, transient stimulation with 100 nM CGRP (1 h) induced slow recycling (~8 h) of the internalized heterodimers, whereas after sustained stimulation with 100 nM CGRP (> 2 h), the CGRP receptors were internalized and then targeted to the lysosomes [111]. These discrepancies in the trafficking of CLR/RAMP het-