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WOJCIECH NOWAK

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By

Wojciech Nowak

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ABSTRACT

REGULATION OF RECEPTOR ACTIVITY MODIFYING PROTEIN EXPRESSION AS A NOVEL MECHANISM FOR MODULATION OF ADRENOMEDULLIN ACTIVITY IN RAT MESANGIAL CELLS

By

Wojciech Nowak

Adrenomedullin (AM), a recently discovered potent vasodilatory peptide, exhibits myriad of physiologic effects by binding to a calcitonin-like receptor (CL receptor). CL receptor is a member of a well-characterized group of G protein-coupled receptors (GPCRs) and exhibits a remarkable capability of ligand specificity switching contingent upon its association with specific member of a novel group of receptor activity modifying proteins (RAMPs). When co-expressed and dimerized with RAMP-1, CL receptor functions as a calcitonin gene-related peptide receptor ($CGRP_1$). Conversely, interaction with RAMP-2 or RAMP-3 renders it a fully functional AM receptor (AM_1 or AM_2, respectively). While the model of ligand-GPCR interaction is generally regarded as well established, the discovery of RAMPs prompted a new look at the paradigm for receptor phenotype determination. It also provided for a plethora of opportunities to recognize and potentially exploit new mechanisms responsible for modulation of receptor activity.

This thesis focuses on the functional interplay between AM and its receptor complex (CL receptor+RAMP-2/3). In particular, in the effort to identify the molecular pathway(s) involved in influencing actions of AM, the herein described experiments investigate the mechanisms and effects of extracellular signals on RAMP gene and protein expression as well as the consequent alteration of AM activity. The experimental model utilized is that of rat glomerular mesangial cells (MC) in culture. MC constitute a

vital structural and functional part of the renal glomerulus and as such, aberration of their normal biology has been shown to directly contribute to the genesis and progression of several glomerulopathies. In addition, MC are the site of production and/or the target for numerous cytokines, hormones, and other extracellular signals of which AM is one of the more recently characterized. AM exhibits potent anti-proliferative and anti-migratory effects on rat MC. These biological responses to AM are receptor mediated, hence amenable to alterations involving any component of the AM receptor complex.

Data from this investigation show that upregulation of RAMP-3 gene and protein expression results in the increase of AM-mediated cyclic AMP production and a concomitant decrease in mesangial cell proliferation. Furthermore, the current study identifies platelet-derived growth factor (PDGF) and lipopolysaccharide (LPS) as extracellular factors increasing RAMP-3 mRNA expression and cell-membrane associated RAMP-3 protein abundance. Both, PDGF and LPS, attain their effects via activation of mitogen-activated protein kinase signal transduction pathway(s). Moreover, the effect of PDGF and LPS on RAMP-3 expression results from stabilization of the RAMP-3 transcript, as observed by an increase in RAMP-3 mRNA half-life, and is independent of new RNA synthesis.

In summary, this study demonstrates that differential expression of RAMP-3 represents a novel mechanism for regulation of mesangial cell's responsiveness to AM. It also identifies the process of RAMP-3 expression as a molecular target for cytokines (PDGF) and other extracellular signals (LPS) known to be involved in the maintenance of normal mesangial cell biology and/or its alteration in disease.

To my parents, who fostered my curiosity

and

my wife whose encouragement allows its growth.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

AM	Adrenomedullin
AM_1	Adrenomedullin receptor (molecular constituents: CL receptor+RAMP2)*
AM_2	Adrenomedullin receptor (molecular constituents: CL receptor+RAMP3)*
AMR	Adrenomedullin receptor (generic)
AMY	Amylin
AMY_1	Amylin receptor (molecular constituents: CTR+RAMP1)*
AMY_2	Amylin receptor (molecular constituents: CTR+RAMP2)*
AMY_3	Amylin receptor (molecular constituents: CTR+RAMP3)*
ANG II	Angiotensin-II
AVP	Arginine-vasopressin
cAMP	Adenosine 3'-5' cyclic monophosphate
CGRP	Calcitonin gene-related peptide
$CGRP_1$	CGRP receptor (molecular constituents: CL receptor+RAMP1)*
CL	Calcitonin-like (receptor/protein); formerly CRLR*
CRLR	Calcitonin receptor-like receptor; currently CL receptor*
CT	Calcitonin
CTR	Calcitonin receptor
EC	Endothelial cells
ERK	Extracellular signal-regulated kinase
ESRD	End-stage renal disease
ET-1	Endothelin-1
GFR	Glomerular filtration rate
GPCR	G protein-coupled receptor
HAoEC	Human aortic endothelial cells
HUVEC	Human umbilical vein cells
IFN	Interferon
IL	Interleukin
JNK	c-Jun N-terminal kinase
L-NAME	Nω-nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
MAP	Mean arterial blood pressure
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
NO	Nitric oxide

* Nomenclature recommended by International Union of Pharmacology (IUPHAR). XXXII (as described by Poyner *et al. Pharmacol Rev* 54: 233-246., 2002.). This naming system extends to all of the established receptors for the calcitonin family of peptides. It assigns the receptor name after the endogenous ligand to which it exhibits the highest binding affinity, following the guidelines of IUPHAR (as described by Ruffolo *et al.* in *The IUPHAR Compendium of Receptor Characterization and Classification*, 2nd ed, pp7-8, IUPHAR Media, London, UK., 2000.).

p38 MAPK	p38 mitogen-activated protein kinase
PAMP	Proadrenomedullin N-terminal 20 peptide
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphotidyl inositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PP2A	Protein phosphatase 2A
RAMP	Receptor activity modifying protein
RBF	Renal blood flow
RCP	Receptor component protein
RMC	Rat mesangial cells
RT-PCR	Reverse transcription-polymerase chain reaction
SAPKs	Stress activated protein kinases
TGF	Transforming growth factor
TLR	Toll-like receptors
TNF	Tumor necrosis factor
UTRs	3'-Untranslated regions
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells

1. INTRODUCTION

The recent discovery of receptor activity modifying proteins (RAMPs) has significantly altered our understanding of mechanisms involved in the regulation of G protein-coupled receptors. RAMP-1, 2, and 3 have been shown to differentially couple to calcitonin receptor (CTR) and calcitonin-like receptor (CL receptor) giving rise to distinct receptor phenotype characteristics (199), (41). Receptor trafficking, glycosylation, and a direct RAMP-receptor interaction have all been proposed as possible mechanisms responsible for the critical role of RAMPs in CTR and CL receptor ligand specificity determination (for reviews see Foord *et al.* (82) and Sexton *et al.* (294)). Accumulating data suggests that the dynamic alteration of RAMP expression levels may provide for yet another mode by which these proteins regulate the function of CL receptor hence changing the cellular responses initiated by adrenomedullin (AM) exposure. Given that AM plays an important role in normal as well as pathological processes in mesangial cells, elucidation of mechanisms responsible for changes in AM-receptor interactions becomes of critical importance. The current study was undertaken to investigate the effects of altered RAMP gene expression on the responsiveness of mesangial cells to exogenous AM. In addition, this thesis characterizes platelet-derived growth factor and lipopolysaccharide as agents regulating AM-mediated cellular responses by their ability to alter RAMP-3 mRNA abundance.

This brief introductory chapter is followed by literature review in chapter two. Chapter two offers an in-depth discussion of the scientific literature describing the biology of mesangial cell, characterization of adrenomedullin and its receptor complex, and our current understanding of the adrenomedullin-receptor-RAMP interaction.

Chapters three through five are organized according to the specific aims of this study and independently consist of brief introduction, experimental data, and specific discussion relevant to the particular aim. Thus, chapter three explores the gene expression profile of RAMP-1, 2, 3, and CL receptor in mesangial cells cultured under basal conditions. It also identifies the effects of RAMP-1, 2, or 3 overexpression on AM-mediated mesangial cell proliferation and adenylate cyclase activity. Chapter four incorporates the major aim of examining PDGF and LPS effects on RAMP-3 mRNA and membrane-associated protein expression. It also describes the effects of PDGF and LPS on AM-induced adenylate cyclase activity in mesangial cells. Chapter five investigates further the effects of PDGF and LPS on RAMP-3 mRNA expression with an aim to characterize the molecular mechanisms responsible for the observed changes. By utilizing specific pharmacological inhibitors, the experiments described in this chapter scrutinize the involvement of PDGF receptor and MAPK pathways in the case of PDGF-induced effects as well as PI3K and MAPK pathways in regards to LPS. Lastly, experiments described in chapter five were also designed to examine the effect of PDGF and LPS on the stability of RAMP-3 mRNA as measured by the mRNA half-life. The closing chapter of this thesis, chapter six, offers a list of major hypothesis tested and the experimental results obtained in the course of this study. It also enumerates the limitations of this study and discusses its positive outcomes focusing on the possible implications of the current findings and anticipated future perspectives.

2. LITERATURE REVIEW

2.1.Mesangial cell.

2.1.1. General characteristics and cell functions.

Since the initial identification of mesangial cells by Zimmermann in 1933 (374), much has been learned about the characteristics of this independent cell type localized mainly to the trigonum of the glomerular tuft (Figure 1). Similarities of mesangial cells to smooth muscle cells (presence of contractile elements and angiotensin II receptors (165), (115), (307), (50)) as well as their architectural arrangement in the glomerulus suggest several important functions.

As specialized contractile smooth muscle cells with a direct contact to the glomerular endothelium, mesangial cells play a pivotal role in the regulation of glomerular hemodynamics. By contracting, mesangial cells reduce blood flow to select capillary loops thus effectively reducing the intraglomerular filtration area. This observation was first supported by the fact that substances capable of reducing the ultrafiltration coefficient would also cause robust contraction of isolated glomeruli and cultured mesangial cells (59).

Another important aspect of mesangial-endothelial cell interaction is underscored by the nature of their intimate apposition. Unlike other renal cell-cell interactions, mesangial cells communicate directly with the endothelial lining of glomerular capillaries without the interference of the glomerular basement membrane. Consequently, endothelial fenestrations allow for free contact of plasma and various macromolecules with mesangial cells. Macromolecular uptake by mesangial cells has been reported by

number of investigators and led to the current understanding of yet another function of these highly specialized renal cells: handling of macromolecules, immune complexes, and advanced glycation end products (72), (306), (333), (87). It has been estimated that a subpopulation of 3 to 7 percent of all glomerular mesangial cells in the rat express Fc and C3b receptors as well as the common leukocyte antigen and Ia determinants (289). These cells, as initially hypothesized by Schreiner *et al.* (289) and subsequently confirmed by Lovett and Sterzel (187), are involved in recognizing and presenting foreign antigens and stimulating lymphocyte proliferation. Thus, through their phagocytic abilities and the expression of above-mentioned surface receptors, mesangial cells aid glomerular resident macrophages in modulation of glomerular inflammatory processes (187). Currently this notion is well established and grossly evident upon considering the pathophysiology of IgA nephropathy and related diseases, where complement activation is thought to be of primary pathological importance (*vide infra*). In addition, several investigators reported pro-inflammatory like responses of mesangial cells while challenged with sub-maximal doses of C5b. Namely, immune complex formation stimulated mesangial cell production of TNF, IL-1, IL-6, various eicosanoids and reactive oxygen species. It also enhanced elaboration of extracellular matrix leading to pan-glomerular expansion of the mesangium (6), (186), (287), (101), (5).

As alluded to previously, mesangial cells constitute a dynamic cell type, which is a site of production and a target for a variety of cytokines and growth hormones (for review see (285), (225), (183)). Of particular interest to this thesis is the production and mesangial cell response to platelet-derived growth factor (PDGF). PDGF, a major cytokine secreted by mesangial cells acts in turn as their most potent mitogen as

established for mouse, rat, and human cell lines (200), (79), (3). By interacting with a specific tyrosine kinase receptor (PDGF receptor, PDGFR), widely expressed by mesangial cells, PDGF has been shown to significantly increase mesangial cell thymidine incorporation rate (by 10 to 15-fold) as well as augment the synthesis of extracellular matrix components (197), (360). In addition, PDGF also induces directed mesangial cell migration (17), (161). Thus, studies suggest that PDGF is not only a causal factor in development of mesangial hypercellularity and excessive matrix accumulation (the fundamental changes observed in several glomerular disease states) but also participates in mesangial cell repopulation following sub-total glomerular damage characteristic to several forms of glomerulonephritis (1). Despite the clear relationship of PDGF effects on mesangial cells and the etiology of a number of glomerular diseases, PDGF plays a vital role in the proper development and function of normal mesangial cells. Genetic knock-out mice for PDGF or PDGFR exhibit failure of mesangial cell growth. In addition, both mutant strains reveal a lack of glomerular tuft architecture due to the absence of mesangial cells resulting in formation of poorly defined, greatly distended capillary sacks (182), (309). Accordingly, these observations establish the need for PDGF in normal mesangial cell growth and development. They also emphasize the importance of another mesangial cell function: the assistance in appropriate generation and maintenance of glomerular capillary loop structure.

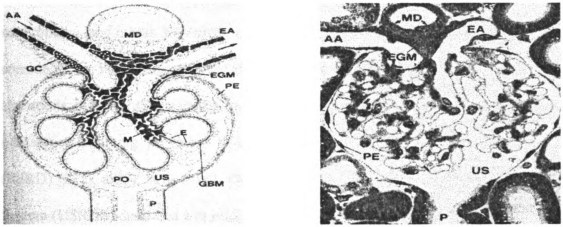
Perhaps the most obvious function of mesangial cells is that of structural support for the entire glomerular unit. Spanning the majority of the central portion of the glomerulus, mesangial architecture provides for the skeletal structure defining the glomerular capillary loop arrangement (see figure 1). The importance of this function is

clearly illustrated by yet another experimental model, the antithymocyte antibody, Thy 1.1-dependent mesangiolysis model. An intravenous administration of rabbit anti-rat thymocyte serum reactive with Thy-1-like antigens present on rat mesangial cells induces an abrupt mesangial cell injury with sequential mesangiolytic and mesangial-proliferative/infiltrative lesions. Concomitant with mesangial injury and lysis, a rapid loss of characteristic glomerular capillary architecture occurs. Furthermore, restoration of the typical capillary pattern follows the progressive mesangial cell recovery (128), (353), (363), (362). Interestingly, neutralization of PDGF or PDGFR directly by injection of antibodies attenuates the pathological findings following anti-Thy 1.1 administration, thus once again providing strong evidence for involvement of PDGF in the etiology of glomerulonephritis (135), (134).

The structural support by mesangial cells is mainly afforded through liberation of extracellular matrix components forming an organized mesangial matrix. The major constituents of the mesangial matrix are type IV and V collagen, the glycoproteins laminin and fibronectin, the basement membrane heparan sulphate proteoglycan (perlecan), as well as chondroitin/dermatan sulphate proteoglycans: biglycan, decorin, and versican (325). In addition, metalloproteinases and their specific inhibitors, also synthesized by mesangial cells, form a part of the extracellular matrix. As initially suggested by Davies et al. (49), these metalloproteinases, while regulated by a variety of growth factors and cytokines, appear to be largely involved in modulation of mesangial matrix turnover via proteolytic as well as cell-signaling capabilities. Mesangial cells, embedded in the mesangial matrix, do not merely synthesize this supporting scaffold but also participate in an extensive "cross-talk" with the extracellular matrix molecules and

other soluble mediator substances. These mesangial cell-extracellular matrix interactions exert major effects on such phenotypic features as cell growth, apoptosis, and differentiation. As a result, they have been causally implicated in influencing mesangial cell biology during embryonic development, tissue repair and glomerular disease (48, 264, 286, 310, 343).

A **B**



AA-afferent arteriole
EA- efferent arteriole
MD- macula densa
GC-granular cell
EGM- extraglomerular mesangium
PE-parietal epithelium
M-mesangial cell
E-fenestrated endothelium
GBM-glom.basement memb.
PO-podocyte
US-urinary space
P-prox. tubule

Figure 1. Longitudinal section through a renal glomerulus. A: diagram. B: Light microscopic view (x ≅ 490). Adapted from Kriz *et al.*,1988 (166).

2.1.2. Mesangial cell in disease.

The aforementioned functions of mesangial cells are tightly regulated in a healthy state. On the other hand, changes in mesangial cell biology are well-documented events participating in the development and progress of diseases like glomerulosclerosis, IgA nephropathy, diabetic nephropathy, hypertensive nephrosclerosis, mesangioproliferative nephropathy, and others. Excessive mesangial cell proliferation and matrix deposition are considered to be pathognomonic for several renal diseases (160), (77), (64). These alterations instigate the maladaptive process of glomerular hypertrophy and capillary obliteration resulting in glomerulosclerosis, often leading to end-stage renal disease (ESRD) (322), (150), (81), (344). The 1999 Annual Data Report of the US Renal Data System (USRDS) described a dramatic increase in ESRD over the past decade reaching a stunning 287 new cases per one million population in the United States in 1997. In view of the current epidemiological data, it becomes apparent that better understanding of the etiology and pathophysiology of renal disease is crucial in order to prevent further rise in the incidence of this ailment. Considering that mesangial cell proliferation and mesangial matrix accumulation are the hallmark of glomerulosclerosis (and related kidney pathologies) and the fact that AM along with other substances regulates mesangial cell biology, it becomes imperative to further investigate the mechanism(s) responsible for AM actions in mesangial cells. While the exact mechanisms responsible for AM-mesangial cell interactions are still not entirely clear, their nature (in particular at the receptor level) may be at least partially revealed by examining the dynamic interplay of AM receptor components in physiogical and pathophysiological states. To that extend, current experiments were designed to characterize the AM receptor complex in mesangial

cells. Furthermore, this study examined the effect(s) of PDGF and lipopolysaccharide (substances of great importance in the development of glomerular injury) on the components of AMR and the associated changes of AM activity in mesangial cells.

2.2. Adrenomedullin.

2.2.1 Adrenomedullin gene.

The first report describing the isolation and initial characterization of adrenomedullin (AM) was published in April 1993. Kitamura and colleagues described a polypeptide obtained from extracts of human pheochromocytoma tissue capable of raising intracellular cyclic AMP levels in rat platelets. It also appeared to have a powerful hypotensive activity through its vasodilatory effect on the resistance vessels (154). Furthermore, Kitamura *et al.* utilized an AM-specific radioimmunoassay (RIA) to demonstrate measurable amounts of this peptide in circulation. A few months later, the same research group presented the human AM gene sequence, thus introducing a new circulating hormone to the expending repertoire of known bioactive molecules inscribed in the human genome (157). Since the initial discovery, approximately one thousand publications and two international symposia have been dedicated to the characterization and better understanding of AM, making it one of the most vigorously studied novel hormones.

Human AM (hAM) gene is found in a single locus of chromosome 11, it encompasses 4 exons and 3 introns, and it's flanked at the 5'-end by RNA polymerase II responsive TATA, CAAT, and GC boxes. It also encodes several binding sites for

activator protein-2 (AP-2) (123), a cyclic AMP-regulated enhancer (75), nuclear factor-κB (125), hypoxia-inducible factor-1 (HIF-1) (44), hypoxia response elements (HREs) (88), and a binding site for steroidogenic factor-1 (SF-1) (210). The presence of such a variety of functional elements on the hAM gene suggests a complex involvement of numerous factors in the regulation of the gene which in its entirety codes for a 185 amino acid long precursor protein: preproadrenomedullin (preproAM). Enzymatic processing of this precursor leads to formation of proadrenomedullin (proAM) and subsequently yields a production of AM as well as another biologically active polypeptide, "proadrenomedullin N-terminal 20 peptide" (PAMP) (155). Interestingly, more recent investigations suggested several other biologically active and structurally stable AM fragments to be present in the circulation. Notably, Gumusel and colleagues proposed that differential endopeptidase-induced cleavage of the proAM gives rise to $AM_{153-185}$, termed adrenotensin; while yet another form of precursor processing results in AM_{11-26} peptide isolated from bovine adrenal medulla by Kitamura *et. al* (93), (159). As described below, the biological activity of these preproadrenomedullin-derived peptides varies significantly and has been proposed to depend on the presence or absence of vital protein structural components.

2.2.2. Adrenomedullin protein structure.

Recognition of the structural homology of AM to calcitonin gene-related peptide (CGRP), a potent vasodilator and central nervous system as well as peripherally acting neurotransmitter, secured AM's membership in the CGRP family of peptides. Despite a rather low overall amino sequence homology (estimated at 24% with CGRP), 52 amino

acid long AM shares common features of a ring structure formed by one intramolecular disulfide bond (between residues 16 and 21) and an amidated carboxyl terminal end with the other members of the family: calcitonin (CT), α- and β-CGRP, and amylin (AMY) (114). The integrity of these structures is essential for the biological activity of AM, as it is also true for the related peptides (63), (156). Conversely, the terminal carboxyl-end fragments devoid of the proximal ring structure serve as selective peptidyl competitive antagonists to their respective full-length counterparts. Accordingly, peptide fragment AM_{22-52} serves as a receptor blocker for AM, while α-$CGRP_{8-37}$ and β-$CGRP_{8-37}$ are utilized as competitive antagonists for the CGRP receptors (63), (37), (184). As expected, the peptidic nature of these antagonists imposes inherent limitations on their use during pharmacological manipulations. Thus, numerous efforts are directed towards the discovery of selective, non-peptide receptor antagonists to the members of the CGRP family. At present, three such compounds have been reported. BIBN4096BS, WO98/11128 (Compound 1), and SB-273779 all have been characterized as highly selective non-peptide antagonists for the subtypes of CGRP receptor (56), (62), (7). Unfortunately, a non-peptide antagonist selective for AM receptors is not currently available.

2.2.3. Sites of Adrenomedullin gene and protein expression.

At present, it has been established that AM exists ubiquitously in a great variety of tissues. With the aid of highly sensitive RIA-s and/or by immunohistochemical studies, AM was identified in the adrenal medulla, heart, aorta, kidney, lung, brain, pancreas, skin, and other tissues (154), (122), (275), (284), (193), (196), (194). AM protein and/or

gene expression were confirmed in many cell types of which cardiac myocytes (44), vascular smooth muscle cells and endothelial cells (311), (313), (312), renal mesangial cells (174), (203), (242), renal distal and collecting tubular cells (14), (283), (137), pulmonary cells (195), and a number of human tumor cell lines (223), (158), (88), (318) are but a few examples suggesting a wide biological role for AM.

2.2.4. Signals responsible for Adrenomedullin secretion.

Complementing the wide cell type and tissue distribution of AM is an equally generous collection of cytokines, hormones, and extracellular factors implicated in the regulation of AM production and secretion. Since AM appears to be secreted constitutively by cells and not stored by secretory granules, regulation of AM production parallels precisely its secretion (317), (127), (146). An extensive collaborative work of several Japanese researchers enumerated a multitude of substances having an effect on AM secretion. Of these, inflammatory cytokines: interleukin-1α and β (IL-1-α/β), tumor necrosis factor α and β (TNF-α/β), and LPS appear to stimulate AM production in human, bovine, porcine, and rat vascular smooth muscle cells (VSMC) (312), (313), (329). Cultured fibroblasts exhibit essentially the same responses to IL-1-α/β, TNF-α/β, and LPS as the VSMC (126), while some remarkable species variation was noted on examination of the endothelial cells (EC). TNF-α and LPS elevated AM secretion from porcine, rat, and bovine EC. IL-1 stimulated only rat endothelial AM production, whereas human derived EC responded negatively to TNF and IL-1 administration (311), (127), (329). Conversely, LPS showed a potent stimulatory effect on AM secretion by macrophages (168), (167), (370). In general, data from several laboratories suggest that

pro-inflammatory and endotoxic factors stimulate significant AM production and release from vascular cell types allowing this potent vasodilatory peptide to contribute to the hypotensive events and vascular collapse of sepsis as well as other inflammatory states. Of the other cytokines tested, transforming growth factor-β (TGF-β) inhibits AM secretion with the greatest potency in VSMC and EC (314), (178). Interferon-γ (IFN-γ) shares the same effect as TGF-β on vascular cell lines (329), (127), (314), whilst exhibiting significant stimulation of AM release from cultured astrocytes (316), (169). Hofbauer *et al.* also suggested a permissive role for IFN-γ, as administration of this cytokine potentiated the additive effect of IL-1β and TNF-α on AM gene expression in rat aortic VSMC (120). Other cytokines: fibroblast growth factor (FGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) demonstrate negligible effect on regulation of AM secretion (312). A broad panel of vasoactive peptides, including arginine-vasopressin (AVP), endothelin-1 (ET-1), angiotensin-II (ANG II), bradykinin, adrenaline, and substance P stimulate formation of AM (270), (269), (146), (206), (336), (314). This effect was also demonstrated for thrombin, where use of submaximal doses led to considerable rise of AM in EC and skin keratinocytes (127), (146). Furthermore, an investigation of hormonal influences on AM production revealed a complex interaction between several classes of steroid and non-steroid derived hormones and a potential for a tissue-specific control of AM gene activation. Dexamethasone, cortisol, aldosterone, thyroid hormone, and retinoic acid all exhibit significant augmentation of AM gene expression and/or AM secretion from a variety of cultured cell types (329), (124), (205). This effect is also correlated with the *in vivo* observations where, for example, raised lung mRNA and plasma levels of AM were

reported in hyperthyroid rats as well as adrenalectomized rats following dexamethasone administration (215), (105). Tissue-specific regulation of AM production studied by Abe *et al.* in ovarian granulosa cells revealed a gonadotropin-sensitive pattern, where presence of follicle stimulating hormone (FSH) achieved a long term inhibition of AM secretion (4). Additionally, progesterone and an anti-estrogen (tamoxifen) augment AM plasma and gene expression levels, respectively (133), (372).

Stress factors, namely hypoxia and fluid shear also have been reported to influence AM gene expression and protein production. Hypoxia as well as oxidative stress induction (via hydrogen peroxide, cobalt chloride or desferrioxamine mesylate) increase AM secretion and its gene expression unanimously in many cell types, including cardiac myocytes (368), (44), coronary artery EC (224), VSMC, Madin-Darby canine kidney (MDCK) cells, mesangial cells (218), retinal pigment epithelial (RPE) cells (338), colorectal carcinoma cell line (DLD-1) (223), and neuroblastoma cell lines (IMR-32 and NB69) (159). Accumulating evidence suggests that this hypoxia-induced production of AM is regulated by the hypoxia-inducible factor-1 (HIF-1) transcription factor present in the promoter consensus sites of the AM gene (88), (226), (44). Regulation of AM secretion by fluid shear stress, on the other hand appears to be somewhat controversial. Chun *et al.* reported a time and shear stress intensity-dependent elevation of AM mRNA in endothelial cells derived from the human umbilical vein (HUVEC) (42). In contrast, Shinoki *et al.* observed a marked decrease in AM gene expression and peptide secretion from cultured human aortic endothelial cells (HAoEC) exposed to shear stress loading apparatus protocol (304). Researchers agree that the effect of physical stress on AM secretion is likely to be a consequence of AM gene regulation, presumably through

activation/deactivation of the shear stress responsive element (SSRE) located in the AM gene promoter region (304); the issue of differential regulation reported for HUVEC and HAoEC, however awaits further investigation.

Considering the diverse nature of factors involved in the regulation of AM production and secretion, perhaps a more inclusive approach is to study the involvement of various signal transduction pathways in the synthesis of this peptide. In general, it appears that stimulation of cAMP-protein kinase A pathway results in a decrease of AM secretion as observed in glomerular epithelial cells (174), VSMC (314), and granulosa cells (4). However, exposure of cultured rat endothelial cells to forskolin, a direct adenylate cyclase (AC) activator, and a cell-permeable analog of cAMP, 8-bromo-cAMP, had no effect on AM production, once again pointing out the cell specific nature of AM regulation (127). On the other hand, activation of phospholipase C-protein kinase C and Ca^{++}/calmodulin pathways leads to AM gene expression and/or accumulation of immunorective-AM (ir-AM) in the supernatants of cultured VSMC (104), cardiac myocytes (336), and others. Since the inflammatory cytokines are known to enhance nitric oxide (NO) formation and their profound effect on AM gene expression has been well established by a number of investigators, Hofbauer and others attempted to characterize the exact relationship between NO pathway and AM expression. As expected, rat aortic VSMC, endothelial cells, hepatocytes, and mesangial cells exhibited marked elevation in AM production induced by NO donation using S-nitroso-N-acetylpenicillamine (SNAP). Furthermore, NO deprivation by Nω-nitro-L-arginine methyl ester (L-NAME) attenuated TNF-α, IL-1β, and INF-γ induced AM gene expression. Interestingly, exposure of these cells to 8-bromo-cGMP, an analog of cGMP,

however had no effect on AM secretion, while pretreatment with a guanylate cyclase (GC) inhibitor, 1H-oxodiazolo-quinoxalin-1 (ODQ), failed to attenuate the SNAP-assisted increase in AM mRNA expression (120). Compatible with these findings are the reports of Isumi *et al.* where exposure of rat EC to 8-bromo-cGMP had no effect on AM synthesis (127) and Dotsch *et al.* who upon incubation of human umbilical vein endothelial cells (HUVEC) with the NO donors (sodiumnitroprusside, SNP; morpholinosydnonimine,SIN-1; and phospodiesterase V inhibitor, zaprinast) observed an increase in both AM secretion and mRNA expression (57). Altogether, these results support that NO directly enhances AM production, as observed in response to the pro-inflammatory cytokine exposure, but the NO-dependent signaling involved appears to be unrelated to the classical GC-cGMP-nitric oxide synthase pathway.

2.2.5. Biological actions of Adrenomedullin.

By far, the best-characterized actions of AM are those related to the cardiovascular system. Initiated by the first report on AM, its potent and long-lasting hypotensive effect has been subsequently studied by a number of laboratories. To date however, it appears that virtually every organ system examined is affected to a significant degree by the biological activity of this circulating hormone. Accordingly, a comprehensive review of all of the actions of AM reaches beyond the scope of this work. Thus, only a limited discussion regarding AM actions in the cardiovascular and renal systems with a particular attention to AM effects in the renal glomerular mesangial cells

will follow. Please refer to table 1 for the generalized overview of the multi-functional properties of AM.

Studies of AM actions on vascular beds of rat, cat, dog, pig, sheep, and human collectively show that AM elicits relaxation of the resistance vessels thereby achieving a long-lasting drop in the mean arterial blood pressure (MAP) (90), (118), (30), (68), (255), (222). In most systems examined, vasodilating effect of AM is attenuated by L-NAME indicating at least partial involvement of the NO-dependent pathway, (73), (208), (116), however regional as well as interspecies variations have been reported (231), (32), (16). As demonstrated by Parkes *et al.*, decrease in MAP induced by intravenous AM administration to conscious sheep is accompanied by an increase in heart rate and a slight drop in stroke volume (collectively resulting in an elevation of cardiac output) along with a marked decrease in total peripheral resistance (253). In addition, a direct positive inotropic effect on the heart has been reported and appears to be cAMP and NO independent (315), (152).

Besides the direct vasomotor activity, AM appears to play an important role in the cell growth regulation. Its effects on cell growth and apoptosis depend largely on the cell type and the experimental conditions examined. Miller *et al.* characterized AM activity in several human tumor cell lines, where AM acts (perhaps in an autocrine fashion) as a potent growth factor promoting neoplastic proliferation (204). Similarly, AM stimulates cell proliferation in Swiss 3T3 fibroblasts (356), human oral keratinocytes (143), rat gastric epithelial cells (346), and human retinal pigment epithelial cells (337). On the other hand, AM has a general anti-proliferative effect on select cardiovascular cell types: it inhibits growth of rat VSMC (142) and hypertrophy of cultured myocytes and

fibroblasts (335), (334). Vascular endothelial cells however depend on the presence of AM for proper growth and morphology as illustrated by the embryonic lethality of AM gene knockout (KO) mice. Independent research laboratories reported marked endothelial cell pathology and a poor development of vitelline vasculature in the KO mice (303), (300). Also, AM was shown to have an anti-apoptotic effect on cultured rat endothelial cells, reflecting its vital role not only during vascular embryogenesis but also as a cell survival factor following the differentiation of the vascular system. The exact mechanism responsible for AM inhibition of apoptosis in EC remains illusive. Despite its parallel to NO-induced anti-apoptotic effects in EC, AM appears to signal through a cGMP-independent pathway (282). Likewise, its effect is not dependent on the ability to elevate intracellular pools of cAMP, however it has been reported to regulate endothelial cell death by activating a known anti-apoptotic gene, MAX (147), (297).

Beginning with the initial studies on renal artery AM administration in the anesthetized dogs, it became clear that AM exerts profound effects on renal function. Ebara and co-workers reported that intrarenal infusion of AM at concentrations sub-optimal for heart rate and MAP alterations resulted in an increase of renal blood flow (RBF), total urine output, and urinary sodium excretion. These findings, unaccompanied by changes in glomerular filtration rate (GFR), suggest a direct glomerular effect of AM (61). As expected, at higher concentrations, AM infusion affords marked depression of MAP but concomitantly it increases GFR, vasodilates afferent and efferent arterioles, and decreases distal tubular sodium reabsorption thus further augmenting fractional sodium excretion (116), (137), (242). Additional studies confirmed these AM-induced renal vasodilatory and natriuretic actions to be NO-dependent (208), (116), (341). Contrary to

these findings and paradoxical to renal effects of AM in general, Leclerc and Brunette reported a cAMP-dependent sodium-sparing capacity of AM. By influencing the sodium/hydrogen exchangers of the distal tubular system, AM effect resembled the well-established mechanism of aldosterone activity (179).

Analogous to the findings for the vascular cell types, AM is expressed by a variety of renal cells where it has also been shown to elicit numerous biological effects including those of growth-regulation. Using reverse transcription-polymerase chain reaction (RT-PCR), Owada *et al.* examined the AM gene localization in microdissected rat nephron segments. RT-PCR demonstrated the presence of AM mRNA in the glomerulus, cortical collecting duct, outer medullary collecting duct, and inner medullary collecting duct but not in proximal convoluted tubule or medullary thick ascending limb. Further analysis by northern blotting revealed especially high AM expression in the glomerular mesangial cells. Mesangial cells not only produced the peptide but responded to its administration by robust generation of intracellular cAMP in the presence and absence of fetal calf serum (242).

Generalized AM actions:	**References:**
Vascular effects:	
• IV administration of AM results in sustained hypotension via NO, cAMP, and/or PG generation depending on the vascular bed studied	(90), (73), (63), (364)
• direct effects on heart: positive ionotropic and chronotropic effects coronary artery dilation	(315),(107), (170)
Renal effects:	
• intrarenal AM infusion increases RBF, GFR, Na^+ excretion, and urine flow	(116), (65), (219)
• stimulates mesangial cell contraction and inhibits PDGF-induced ET-1 production	(40)
• inhibits PDGF-induced MAPK-dependent mesangial cell proliferation	(328), (292)
• stimulates p38 and PI3-K dependent mesangial cell hyaluronic acid release	(251)
• stimulates intrarenal renin release	(132)
Endocrine effects:	
• inhibits ACTH release	(278), (254)
• inhibits aldosterone production/secretion	(361)
• inhibits insulin secretion	(196)
Effects in Bone:	
• promotes osteoblast growth and protein synthesis; increases the relative area of mineralized bone	(46), (45)
Effects in Lung:	
• inhibits histamine-induced bronchoconstriction	(364)
• inhibits LPS-induced alveolar macrophage release of neutrophil chemoattractants	(140)
Immunologic effects:	
• bacteriocidal/static against gram+/- bacteria (at supra-physiologic concentrations)	(11)
CNS effects:	
• ICV AM administration attenuates AVP release	(367)
• ICV AM administration inhibits thirst drive	(216)
• ICV AM administration is pro-anorexic	(323)
• ICV AM administration results in hypertension and increased HR (in contrast to peripheral vasodilitory effects)	(279), (274)

Table 1. Biological Actions of Adrenomedullin.

2.2.6. Actions of Adrenomedullin in mesangial cells.

Of particular interest to this work are the actions of AM on the glomerular mesangial cells. As mentioned previously, mesangial cells elaborate basal levels of AM but they have also been reported to produce AM in response to various cellular/exogenous factors (174). The basal rate of AM secretion from rat mesangial cells was estimated at 0.26±0.05 fmol/10^5 cells/h and approximates one-third to one-fifth of the rate deduced for myocytes and EC (203). In addition, mesangial cells have been shown to express AM-sensitive receptors and are a prime target for AM autocrine and/or paracrine activity (240). While the fact that AM modulates mesangial cell contraction is unequivocal, its bearing on the direct regulation of RBF remains highly controversial (40), (362). On the other hand, the effects of AM on mesangial cell growth, apoptosis, cell migration, free radical generation, and extracellular matrix production have been extensively studied and are well established. We have recently reviewed in detail the current understanding of AM actions on mesangial cells (249). In general, the autocrine/paracrine nature of AM actions affords its role as a local modulator of mesangial function while the ability to stimulate production of intracellular cAMP, initially described by Khono *et al* and Chini *et al*, serves as its *modus operandi* (40), (163). Several research groups, including our laboratory, documented potent inhibitory effect of AM on mesangial cell growth (40), (162), (292), (203), (247). This anti-proliferative action is evident in the quiescent mesangial cell culture as well as in the cells exposed to such mitogenic factors as PDGF, epidermal growth factor (EGF), elevated transmural pressure, and to a lesser degree endothelin-1 (38), (239). AM achieves the anti-proliferative effect via inhibition of the extracellular signal-regulated

kinase (ERK) activity in a cAMP/PKA-dependent manner (40), (100), (247). This effect on growth regulation is complemented by AM-induced apoptosis in mesangial cells, which is compatible with a general finding that elevation of intracellular cAMP leads to an increase in programmed mesangial cell death (214), (229). Parameswaran *et al.* demonstrated an AM-mediated increase in the activity of protein phosphatase 2A (PP2A) that leads to a concerted inhibition of ERK, thus a decrease in cell proliferation, and a concomitant AM-induced stimulation of apoptosis in mesangial cells (248). Further characterization of the pro-apoptotic action of AM determined it to be p38 MAPK dependent and to coincide with a significant caspase-3 and caspase–8 activation (250), (247), (246). In addition to counteracting the effects of PDGF on mesangial cell proliferation, AM also inhibits PDGF-induced mesangial cell migration and ET-1 production, which are both implicated in the pathogenesis and progression of glomerular disease (161), (162). Other actions of AM in mesangial cells include inhibition of reactive oxygen metabolite formation (38) and ANG-II stimulated mesangial cell migration (161). In general, apart from its stimulatory effect on mesangial cell hyaluronic acid (a major glycosaminoglycan component of the extracellular mesangial matrix) release (251), AM appears to play an important protective role in pathogenesis and progression of glomerular disease. In that respect, besides its antiproliferative and pro-apoptotic functions, the antagonistic nature of functional interplay between AM and PDGF (a prime cytokine implicated in renal disease) deserves further investigation. The present study will, in part, characterize the effect of PDGF on the AM receptor system and its consequence in regards to AM actions in mesangial cells (see chapter 3).

2.2.7. Effects of Adrenomedullin gene alteration and somatic Adrenomedullin gene delivery.

Much has been learned from the recent advances in gene engineering regarding the role of AM in normal development as well as its involvement in pathogenesis and progress of various diseases.

Caron and Smithies first reported generation of the AM gene knockout (KO) in mice. The complete lack of the preproAM gene resulted in a 100 percent mortality of mice at midgestation and characteristic cardiac abnormalities with a presence of extreme hydrops fetalis (28). Findings from another laboratory confirmed the need of AM gene for survival past mid-embryonic age, however the selective disruption of AM gene with salvage of the PAMP encoding region led only to a mild form of subcutaneous edema without previously reported frank hydrops (300). Others suggested the presence of poor placental circulation, namely compromised vitelline vascularity and abnormal umbilical artery contraction, as a cause for lethality in a complete AM gene KO (86), (303). Interestingly, AM gene KO mice were rescued by osmotic pump-assisted infusion of AM to embryos prior to the midgestational age (106). Altogether, these findings emphasize the importance of AM in embryogenesis, in particular with regard to the cardiovascular system. Since the heterozygote mice for the AM gene KO ($AM^{-/+}$) are viable and fertile, further studies granted additional information on the role of AM in the maintenance of the circulatory system. Using angiotensin II and salt loading as a model previously described to cause severe end-organ damage in rodents (13), (190), Shimosawa *et al.* suggested a cardioprotective role for AM against oxidative stress and ANG II-induced coronary artery injury. They reported a marked increase in coronary artery lesions and

elevation in all oxidative stress indices studied in $AM^{-/+}$ mice as compared to controls. In addition, ANG II/salt loading failed to upregulate cardiac AM production in the KO but not the sham mice (300). The $AM^{-/+}$ mice also showed a diminished nitric oxide production and a significant increase in the basal blood pressure as compared with wild-type littermates (303). As expected, transgenic mice overexpressing the AM gene had a significantly lower mean blood pressure, which was normalized by intravenous injection of L-NMMA, a NO synthase inhibitor. In addition, mice overexpressing AM exhibited an increase in the survival rate following induction of septic shock by lipopolysaccharide (LPS) administration. The transgenic mice were less sensitive to LPS-induced hemodynamic changes and showed a remarkable resistance to organ damage clearly associated with septic shock and grossly evident in the wild-type animals (302). These observations together with the finding that AM concentrations are greatly elevated during septic shock (more so that in any other pathological state reported (117)) establish the protective role for AM and possibly point towards a new therapeutic approach in the treatment of sepsis.

Several laboratories described a successful adenovirus-assisted AM gene transfer. In order to better characterize the role of AM in maintenance of a healthy cardiovascular system, human AM (hAM) gene was administered intravenously to the following hypertensive rat models: spontaneously hypertensive rats (34), Deoxyxorticosterone acetate (DOCA)-salt hypertensive rats (55), Dahl salt sensitive rats (371), and Goldblatt hypertensive rats (345). For all rat models, researchers reported expression of human AM gene in the heart, aorta, kidney, lung, liver, and adrenal glands following the gene delivery. Detectable levels of hAM were also present in plasma and estimated by Zhang

et al. at 6.4±1.4 ng/ml three days post-injection (371). Introduction of hAM gene caused a robust and long lasting reduction in the mean arterial blood pressure in all hypertensive rat models. In addition, left ventricular hypertrophy, interstitial fibrosis and cardiomyocyte diameter were significantly reduced as compared with non-manipulated animals (55), (345), (371). A direct protection of vascular walls was also demonstrated by local hAM gene delivery into the carotid artery following a balloon-injury procedure. Compared to the control animals, carotids of rats exposed to hAM gene demonstrated considerable decrease in neointimal formation and a significant reduction in intima/media ratio, indices of healthy vascular wall regeneration (35). These findings underscore the importance of AM in post-injury re-endothelialization and complement previous reports suggesting its function as an endothelial cell survival factor (147). While this pro-endothelial effect of AM, hypothetically also beneficial in the ischemia/reperfusion injury has not been yet directly tested, Chao *et al.* investigated hAM gene delivery in the rat model of myocardial infarction by coronary occlusion/reperfusion. Introduction of hAM gene greatly reduced the infarct size, rate of sustained ventricular fibrillation, and the extent of apoptosis of myocytes confined to the ischemic area (35).

The protective effects of AM gene delivery also extend to the kidney, where elevation in renal hemodynamic parameters (GFR and RBF), reduction in glomerular sclerosis, attenuation of tubular and interstitial damage, and urinary protein excretion were all observed (55), (345). In addition, the Dahl salt sensitive rats showed marked increase in tubular and interstitial cell proliferation that correlated with renal injury but was significantly reduced by hAM gene delivery (371).

Several lines of evidence suggest strongly an involvement of AM in a variety of disease states: plasma AM levels are elevated in a broad range of diseases (see table 2), AM gene KO/overexpression studies emphasize its importance in proper embryogenesis and normal cardiovascular/renal system function, somatic AM gene delivery as well as intravenous AM administration (151), (227), (354), (148), (136) protect against multifaceted nature of pathologies.

These findings, especially the evident protective cardio-renal role of AM, prompted a recent sprout of interesting clinical trials examining the effects of exogenous AM administration in healthy as well as disease-affected human subjects (198), (259), (355). Intravenous (i.v.) infusion of AM in healthy volunteers, patients with essential hypertension, and patients diagnosed with IgA nephropathy confirmed potent hemodynamic and neurohumoral effects of this polypeptide. In all subjects AM administration produced significant fall in arterial pressure with concomitant increase in heart rate and cardiac output. Stimulation of the sympathetic nervous system and renin release were also noted (332). Nagaya *et al.* investigated i.v. AM effects in patients with congestive heart failure and pulmonary hypertension. AM treatment appeared to have beneficial effects in both groups of patients. It significantly improved cardiac function by eliciting an increase in cardiac stroke index, ejection fraction, left ventricular posterior wall thickening and urinary sodium excretion (221). AM administration also improved pulmonary parameters by causing a significant decrease in pulmonary vascular resistance and an appreciable reduction in mean pulmonary arterial pressure (220).

Disease state:
• Cardiovascular disorders: Essential hypertension Acute myocardial infarction Cerebrovascular disease Heart failure Preeclampsia Hemorrhagic shock Pulmonary hypertension Mitral stenosis Subarachnoid hemorrhage Raynauds disease • Renal disorders: Chronic renal failure Renal failure of mixed etiology End-stage renal failure IgA nephropathy Glomerulonephritis • Respiratory disorders: Chronic obstructive pulmonary disease Asthma (acute only) • Endocrine disorders: Type I diabetes NIDDM Primary adrenal insufficiency Thyrotoxicosis (Grave's disease) Primary hyperaldosteronism • Other conditions: Hepatic cirrhosis Cancer of lung, GI tract, ACTH-secreting adenoma Sepsis Wegener's granulomatosis

Table 2. Pathophysiological states associated with elevated plasma adrenomedullin levels. Adapted from Hinson *et al.* 2000 (114).

2.2.8. Actions of other preproadrenomedullin gene products.

Like AM, PAMP exhibits a potent hypotensive effect, albeit the exact mechanism of its action is related to an inhibition of catecholamine secretion from sympathetic nerve endings rather than a direct vasodilatory mechanism akin to AM (153), (299), (321), (320). In the central nervous system PAMP appears to regulate blood glucose, food intake and gastric empting. Ohinata et al. showed that a significant elevation in blood glucose as well as inhibition of food intake and gastric empting occurred within 30 minutes or less following central administration of PAMP (233), (234). These effects seem to be independent of the CL receptor system and have been shown to rely on bombesin (BN) receptor activation in the case of blood glucose regulation and a BN-independent, PAMP-specific receptor responsible for food intake and gastric empting suppression (235). PAMP-specific receptors, while currently not cloned, are likely extensively distributed as suggested by ^{125}I-PAMP binding studies (130). Their activation by PAMP presumably leads to peripheral vasodilatation (235). PAMP has been also reported to stimulate aldosterone secretion from the rat adrenal glomerulosa cells in a cAMP-dependent manner (113), (326), inhibit ACTH secretion from cultured pituitary cells (280), and regulate renin synthesis and secretion from the juxtaglomerular cells of the kidney (185).

Another protein akin to AM, adrenotensin appears to have vascular effects opposite to those of AM (93). In particular, cat pulmonary arterial rings and isolated rat aortas showed a dose-dependent contractile response to adrenotensin (92), (373). In addition, intravenous injection of adrenotensin significantly elevated the mean arterial pressure in anesthetized rats and induced the proliferation of cultured vascular smooth

muscle cells. Interestingly, these effects were attenuated by the concomitant exposure to AM. Also, there was a significant reciprocal inhibition noted in the endogenous release of adrenotensin and AM from the rat aorta (373).

More recently another endogenous, biologically active peptide derived from differential processing of AM or its pre-pro form has been characterized by Kitamura *et al.* AM $_{11\text{-}26}$ was isolated from bovine adrenal medulla and produced strong pressor effect when administered to conscious, unanesthetized rats. Surprisingly concomitant with the hypertensive effect, $AM_{11\text{-}26}$ dose-dependently increased the heart rate presumably overriding the baroreceptor response by a potent stimulation of catecholamine release (159).

The antagonistic nature of the biological effects observed in the peptides derived from a differential enzymatic processing of the precursor protein suggests an interesting system of intra-molecular regulation of preproadrenomedullin. Clearly, further studies are necessary to better characterize the newly proposed products of preproAM and their relationship to AM and PAMP.

2.3. Adrenomedullin receptor complex.

2.3.1 Adrenomedullin receptor system discovery.

The vast array of AM activities described in a number of tissues (for review please see table 1) is thought to be mediated by AM interaction with a cell-surface receptor. Until recently, the identity of this receptor has been elusive and particularly confusing due to numerous inconsistent reports on the pharmacological inhibition of AM activities. For example, the vasodilatory effects of AM were shown to be blocked by

$CGRP_{8-37}$ in a number of tissues, thus thought to be signaled through the CGRP receptor (232), (258), (94), (68). To the contrary, some reported that $CGRP_{8-37}$ had no effect on AM-mediated actions although, at the concentrations used, it potently inhibited responses to CGRP administration thus arguably pointing to the existence of AM-specific receptors. For example, unique AM receptors were implicated in AM-induced vasodilatation of the guinea pig pulmonary artery, hypotensive effects in Long-Evans rats, control of aldosterone production in rat adrenals, and many other AM-mediated actions (108), (31), (260), (319). Yet others documented AM-induced responses that were sensitive to both peptidal inhibitors: $CGRP_{8-37}$ and AM_{22-52} (240).

The initial attempts at molecular cloning of AM receptor resulted in data comparably confusing to that of the pharmacological studies described above. Kapas et al. first reported cloning of AM receptor from rat lung that was capable of binding ^{125}I-AM and causing elevation in intracellular cAMP when transfected into COS-7 cells (144). Further analysis of this receptor revealed its similarity to previously described orphan receptor called L1 or G10d (69), (103). Several months later, Kapas and Clark documented that a previously identified canine receptor, RDC-1 also had a CGRP and AM-like characteristics and with a considerable homology to the L1 receptor, it too was capable of supporting CGRP and AM-induced increase in cAMP when transfected into COS-7 cells (145). Later, a human counterpart to the rat AM receptor identified by Kapas *et al.* was cloned and the characterization of the AM receptor biology seemed to be well underway (102). Disappointingly, subsequent attempts by other laboratories to further examine the nature of the proposed AM receptors failed to reproduce previously reported observations (149), (29), (199).

A viable alternative for an AM receptor emerged shortly after the initial finding by Aiyar and co-workers that an orphan receptor, calcitonin receptor-like receptor (CRLR) exhibited a well-characterized $CGRP_1$ receptor pharmacology with a weak AM cross-reactivity. Expression of human CRLR in human embryonic kidney 293 (HEK 293) cells afforded a robust increase in CGRP-induced cAMP accumulation. Both, specific binding of ^{125}I-CGRP and CGRP-stimulated cAMP production were dose-dependently inhibited by pre-treatment with $CGRP_{8-37}$ (9). Later, similar results were obtained for rat CRLR (99) and porcine CRLR (66). Interestingly, not all cell lines examined supported the findings reported by Aiyar *et al.* and the laboratories that originally documented cloning the rat (33), (230) and human (80) CRLR sequences classified this receptor as an orphan. They recognized that CRLR is a seven-transmembrane-domain G-protein-coupled receptor (GPCR), however they failed to identify its native ligand. Together with receptors for calcitonin, gastric inhibitory peptide, glucagon, pituitary adenylate cyclase activating hormone, vasoactive intestinal peptide, secretin, growth hormone releasing hormone and parathyroid hormone CRLR forms the family B of GPCR-s. It consists of 464 and 461 amino acids and has a remarkable 50% and 54% amino acid sequence identity with rat and human calcitonin receptor, respectively (33), (80). Its identity to the calcitonin receptor is even greater in the transmembrane regions where it is estimated at almost 80%, further implying possible commonalities in structure and/or function between these to receptors (41).

In 1998, publication by McLatchie and colleagues presented new evidence that did not only explain the elusiveness of the AM and CGRP receptor but also introduced a novel concept for the receptor phenotype determination (199). In the effort to clone the

gene encoding the human CGRP receptor, the group utilized a DNA library from human neuroblastoma SK-N-MC cells previously reported to bind ^{125}I-CGRP and show CGRP-mediated cAMP accumulation (375). Systematic analysis of complementary RNA-s derived from the DNA library using a Xenopus oocyte system (expressing an endogenous CGRP receptor and an exogenous cAMP-sensitive cystic fibrosis transmembrane regulator, CFTR) yielded a 148 amino acid protein capable of significant induction of CGRP-mediated cAMP production. This protein, named receptor-activity modifying protein 1 (RAMP-1), however did not by itself constitute a functional CGRP receptor since its expression in HEK 293T, COS-7, or Swiss3T3 cells did not result in pharmacological responses characteristic of a CGRP receptor. Conversely, concomitant expression of RAMP-1 and CRLR in HEK 293T cells and oocytes restored the pharmacological findings of the CGRP-specific receptor. Since HEK 293T, COS-7, and Swiss3T3 cells do not express endogenous CRLR, co-expression of both proteins (CRLR+RAMP1) was necessary to reconstitute a functional CGRP receptor. Further database search identified two more RAMP-1-like proteins: RAMP-2 and RAMP-3, which as a group exhibit approximately 31 percent identity and 56 percent similarity to each other. Evaluation of RAMP-2 and RAMP-3 in the similar fashion to that described for RAMP-1 led McLatchie and her colleagues to an astounding conclusion. For the first time a member of a well-characterized GPCR family, CRLR, was shown to change its receptor phenotype and recognize a different ligand based on a specific association with one of the RAMP proteins. Explicitly, co-expression of CRLR with RAMP-1 reconstitutes a fully functional CGRP receptor, while concomitant transfection of CRLR/RAMP-2 or CRLR/RAMP-3 establishes an AM receptor (199). Other laboratories

extended the above findings to multiple cell lines reporting that RAMP-2/CRLR complex functions as an AM receptor in human endothelial and vascular smooth muscle cells (139), rat osteoblast-like UMR-106 and COS-7 cells (26), and Drosophila Schneider 2 cells (10) while RAMP-1/CRLR interaction leads to the formation of a CGRP receptor in the same cell lines. Interestingly, although RAMP-2 and RAMP-3 have a relatively low homology with an estimated 30% sequence identity, they generate essentially a pharmacologically indistinguishable adrenomedullin receptor when co-expressed with CRLR in HEK 293T cells (83). While other laboratories reported no significant differences between RAMP-2/CRLR and RAMP-3/CRLR receptor phenotypes, it may be speculated that variations in their regulatory system exist. Supportive of this notion are the findings from RAMP expression studies in animal disease models where variable RAMP-2 and RAMP-3 mRNA expression was observed (see section 2.3.6). In addition, the current study offers convincing evidence that RAMP-2 and RAMP-3 expression in differentially regulated by platelet-derived growth factor (PDGF) and lipopolysaccharide (LPS) in the rat glomerular mesangial cells (see section 4.3).

In contrast to the effects of coupling with CRLR, notable differences between RAMP-2 and RAMP-3 were found in their interaction with the calcitonin receptor (CTR). As described in the following section, RAMP-2 and RAMP-3 co-expressed with CTR generate receptors characterized by marked phenotype variation and receptor specificity (41) (327) (376).

A recent revision of nomenclature for the receptors of the calcitonin family of peptides has been recommended in order to reflect the current state of knowledge regarding the molectual profile of these receptors as well as their name compliance with

the present guidelines of the International Union of Pharmacology (for details, please refere to footnote on page XI). The revised nomeclature will be adopted for the remainder of this work.

2.3.2. Other receptors that interact with RAMPs.

Following the discovery of RAMPs and their interaction with calcitonin-like receptor, CL receptor (formerly known as calcitonin receptor-like receptor, CRLR), an obvious question was posed: can RAMPs interact with other receptors? Considering the remarkable molecular identity between CL receptor and calcitonin receptor (CTR), CTR seemed to be a plausible receptor target for the novel family of RAMPs. Overwhelming evidence suggests that RAMP expression is not required for the biological responses induced by calcitonin binding to its receptor (41) (295). However, shortly after the publication by McLatchie *et al.* several independent investigators reported that coexpression of RAMPs with CTR results in generation of the amylin receptor. Cotransfection of human CTR with RAMP-1 (AMY_1) or RAMP-3 (AMY_3) engendered an amylin receptor phenotype in COS-7 and rabbit aortic endothelial cells. RAMP-2 appeared to have only minimal effect on amylin receptor generation (AMY_2) (41) (212). Likewise, transfection of RAMP-1 and RAMP-3 into Chinese hamster ovary (CHO)-K1 cells, which endogenously express a CTR, produce an amylin receptor (41). Further studies showed that RAMP-2 is also capable of conferring an amylin receptor phenotype upon CTR, however the cellular background and the subtype of CTR present greatly influence this finding. Namely, the most commonly abundant isoform of the human CTR (the insert negative $hCTR_{I1-}$), unlike the $hCTR_{I1+}$ isoform, is not capable of interacting

with RAMP-2 and consequently fails to establish an amylin receptor in COS-7 cells. On the other hand, cotransfection of RAMP-2 with either isoform of CTR in CHO-P cells generated a high affinity amylin receptor (AMY_2) (327) (376). Tilakaratne *et al.* theorized that since the endogenous RAMP expression is similar in both cell lines, cellular factors other than CTR and RAMPs might contribute to the final receptor phenotype generation. It is worth noting that unlike their interaction with CL receptor, RAMP-2 and RAMP-3 couple differentially to the CTR. The significance of these differences is currently poorly understood.

While to date there is no direct evidence that RAMPs interact with receptors other than CTR and CL receptor, several observations make this an attractive speculation. The ubiquitous abundance of RAMPs in a great number of tissues far exceeds that of CTR and CL receptor. For example, RAMP mRNA expression was reported in the brain regions that are devoid of any CTR or CL receptor presence. Interestingly, these areas (specifically the subfornical organ and area postrema) express RAMP-1 and RAMP-3 and have been previously shown to be under the control of both adrenomedullin and amylin (266) (267) (273) (296) (339). Furthermore, the expression of RAMPs in brain correlates to that of the other members of the group B family of GPCRs, notably the pituitary adenylate cyclase-activating polypeptide receptor and the glucagon receptor (342) (201). These findings suggest that RAMPs may couple with other, yet unidentified, molecules to form functional receptors for adrenomedullin and amylin. They also hint at the ability of RAMPs to interact with the other members of the B family of GPCRs. Latest analysis of several GPCRs as potential partners for RAMPs however failed to detect any concerted cell-surface expression or a direct physical interaction between

RAMPs and selected receptors. All receptors tested in this study belong to the family B of GPCRs. While two of them exhibit marked degree of amino acid homology with CL receptor, PTH/PTHrP-R and GluR (approx. 45% homology), the others, vasopressin V1aR and V2R are only vaguely related to the RAMP-interacting receptors: CL receptor and CTR (76).

A comparison between RAMP tissue distribution and that of 150 known receptors was performed in the effort to identify other potential targets for RAMP interaction. A statistically significant correlation was found between RAMPs and two receptors: the EP4 prostanoid receptor and an orphan neuropeptide-like receptor, nonetheless further studies are needed to elucidate if RAMPs indeed interact with these and/or other receptors (106).

Recently, another receptor accessory protein has been investigated for its ability to interact with CL receptor. CGRP-receptor component protein (RCP), an intracellular peripheral membrane protein that is not related to the RAMP protein family, was reported to potentiate the CGRP-mediated responses in *Xenopus laevis* oocytes (189). While RCP appears to physically interact with CL receptor affecting both CGRP- and AM-evoked responses, it does not exhibit a chaperone-like activity nor does it affect the ligand-binding pharmacology. Instead, it is proposed to couple CL receptor directly to the downstream signaling molecules, in particular the subunits of G-proteins and/or adenylate cyclase complexes (70) (271). Since the data characterizing RCP and its receptor interactions are relatively scarce, it is especially difficult at present to define the role of RCP as it may pertain to the receptor biology of CL protein.

2.3.3. Characteristics of RAMP gene and protein structure.

RAMP-1 and RAMP-2 sequences were originally cloned from human neuroblastoma SK-N-MC cell cDNA library, whereas RAMP-3 cDNA was isolated from human spleen. Sequential comparison of RAMP cDNA-s, proposed by McLatchie and co-workers, with the genomic map revealed their chromosomal location and gene organization. RAMP-1 gene resides on chromosome 2, the gene for RAMP-2 is on chromosome 17, and that of RAMP-3 localizes to chromosome 7 (199). Since the scan of approximately 95% of the human genome revealed no more RAMP-like sequences, it is likely that RAMP1, 2, and 3 are the only unique members of this gene family (106). The genes encoding RAMP-1 and RAMP-3 share several characteristics. Composed of three exons and divided by large introns, they span an access of 24 kilobases (Kb). The RAMP-2 gene on the other hand is relatively small (approx. 5 Kb) and consists of four exons. Common to all of the genes is the localization of the 5'UTR and the signal peptide sequences on the first exon and the C-terminal and transmembrane domains on the last exon of the corresponding RAMP genes (294).

The analysis of hydrophobicity plots suggest a substantial similarity of protein topology among the RAMPs despite their relatively low amino acid sequence identity which is estimated at 30%. The RAMP interspecies sequence similarity however is well conserved with approximately 90% identity between rat and mouse. Likewise, the rodent sequences show roughly 70%, 65%, and 85% identity with human RAMP-1, RAMP-2, and RAMP-3, respectively (217) (121). RAMP-1 and RAMP-3, both 148 amino acid proteins, have a 26 amino acid long N-terminal signal peptide followed by an approximately 90 amino acid extracellular domain, a single 20 amino acid

transmembrane segment, and a short 10 amino acid C-terminal tail (199). RAMP-2, composed of 175 amino acids, has essentially the same protein structure organization. As intrinsic membrane proteins, RAMPs are relatively small and have a predicted size of approximately 14,000-17,000 molecular weight (M_r). Several residues are uniformly conserved in all RAMP-s across many species suggesting their importance in preservation of a common secondary structure and/or function. These include four cysteine residues localized to the extracellular domain and two amino acid sequences: DPPXX and LVVWXSK present in the N-terminal and C-terminal parts of the transmembrane domain, respectively. Two additional cysteine residues are found in the human, rat, and mouse RAMP-1 and RAMP-3 (199). Furthermore, several consensus sites for N-glycosylation have been identified on RAMP-2 and RAMP-3. Analysis of human, rat, and mouse RAMP-3, for example consistently identify four N-glycosylation sites of which all four are also present in mouse RAMP-2. Interestingly, RAMP-1 amino acid sequence is devoid of any consensus sites for N-glycosylation, perhaps implicating a different mechanism for post-transcriptional modifications among the RAMP subtypes (211). Other sequences possibly involved in the regulation of RAMP-s include protein kinase C and protein kinase A phosphorylation sites present in the intracellular C-terminus of RAMP-1 and -3 (but not RAMP-2) and the unique NHERF motif in the C-terminus of RAMP-3, which previously has been implicated in the activation of the sodium-hydrogen ion exchanger (96) (95) (217). Clearly, the functional importance of these sequences requires further investigation for it may provide us with a better understanding of RAMP regulation and their interaction as a functional part of the adrenomedullin receptor complex.

2.3.4. Tissue and cell specific RAMP expression and RAMP subtype-CL receptor selectivity.

The distribution of RAMP mRNA has been partially examined in the human, rat and mouse tissues. While species differences have been shown to exist, in general, RAMP-1 is abundantly present in the heart, brain, skeletal muscle, thymus, spleen, fat and kidney, while RAMP-2 is in the heart, aorta, kidney, spleen, fat, and lung. RAMP-3 exhibits the most ubiquitous distribution with the greatest abundance in the kidney, heart, brain, and lung (199) (217) (294) (121) (237) (339). Cell specific RAMP mRNA expression has also been observed as originally presented in human HEK193T, Kelly, and SK-N-MC cells (199) and subsequently extended to other cell lines studied (277) (139) (339) (159) (8). In fact, the existence of RAMPs in certain cell types but not others led to the initial classification of CL receptor as an orphan receptor following a functionally unsuccessful expression of CL receptor in COS-7 cells which do not express RAMPs in basal conditions (80). At present, it is well understood that the cellular background of endogenously expressed RAMPs defines the functional character of the observed receptor subtype; moreover, it may have a significant influence on the receptor phenotype detected following transfection of RAMPs and/or CL receptor into various cell types. For example, rabbit aortic endothelial cells (RAECs) which endogenously express rabbit RAMP-2 (rRAMP-2) but not rRAMP-1, exhibit selective responses to AM which are blocked by AM_{22-52} but not $CGRP_{8-37}$. Transfection of human RAMP-1 (hRAMP-1) into the endogenous background of RAECs affords CGRP-mediated responses selectively inhibited by $CGRP_{8-37}$ but not AM_{22-52}. Cotransfection of hRAMP-1 and hRAMP-3 into RAECs however significantly diminishes CGRP-evoked responses

suggesting greater affinity of hRAMP-3 for the endogenous CL receptor than that of hRAMP-1. Conversely, transfection of hRAMPs into RACEs does not alter their responsiveness to AM possibly implying a stronger interaction between rRAMP-2 and rCL receptor (constituting an endogenous rabbit AM_2) than any interaction of rCL receptor and hRAMPs (213). The notion of intra- and inter-species differences in affinities for CL receptor among RAMP subtypes has also been documented in other cell types. Buhlmann *et al.* studied the rat osteogenic sarcoma UMR 106-06 cells, which endogenously express rCL receptor and rRAMP-2 but little rRAMP-1, as well as the COS-7 cells which lack native CL receptor or RAMP expression. Transfection of hRAMPs into these cell lines revealed a higher affinity interaction between RAMP-1 and CL receptor than RAMP-2 and the receptor (26). As exemplified above, the concept of variable RAMP subtype-CL receptor affinity affords additional possibilities for modulation of receptor phenotype by the RAMP family of proteins. Importantly, it also hints that a well-regulated mechanism for RAMP-RAMP interaction may exist and it too, along with the RAMP-CL receptor interaction, could be responsible for the final receptor type determination. To that extent, a competitive and/or an inhibitory relationship among the RAMP subtypes could be theorized. Corroborative with this hypothesis are the findings that RAMP-1 (199) and RAMP-3 (294) form stable homodimers. To date, formation of RAMP heterodimers however has not been reported.

Of particular interest to this study is the renal expression of RAMP mRNA. Abundant expression in the renal tissue was reported for RAMP-2 and RAMP-3 with similar expression in both cortical and medullary parts of the rat kidney (369). Utilizing competitive, quantitative RT-PCR, Totsune *et al.* measured the mRNA expression levels

for RAMP-2 and RAMP-3 in a normal Munich-Wistar rat kidney. RAMP-2 mRNA was detected at 26.5±1.9 mmol per mole of GAPDH and RAMP-3 at 7.7±0.4 mmol per mole of GAPDH (mean±SE). CL receptor was also present in the renal tissue, albeit at significantly lower concentrations. (330). As previously described, RAMP-2 and RAMP-3 can independently constitute a functional AM receptor when associated with CRLR. Renal expression of both RAMP-2 and RAMP-3 affords a conceivably complex mechanism for regulation of AM activity in the kidney during the basal as well as disease states (for further discussion see section 2.3.6). A more precise cellular localization of RAMP expression in the renal tissue could aid our understanding of the molecular mechanisms involved. To date, however there is only one report on RAMP expression profile in a non-embryonic kidney cell line. Robert-Nicoud and co-workers reported basal RAMP-3 expression and its marked upregulation by vasopressin in mouse clonal cortical collecting duct (CCD) principal cells (268). Since these cells participate in sodium and water reabsorption under a direct influence of vasopressin and because adrenomedullin has a known natriuretic activity, the effect of vasopressin on RAMP-3 expression may represent a feedback mechanism by which CCD ensure the fluid balance. With an aim to further elucidate the ambiguous kidney cell profile of RAMP expression, the present study characterizes the mRNA expression of RAMPs in the renal mesangial cells and their effect on AM activity.

2.3.5. Mechanism of RAMP-receptor interaction.

As initially postulated by McLatchie *et al.* and further theorized by Foord and Marshall, the consequences of RAMP-CL receptor interaction are at least threefold:

1. RAMPs participate in the trafficking of CL receptor, thus regulating its appearance at the cell surface. 2. RAMPs influence the state of CL receptor glycosylation. 3. RAMPs, at least in part, determine the distinct pharmacology of CL receptor by their differential association with the receptor. Furthermore, they hypothesized that the particular pharmacological profile may be imposed upon CL receptor by RAMP-directed glycosylation, induction of conformational change, and/or direct physical association between CL receptor and a RAMP family member (199) (82).

To date, the most convincing evidence clearly defining the role of RAMPs in directed trafficking of CL receptor to the cell-surface plasma membrane comes from the confocal microscopic studies of the epitope-tagged CL receptors and RAMPs along with the fluorescence-associated cell-sorting (FACS) analysis. Several independent laboratories reported significant increase in the cell membrane presentation of CL receptors following co-expression with all subtypes of RAMPs. RAMP-1, 2, and 3 induce CL receptor translocation with a comparable efficacy resulting on average in the cell membrane presentation of 25% of total CL receptor protein. Moreover, a strict requirement for CL receptor presence was detected for RAMP cell membrane translocation (199) (83) (41) (173). This reciprocal need for co-expression strongly suggested a direct physical interaction between CL receptor and RAMPs, which was shortly demonstrate by co-immunoprecipication studies (111). In contrast to CL receptor, CTR localizes to cell membranes independent of RAMPs presence (41) (119) (36). Also, as previously discussed, CTR does not interact with RAMPs in order to function as a classical calcitonin receptor. Nonetheless, when co-expressed with CTR, RAMP-1 cell-surface translocation is readily observed and it correlates with an increase in specific

binding of amylin to CTR as examined in COS-7 cells. Similar findings were reported for RAMP-3/CTR interaction (41) (294). Accordingly, while RAMPs regulate CL receptor protein trafficking as well as its receptor pharmacology, their interaction with CTR is currently understood only at the level of receptor phenotype determination.

Initial findings pointing to a change in molecular mass of CL receptor when cotransfected with RAMP-1 prompted a thorough investigation of the CL receptor protein structure expressed at the cell surface. HEK 293T cells transfected with Myc-tagged CL receptor presented with a single immunoreactive band of approximately 58K relative M_r. When transfected together with RAMP-1, CL receptor immunoreactivity shifted to M_r=66K and a concomitant disappearance of the original 58K band was observed. Parallel to these findings, ^{125}I-labelled CGRP cross-linking to the surface receptors was found to exist only in the RAMP-1/CL receptor transfected HEK 293T cells. Furthermore, endoglycosidase F and H aided analysis of the two CL receptor forms determined that unlike the 58K species, the 66K form represents a terminally glycosylated, hence endoglycosidase H resistant, CL receptor. Since the 66K form is the species capable of ^{125}I-CGRP binding and present only when co-expressed with RAMP-1, it was proposed that mature glycosylation of CL receptor is directed by RAMP-1 and results in acquisition of CGRP binding specificity (199). Corroborative with this hypothesis were the findings from coimmunoprecipitation studies, where the cytosolic and membranous RAMP-1/CL receptor complexes were identified. While investigators reported both immature (core-glycosylated) and terminally glycosylated CL receptor in the cytosol, only the fully glycosylated, mature form of CL receptor appeared at the cell membrane as a RAMP-1/CL receptor complex. In contrast to RAMP-1, cotransfection of

RAMP-2 or RAMP-3 with CL receptor does not change the receptor's glycosylation status preserving its native core-glycosylation and the associated selective AM specificity (199) (83). As documented by Fraser *et al.*, the N-terminal extracellular domain of RAMPs is responsible for RAMP-directed differential glycosylation of CL receptor. Using RAMP-1/RAMP-2 chimeras, they showed that only RAMP-1 N-terminus containing hybrids are capable of inducing terminal glycosylation of CL receptor thus securing the CGRP-specific receptor phenotype (83). Altogether, these initial findings suggested that RAMP-specific CL receptor glycosylation might provide a mechanism for their ability to determine the receptor phenotype. Born and co-workers (91) offered a closer look at the glycosylation of CL receptor by performing site-directed mutagenesis of the three predicted N-glycosylation sites for the human CL receptor. They reported that N-glycosylation at residues Asn^{60} and Asn^{112} is required for proper transport of the hCL receptor to the cell membrane. Surprisingly, while mutations at these residues significantly decreased the frequency of receptor complex-ligand binding, they did not preclude RAMP-1-CL receptor and ^{125}I-CGRP interactions resulting in an adenylate cyclase activity with an EC_{50} comparable to that of the controls (91) (25). Analysis of asparagine at position 117 revealed that despite its predicted potential for N-glycosylation, this modification does not occur in the wild type hCL receptor. Nonetheless, site-directed mutations at Asn^{117} position resulted in a close to complete ablation of CGRP-evoked adenylate cyclase activity. This functional receptor defect was accompanied by an unaffected receptor cell surface expression and a slight instability of the RAMP-1-CL receptor complex (91). Accordingly, it implicates Asn^{117}, and perhaps the neighboring amino acid residues, in facilitating a direct protein-protein interaction

between RAMP and CL receptor in order to establish a stable and functional receptor complex. As pointed out by Gujer *et al.*, the amino acid sequence flanking Asn117 residue is highly conserved among several receptors belonging to the same receptor class as CL receptor, possibly emphasizing the apparent importance of the sequence for the proper receptor function. Unfortunately, there are no reports on the effects of the above-mentioned mutations on the RAMP-2- or RAMP-3-CL receptor interaction and the AM receptor complex function. Taken together, the site-directed mutagenesis studies suggest a crucial role for CL receptor N-glycosylation in proper cell surface delivery and hint at other, glycosylation independent, events regulating the direct RAMP-CL receptor interaction and the resultant specific ligand binding ability.

Interestingly, more recent reports presented additional evidence undermining the importance of CL receptor glycosylation in receptor phenotype regulation while focusing on the significance of the direct RAMP-CL receptor interaction. Contrary to the initial findings in the HEK 293T cells, cotransfection of CL receptor and RAMP-1 or RAMP-2 in *Drosophila* Schneider 2 (S2) cells resulted in mature glycosylation of CL receptor which was independent of the expressed RAMP subtype. In addition, radioligand binding studies revealed that albeit uniformly glycosylated, CL receptor retains CGRP or AM receptor characteristics, identical to those found in the previous studies, based on the presence of RAMP-1 or RAMP-2, respectively (10). Hilairet *et al.* extended the analysis of CL receptor glycosylation to mammalian cells by finding that indeed the direct RAMP-CL receptor protein interaction and not the glycosylation state of CL receptor determine the receptor ligand specificity. They found that while the core-glycosylated CL receptor form predominates when cotransfected with RAMP-2 or RAMP-3, AM appears

to bind only to the mature (N-glycosylated) form of the receptor on the cell surface. Furthermore, the cross-linking analysis showed that RAMP-2 and RAMP-3 form stable heterodimers with CL receptor. Together, as a ternary protein receptor complex, RAMP-CL receptor participate in the ligand binding as depicted by the consistent incorporation of ^{125}I-AM into the RAMP-2 and RAMP-3 protein structure. Analogous interaction was also found for the RAMP-1-CL receptor heterodimer complexes and their selective binding of ^{125}I-CGRP (112). To further analyze the nature of RAMP-CL receptor interaction, Kuwasako *et al.* utilized RAMP deletion mutant constructs and identified the key RAMP residues responsible for the generation of the functional receptor complex. The deletion of human RAMP-2 residues 86-92 and RAMP-3 residues 59-65 resulted in a significant decrease in specific ^{125}I-AM binding, hence an attenuated AM-directed cAMP production in HEK 293 cells. Interestingly, the cell surface expression of human RAMP-CL receptor complexes was not affected by the mutations, suggesting that the identified RAMP residues directly interact with CL receptor to form a high affinity agonist-binding site and/or evoke necessary conformational change of the receptor complex associated with the acquisition of ligand specificity (171). The same group obtained similar findings when they co-expressed rat CL receptor with rat RAMP deletion mutants in HEK 293T cells. Here, the deletions of residues 93-99 and 58-64 from rRAMP-2 and rRAMP-3, respectively, significantly inhibited high-affinity ^{125}I-AM binding and AM-evoked cAMP production despite full cell surface expression of the receptor heterodimers (172). It is worth noting that the sequence identity between the key residues identified for RAMP-2 and RAMP-3 is minimal in both human and rat sequences. Also, single substitutions within these sequences do not result in a significant loss of ligand recognition, thus

emphasizing again the importance of the entire sequence as a possible structural determinant of the ligand-binding pocket (171). The direct involvement of RAMPs in defining the ligand recognition site was also suggested by the observations of Mallee and colleagues. Intrigued by two independent reports on marked species selectivity of non-peptidal CGRP receptor antagonists, BIBN4096BS and Compound 1 ((56) (62)), they postulated that precise species variations in RAMP-1 dictate the observed variability in affinities reported for these antagonists of CL receptor-RAMP-1 complex. Using recombinant human/rat CL receptor-RAMP-1 complexes they showed that hCL receptor-rRAMP-1 heterodimers presented with the rat receptor pharmacology, while those composed of rCL receptor-hRAMP-1 retained human CGRP receptor characteristics. Furthermore, they identified lysine at position 74 in the RAMP-1 sequence as the key amino acid involved in modulation of antagonist affinity for CL receptor-RAMP-1. A single lysine to tryptophan substitution in rat RAMP-1 at this position resulted in a greater then 100 fold increase in antagonist affinities, resembling those native to the human receptor complex (191). Considering the fact that both BIBN4096BS and Compound 1 are competitive antagonists at the ligand binding site of the CL receptor-RAMP-1 receptor (56) (62), the above findings strongly imply that RAMP-1 forms an integral structural part of the receptor binding site responsible for the recognition of both CGRP and the small molecular antagonists.

Similar to other related GPCRs, CL receptor has been shown to undergo receptor internalization following ligand exposure (173). Several laboratories scrutinized the role of RAMPs in the ligand-induced CL receptor cycling phenomenon. Notably, Kuwasako and colleagues visualized RAMP and CL receptor localization and trafficking by

fluorescent microscopy in HEK 293 cells. They confirmed the previous findings suggesting a requirement of RAMPs for the appearance of CL receptors at the plasma membrane. Green fluorescent protein (GFP) labeled CL receptor was not found in the plasma membranes and it failed to generate CGRP or AM-evoked responses without concomitant RAMPs expression. On the other hand, co-expression of RAMPs and CL receptor resulted in ample cell surface appearance of both proteins restoring ligand-induced intracellular cAMP production and calcium mobilization. Furthermore, AM-mediated internalization of CL receptor-GFP was noted in association with all three RAMP subtypes. This internalization process appears to be largely clathrin-coated pit-mediated and involves specific targeting of both CL receptor and RAMPs to lysosomes. Again, no CL receptor-GFP internalization occurred in the absence of RAMP expression regardless of the ligand presence (173). Further examination of the sub-cellular localization of CL receptor and RAMPs revealed that unlike CL receptor, which remains in the endoplasmic reticulum (ER) when expressed alone, RAMP-1 is present both in the ER and the Golgi predominantly in a disulfide-linked homodimer form. When co-expressed with CL receptor, RAMP-1 acquires several intramolecular disulfide bonds and in association with CL receptor it co-localizes to cell membrane with a 1:1 heterodimer stoichiometry. Upon agonist exposure, CL receptor undergoes rapid phosphorylation and the entire heterodimer complex associates with β-arrestin and becomes endocytosed via clathrin-mediated process (111). Altogether these findings establish unequivocally a direct association between CL receptor and RAMPs and suggest that RAMPs may also play a role in regulation of ligand-induced CL receptor internalization and/or a chaperone like activity during the receptor trafficking.

2.3.6. Regulation of RAMP gene expression.

A large number of recent publications demonstrated the dynamic nature of RAMP mRNA expression. Indeed, RAMP gene expression is not constitutive and undergoes dramatic changes in a number of pathophysiological states and as a consequence of exogenous substance administration.

For example, a high expression of CL receptor and RAMP-2 mRNA is observed in lungs of normal mice, but it is markedly decreased in endotoxemic mice treated with LPS. In contrast, LPS treatment significantly increases RAMP-3 gene expression in lungs, spleen, and thymus (237). Furthermore, Frayon *et al.* evaluated glucocorticoid regulation of RAMPs and CL receptor by dexamethasone administration in human coronary arteries vascular smooth muscle cells (VSMC). Untreated cells expressed detectable levels of CL receptor, abundant amount of RAMP-2 and lesser amount of RAMP-1. Dexamethasone treatment elevated RAMP-1 mRNA levels significantly while those for RAMP-2 remained unchanged, resulting in a 5-fold increase in the ratio of RAMP-1 to RAMP-2 expression. Slight variations in CL receptor expression were also noted, albeit these appeared to be inconsistent with various dexamethasone concentrations used. Altogether, these results suggest that glucocorticoids may be involved in the regulation of RAMP expression and preferentially lead to the generation of CGRP-responsive receptors in the human coronary VSMC (84). As previously mentioned, vasopressin also has been shown to affect RAMP expression, leading to a 17-fold increase in RAMP-3 mRNA levels in mouse kidney cortical collecting duct principal cells (268). This observation, while intriguing, currently remains only poorly understood

due to the lack of any well-established interaction between vasopressin and the AM receptor system.

Changes in RAMP mRNA expression have been also clearly demonstrated in a variety of pathophysiological conditions. A concerted upregulation of AM, CL receptor, and RAMP-2 mRNA expression was shown in the rat heart following myocardial infarction (236), as well as in the state of congestive heart failure induced by coronary ligation (331). In the chronic model of heart failure, RAMP-1 and RAMP-3 mRNA as well as protein levels were significantly increased in both atria and ventricles of rats 6 months after an aortic banding procedure (47). Furthermore, studies of the LPS-induced mouse sepsis model documented an elevation of AM and RAMP-3 with a concomitant decrease in CL receptor and RAMP-2 mRNA expression in the lung tissue (237).

The alterations in the AM-CL receptor-RAMP axis have also been noted in the pathological states of the kidney where modified RAMP expression pattern was reported following ureteral obstruction and a sub-total renal mass ablation. In the rat model of nephropathy caused by unilateral ureteral obstruction (UUO), RAMP-1, RAMP-2, and CL receptor gene expression were significantly upregulated, whereas RAMP-3 expression remained unaltered (217). Contrary to these findings, Totsune *et al.* (330) demonstrated a 50% and 70% decrease in RAMP-3 and 30% to 43% decrease in CL receptor mRNA expression on the fourth and fourteenth day following 5/6 nephrectomy in rats, respectively. No significant changes in RAMP-2 and AM mRNA levels were noted in the nephrectomized animals as compared to the sham-operated controls. An interesting side observation was the attenuation of RAMP-2 expression by sodium intake restriction, which occurred irrespective of the nephrectomy procedure. The varied

expression of the AM receptor complex components reported by the two laboratories is likely to represent the principal differences of the pathophysiological changes occurring in these two models of nephropathy. Specifically, while UUO leads to primary renal interstitial fibrosis perpetuated by elevated concentrations of TGF-β, the sub-total renal mass ablation results in renal failure with a compensatory renal hypertrophy and elevated sodium excretion in the remnant kidney (21), (141). Despite these differences however both studies show dynamic changes within the AM receptor system (with a particularly impressive quantitative changes in the RAMP expression profile). Taken together with the previously discussed actions of AM in the kidney (see section 2.2.5-6), these observations strongly suggest an important role for AM in the pathophysiology of renal disease as it may be regulated at the level of RAMP mRNA expression.

3. RAMP mRNA expression profile and the effects of RAMP over-expression on AM-induced adenylate cyclase activity and [^{3}H]thymidine incorporation in rat mesangial cells.

3.1. Introduction.

The recent discovery of receptor activity modifying proteins (RAMPs) by McClatchie *et al.* (199) has significantly altered our understanding of mechanisms involved in the regulation of G protein-coupled receptors (GPCRs). RAMP-1, 2, and 3 are distinct gene products and have been characterized as single-transmembrane domain proteins capable of direct interaction with two related members of GPCR: calcitonin receptor (CTR) and calcitonin-like receptor (CL receptor) (41), (199), (26), (139). While the exact nature of RAMP-CTR and RAMP-CL receptor interactions remains elusive, it has been clearly documented that RAMPs facilitate trafficking and determine the phenotype of these receptors (199), (41), (173). In particular, with regard to CL receptor, RAMP-1 and CL receptor co-expression renders the receptor a fully functional calcitonin gene-related peptide receptor ($CGRP_1$). Co-transfection of CL receptor with RAMP-2 or RAMP-3, on the other hand, confers upon CL receptor adrenomedullin receptor characteristics (AM_1 and AM_2, respectively) (199). Consequently, differential expression of RAMPs can potentially function as a regulatory step for CL receptor activity and its ligand specificity towards CGRP and/or AM.

AM, a 52 amino acid peptide recently isolated from a pheochromocytoma (154), has been shown to activate CL receptor causing an elevation in intracellular cAMP in several systems including rat mesangial cells (40), (163), (240). In particular, by

activation of cAMP-PKA pathway, AM exerts anti-proliferative, pro-apoptotic, and anti-migratory effects on rat mesangial cells (38), (247). Since disproportionate mesangial proliferation and matrix deposition are a hallmark pathological change accompanying several glomerular diseases (64), (160), the anti-proliferative effects of AM suggest an attractive, reno-protective role for this hormone.

As thoroughly discussed in section 2.3.4, abundant expression of AM receptor complex components was reported in the rat kidney tissue, where expression of both RAMP-2 and RAMP-3 predominates (369), (330). To date however there is no report on the specific non-embryonic renal cell type RAMP and CL receptor expression. Here we have investigated the mRNA expression profile of AM receptor complex and the functional consequence of RAMP overexpression on the AM responsiveness in rat mesangial cells.

3.2. Materials and methods.

3.2.1. Materials.

Adrenomedullin and adrenomedullin(22-52) fragment were purchased from Sigma® RBI® (St. Louis, MO). RPMI-1640, fetal bovine serum, penicillin/streptomycin, trypsin-EDTA were from GibcoBRL® (Grand Island, NY).

All other reagents were of highest quality available.

3.2.2. Cell Culture.

Rat mesangial cell (RMC) cultures were established from glomeruli obtained from kidney cortex of 55 to 70 g male rats (Sprague-Dawley, Charles River, MA). Glomeruli were isolated by sequential sieving which removes tubules (300 to 150 m sieves), then retains glomeruli on the 63 m sieve, as described by Wolthuis *et al.* (358). Isolated glomeruli were incubated for 10 min at 37 °C in collagenase (750 U/ml), then plated in flasks in RPMI 1640 medium supplemented with 0.6 U/ml of insulin, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 15% fetal bovine serum. Cells were grown at 37 C in 5% carbon dioxide with medium changed twice a week. At confluency, cells were sub-cultured by rinsing with calcium and magnesium free phosphate buffered saline and then incubating with 0.05% trypsin supplemented with 20 mM EDTA. The correct cell type was confirmed by the following criteria:1. a stellate morphology using phase contrast microscopy; 2. microfilaments and sub-plasmalemmal cytoplasmic densities using transmission electron microscopy; 3. insensitive to puromycin aminonucleoside; 4. positive immunofluorescence staining for actin and desmin but negative for keratin and factor VIII antigens and 5. positive contraction reaction with angiotensin II (15). For the

experiments, passages 15-24 were used. Specific confluency for different experiments was determined by preliminary studies.

3.2.3. RT-PCR analysis.

Total RNA was isolated from rat mesangial cells using TRIzol® reagent (GibcoBRL®, Grand Island, NY). Following sodium acetate/ethanol precipitation and several ethanol washes, RNA was used as a template in RT-PCR amplification procedure. RT-PCR reaction was carried out using SUPERSCRIPT™ One-Step RT-PCR with Platinum® *Taq*. (GibcoBRL®, Grand Island, NY) in accordance with manufacturer's specifications. Specific primers used were as follows:

RAMP-1:	sense: 5'-GGG GAG ACG CTG TGG TG-3'
	antisense: 5'-ATG CCC TCA GTG CGC TT-3'
RAMP-2:	sense: 5'-GCA ACT GGA CTT TGA TTA GCA G-3'
	antisense: 5'-GGC CAG AAG CAC ATC CTC T-3'
RAMP-3:	sense: 5'-ACC TGT CGG AGT TCA TCG TG-3'
	antisense: 5'-CTT CAT CCG GGG GGT CTT C-3'
CRLR/CL receptor:	sense: 5'-GCA GCA GAG TCG GAA GAA GG-3'
	antisense: 5'-GCC ACT GCC GTG AGG TGA-3'
GAPDH:	sense: 5'-AGA CAG CCG CAT CTT CTT GTG C-3'
	antisense: 5'-CTC CTG GAA GAT GGT GAT GG-3'

Reactions were carried out, using Perkin-Elmer Model 9600 thermal cycler, in 50 µl total reaction volumes subjected to the following conditions: 1) 50°C for 30 min (1 cycle), 2) 94°C for 5 min (1 cycle), 3) 94°C for 30 s; 50-55°C for 30 s; 72°C for 30 s (40 cycles), 4)

72°C for 15 min (1 cycle). Products were separated by gel electrophoresis and subsequently visualized by ethidium bromide staining and UV illumination. Photographs of the gels were taken and digitalized with UMAX Astra 2000P flat-bed scanner. To assure the identity of the products, cDNA was extracted from the gel and sequenced by standard dye-termination DNA sequencing procedure. Sequences of cDNAs obtained in that fashion were analyzed for similarity to published full-length sequences of RAMP1, 2, 3, and CL receptor via two-sequence comparison method from BLAST database (http://www.ncbi.nlm.nih.gov/BLAST). These analyses revealed identity of RT-PCR products to the corresponding full-length cDNA sequences. Several cDNAs acquired by this method were used as probes in northern blot hybridizations.

3.2.4. RAMP 1, 2, 3, and CRLR cloning and expression.

Full-length cDNA of human RAMP-1, 2, 3, and bovine CL receptor were cloned into Myc-tagged and pCDN vectors obtained from Clonetech (Palo Alto, CA). Multiple DNA preparation batches were used. Expression vectors were transfected into RMC using LipofectAMINE PLUS™ Reagent (GibcoBRL®, Grand Island, NY) following the suggested protocol. In brief, RMC were plated on P-100 tissue culture plates a day prior to the transfection to achieve an approximate 60-70% confluency. On the following day, cells were transfected with a total of 2μg of each vector-cDNA in a serum-free transfection medium for 4 hrs at 37°C/5% CO_2. The total amount of transfected cDNA was kept constant by adding empty Myc or pCDN vector. Afterwards, 4ml of serum-containing medium was added and cells were incubated for an additional 24-36 hrs. This transient transfection protocol resulted in an approximate 70% transfection efficiency as

assessed by green fluorescent protein (GFP). Successful introduction of RAMP-1, 2, and 3 cDNA was further verified by western blot analysis revealing a significant increase in appropriate membrane-associated RAMP protein expression as compared to vector-transfected cells.

3.2.5. Membrane preparation and adenylate cyclase assay.

Cells were harvested from P150 plates and homogenized in Tris HCl, pH 7.4, (10mM)/EDTA (10mM) buffer. Membranes were prepared by homogenization in a Dounce ground glass homogenizer, centrifuged for 20 min at 12,000*g* at 4°C and washed in Tris HCl, pH 7.4, (50mM)/MgCl (10mM) buffer. Final concentration of 40μg of protein/assay tube was obtained and the membranes were immediately subjected to adenylate cyclase assay, as follows. Membrane associated adenylate cyclase activity was measured as the rate of conversion of [α^{32}P]ATP to [^{32}P]cAMP as described by Elshourbagy et al. (67). Accordingly, membranes were incubated for 20 min at 30°C with appropriate drugs and assay mix containing ATP regeneration system (50mM Tris-HCl, pH 7.4, 10mM $MgCl_2$, 1.2 mM ATP, 0.1 mM cAMP, 2.8 mM phosphoenolpyruvate, and 5.2 μg/ml myokinase) and 1.0 μCi of [α^{32}P]-ATP. Total reaction volume was 100μL, drug as well as AM concentrations were as described for particular experiments. Reactions were stopped with 1 ml of stop solution containing 0.28mM "cold" cAMP, 0.33mMATP, and 22,000dpm of ^{3}H-cAMP. Contents of the assay tubes were washed through a Dowex and subsequently alumina columns to separate the degradation products of ATP as previously described by Salmon *et al.* (276). Elution profiles were performed prior to experiments to determine the amount of water (for Dowex columns) and

imidazole (for alumina columns) needed to wash and elute the products. Products eluted from alumina column were counted for the presence of ^{3}H-cAMP and α^{32}P-cAMP. Each experiment was done in triplicates, repeated at least 3 times, and expressed as percentage of AM-mediated adenylate cyclase activity compared to basal.

3.2.6. [^{3}H]thymidine incorporation.

Cells were plated in 24 well plates (30000 cells/well) and grown for 2 days with subsequent serum starving for 48 hrs. Then, they were treated with the compounds for a period of 16 hrs and pulsed with [^{3}H]thymidine for 4 hrs. The radioactivity was counted in Beckman LS counter, after washing the cells and stopping the reaction with 5% trichloro acetic acid and solubilizing the cells in 0.5 ml of 0.25 N sodium hydroxide. Each experiment was performed in quadruplicates and repeated at least three times.

3.2.7. Statistical analysis.

Data are presented as mean±S.E.M. Multiple group comparisons were made using a two-way analysis of variance (ANOVA). Single group comparisons exercised Student's t-test method. Statistical significance was set at $P<0.05$

3.3. Results.

3.3.1. AM receptor components in rat mesangial cells.

We first characterized the expression of AM receptor components in quiescent RMC by RT-PCR as delineated in materials and methods. Under basal conditions, RMC express CL receptor (formerly known as CRLR) as well as all three subtypes of RAMP-s: RAMP-1, RAMP-2, and RAMP-3 (Fig. 2).

3.3.2. Effects of RAMP over-expression on AM-induced adenylate cyclase activity and [^{3}H]thymidine incorporation in RMC.

To investigate the effect of RAMP over-expression on mesangial cell responsiveness to AM, we transiently transfected RMC with RAMP-1, RAMP-2, and RAMP-3 and subsequently examined AM-mediated adenylate cyclase activity and [^{3}H]thymidine incorporation.

Transfection of RAMP-2 or RAMP-3 resulted in 85.4 ±3.02 and 54.5 ±2.63 percent increase in AM-induced adenylate cyclase activity, respectively (Fig. 3). These responses were further potentiated by co-transfection of CL receptor with RAMP-2 or RAMP-3 (136.6±7.9 and 90.71±3.8 percent increase, respectively). AM-induced adenylate cyclase activity in RAMP-2 and RAMP-3 transfected cells was also inhibited by at least 50% following pretreatment of the membranes with 10 μM AM_{22-52}, AM receptor antagonist, indicating that this effect is AM receptor mediated (table 3). Transfection of RAMP-1, on the other hand, had no effect on AM-mediated responses (Fig. 3) despite successful introduction of RAMP-1 cDNA and subsequent elevation in RAMP-1 protein expression as verified by western blotting.

We further hypothesized that in addition to increasing adenylate cyclase activity, RAMP overexpression will also potentiate an AM-mediated decrease in [^{3}H]thymidine incorporation. As predicted, transfection of RAMP-2 or RAMP-3 significantly potentiated AM-evoked inhibition of [^{3}H]thymidine incorporation (Fig. 4). Again, RAMP-1 transfection had no significant effect on mesangial cell responsiveness to AM as assessed by [^{3}H]thymidine incorporation assay (–41.6±4.9 and -29.8±5.4 inhibition of [^{3}H]thymidine incorporation for RAMP-1 and vector alone, respectively; $p>0.1$).

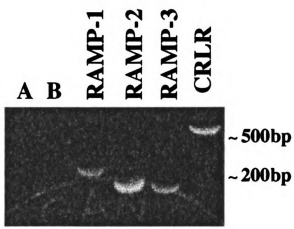


Figure 2. Receptor activity modifying protein (RAMP) and calcitonin receptor-like receptor (CRLR or CL receptor) expression profile in quiescent rat mesangial cells (RMC). RT-PCR was performed on total RNA isolated from RMC using primer sequences designed from published sequences as described in materials and methods. The identity of PCR products resolved by electrophoresis was confirmed by DNA sequencing. The product of complete reaction devoid of RNA was loaded in *lane A* (negative control). *Lane B* contains the product of reaction carried in the absence of reverse transcriptase (no-RT control). RMC express detectable levels of CL receptor as well as all 3 subtypes of RAMPs.

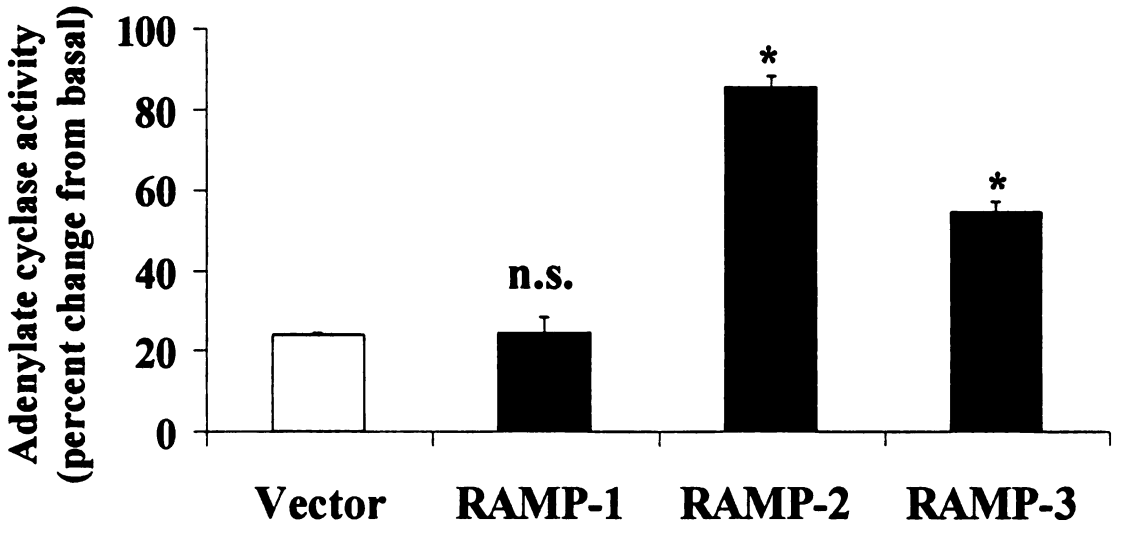


Figure 3. Effect of RAMP-1, RAMP-2, and RAMP-3 overexpression on AM-stimulated adenylate cyclase activity in RMC. RMC were transfected with vector or vector+RAMP-1, RAMP-2, or RAMP-3 at ≈60% confluence. Cells were allowed to grow for another 24-36 hours. Adenylate cyclase (AC) assay in response to 100 nM AM was performed as described in materials and methods. Overexpression of RAMP-2 and RAMP-3 significantly enhanced AM-stimulated AC activity in RMC. Overexpression of RAMP-1 had no significant effect on AC activity. *$p \leq 0.01$, n.s.-statistically not significant, experiments performed in triplicates, $n \geq 3$.

	Vector	RAMP-2 over-expressed	RAMP-2 over-expressed + AM_{22-52} pretreated	RAMP-3 over-expressed	RAMP-3 over-expressed + AM_{22-52} pretreated
AC activity in response to 100 nM AM (percent from basal ±SE)	24.01 ±0.64	85.37 ±3.02	26.77 ±4.44	54.54 ±2.63	30.12 ±4.60

Table 3. Effect of AM_{22-52} on AM-mediated AC activity in RAMP-2 or RAMP-3 transfected RMC. Prior to performing the AC activity assay membranes were pretreated with 10 μM of AM_{22-52}, an AM receptor antagonist. AM_{22-52} significantly inhibited AM-mediated AC activity in RAMP-2 and RAMP-3 overexpressed RMC. $p \leq 0.01$, experiments performed in triplicates, n=3.

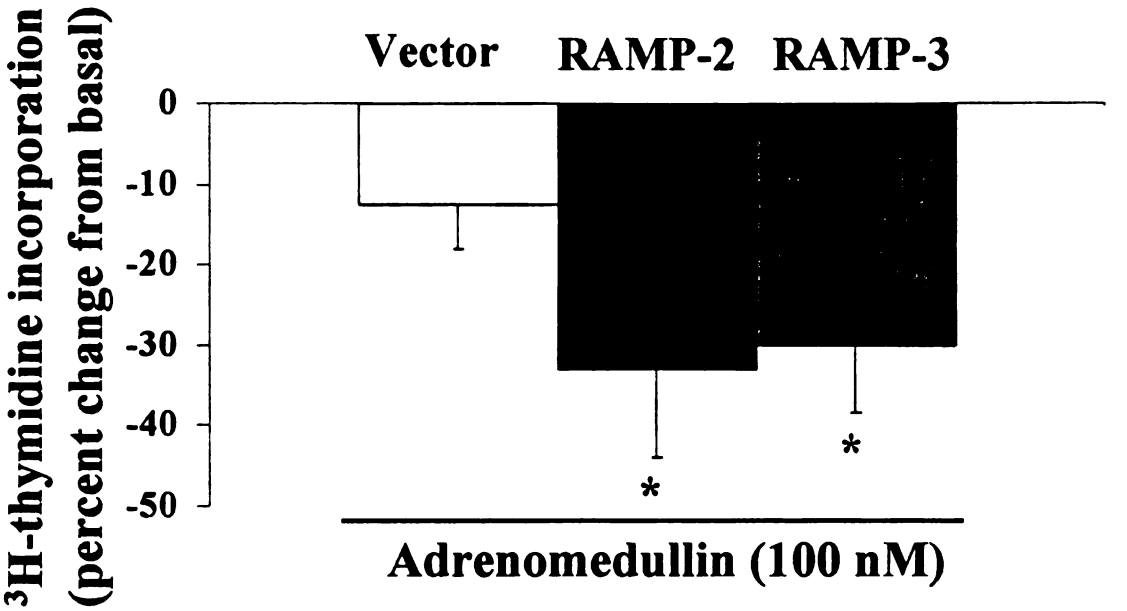


Figure 4. Effect of RAMP-2 and 3 over-expression on [^{3}H]thymidine incorporation in RMC. Cells were transiently transfected with RAMP-2 or RAMP-3 and allowed to grow for additional 24-36 hours. Subsequently, labeled thymidine incorporation in response to 100 nM AM was followed as per protocol described in materials and methods. RAMP-2 and RAMP-3 over-expression significantly potentiated AM-mediated decrease of [^{3}H]thymidine incorporation in RMC; *$p<0.05$ compared with vector; experiments performed in quadruplicates; n=4.

3.4. Discussion.

Several laboratories reported the RAMP gene expression profiles for a number of cell lines and tissues (see section 2.3.4). It is now clear that RAMP mRNA expression varies among cell lines and can be dramatically altered during different physiological states. Since RAMPs constitute a structural part of the AM receptor complex and are capable of modifying its ligand specificity, it becomes crucial to identify the expression patterns of these receptor-associated proteins in order to fully understand the biology of the ligands involved. Here, we have identified for the first time the expression profile of RAMPs in the rat mesangial cells (RMC). Cultured RMC express in basal conditions all subtypes of RAMPs as well as CL receptor. This theoretically allows for generation of both CGRP ($CGRP_1$: CL receptor+RAMP-1) and AM (AM_1: CL receptor+RAMP-2 and AM_2: CL receptor+RAMP-3) specific receptors and correlates well with previously reported findings on the responsiveness of cultured RMC to the exogenous administration of the CGRP family of peptides. Osajima *et al.* (240) observed a 7-fold increase in AM-mediated cAMP generation as compared to only 2-fold rise in cAMP levels following CGRP administration in RMC. In addition, the IC_{50} calculated for the inhibition of peptide-induced cAMP formation was approximately 10^{-8} M for AM_{22-52} as compared to 10^{-6}M for $CGRP_{8-37}$. Accordingly, these data point out that both CGRP and AM-specific receptors are present in RMC but the AM-sensitive receptors are preferentially expressed, suggesting the predominant role for AM in regulating mesangial cell biology. Of note is the finding that amylin had no effect on cAMP generation in RMC even at high concentrations (240). Considering the current understanding of the amylin receptor biology, this finding together with our RAMP profile data implies the lack of functional

calcitonin receptor expression in RMC; this hypothesis however awaits future confirmation.

The expression of all three subtypes of RAMPs offers an additional theoretical possibility for AM receptor modulation in RMC. As previously described, compelling evidence exists to suggest a competitive interaction and homodimer formation among different RAMP subtypes. This leads to a variable ligand specificity of the membrane-associated receptors, as it may depend on the quantitative as well as the qualitative nature of the RAMPs available for the receptor complex formation. For example, transient expression of CL recepors in UMR-106 cells revealed that AM receptor expression and AM-specific receptor binding were enhanced with RAMP-2 co-expression but precipitously reduced by 50 percent with concomitant introduction of RAMP-1 cDNA (26). Similarly, in rabbit aortic endothelial cells co-expression of RAMP-3 along with RAMP-1 decreased the functional response to CGRP by 50 percent (212). These findings may be explained by the direct competition of RAMP interaction with CL receptor, or alternatively they may reflect a process of RAMP-RAMP hetero- and/or homo-dimerization, with the latter recently reported by Sexton *et al.* (294).

The AM-dependent cAMP formation and the related antiproliferative effect of AM on mesangial cells have been previously demonstrated by our laboratory as well as others (40), (247), (292). Activation of PKA has been shown to induce an antimitogenic effect on mesangial cells. Because AM increases adenylate cyclase activity and hence PKA, we hypothesized that overexpression of RAMP-2 and RAMP-3, in addition to increasing adenylate cyclase activity, will also potentiate an AM-mediated decrease in [^{3}H]thymidine incorporation. As predicted, transfection of RAMP-2 or RAMP-3

significantly potentiated AM-evoked inhibition of [^{3}H]thymidine incorporation (Fig.4). Since the excessive mesangial cell proliferation (as indexed by [^{3}H]thymidine incorporation) is a hallmark pathological change present in several renal diseases (160), (77), (64), these findings suggest that the postulated reno-protective effects of AM may at least in part be modulated at the receptor level. In particular, the upregulation of RAMP-2 and RAMP-3, the integral components of AM receptor complex, increases the cell's responsiveness to AM-mediated antiproliferative effects.

The overexpression of RAMP-1 had no significant effect on mesangial cell responsiveness to AM as assessed by adenylate cyclase activity and [^{3}H]thymidine incorporation assays. These findings are consistent with previously reported data describing CL receptor-RAMP-1 as a receptor complex with low AM binding specificity (26), (83). Also, it is worth noting that the overexpression of RAMP-1 had no significant effect on the basal adenylate cyclase activity as the vector transfected and the RAMP-1 transfected cells exhibited a comparable response to AM stimulation (Fig. 3). This observation comes as a contrast to the proposed hypothesis of competitive interaction among the RAMP subtypes, perhaps once again underscoring the importance of cell-specific differences in RAMP behavior, which has been previously discussed in section 2.3.4.

In summary, experimental results presented herein demonstrate that RMC express all three subtypes of RAMPs in basal conditions. Overexpression of RAMP-2 or RAMP-3 (but not RAMP-1) leads to a predicted change in functional responsiveness of RMC to AM. Namely, RMC transfected with exogenous RAMP-2 or RAMP-3 cDNA exhibit a significant decrease in AM-mediated cell proliferation as assessed by [^{3}H]thymidine

incorporation. These results suggest that the effects of AM on RMC proliferation may at least in part be dependent on the profile of RAMP expression and/or cellular availability for intermolecular interactions. Moreover, the membranous adenylate cyclase activity in response to AM is also significantly increased in RAMP-2 or RAMP-3 transfecter RMC. This response appears to be AM receptor specific as pretreatment with AM_{22-52}, an AM receptor antagonist, abolishes it (Table 3).

Since alteration in RAMP-1 gene expression does not appear to affect the actions of AM in mesangial cells, we will refrain from further consideration of RAMP-1 gene regulation.

4. Effects of platelet-derived growth factor and lipopolysaccharide on RAMP expression in rat mesangial cells.

4.1. Introduction.

Platelet-derived growth factor (PDGF) is a well-characterized cytokine secreted by many cell types including the glomerular mesangial cells. In mesangial cells PDGF acts in an autocrine and paracrine fashion as their most potent mitogen (2), (200), (79), (3). By interacting with a specific tyrosine kinase receptor (PDGF receptor, PDGFR), widely expressed by mesangial cells, PDGF has been shown to significantly increase mesangial cell thymidine incorporation rate as well as augment the synthesis of extracellular matrix components (197), (360). In addition, PDGF also potently induces directed mesangial cell migration (17), (161). A number of studies suggest that PDGF is not only a causal factor in development of mesangial hypercellularity and excessive matrix accumulation (the fundamental changes observed in several glomerular disease states) but also participates in mesangial cell repopulation following sub-total glomerular damage characteristic to several forms of glomerulonephritis (1). Consequently, PDGF has been recognized as a prime cytokine responsible for mediating both proliferative and migratory responses in the mesangium during glomerular injury (1), (241), (188). The clearly opposing effects of AM and PDGF on cell growth were examined during the initial characterization of then newly discovered AM polypeptide. AM was found to potently decrease PDGF-induced mesangial cell proliferation (39), (292). Our current understanding of AM biology, especially at the receptor level, offers a new angle on investigation of the regulation of proliferative/anti-proliferative events in mesangial cells.

In particular, the mechanisms responsible for the functional interplay between PDGF and AM receptor deserve further examination. Hence, the present study was undertaken to examine the possible effect(s) of PDGF on AM receptor complex expression in rat mesangial cells.

The endotoxin, lipopolysaccharide (LPS), is a known component of the outer membrane of Gram-negative organisms that plays a crucial role in mediating sepsis-evoked circulatory failure. Recently it has been argued that in addition to inducing generalized nitric oxide formation (256), (51), (209) and downregulating the angiotensin-aldosterone system (24), LPS achieves its effects by directly influencing AM activity. In fact, LPS has been proposed as a leading mediator responsible for stimulation of AM production during sepsis *in vivo* (366), (305), (301), (308) and in a cell culture setting (312). The dramatic rise in intravascular levels of AM during sepsis has been reported in humans as well as animals and is estimated to be greater than in any other pathological condition studied (117), (228), (351). Careful examination of the circulating levels of AM revealed its robust increase during the early, hyperdynamic phase of sepsis characterized by an increase in cardiac output, decrease in total peripheral resistance, hyperglycemia, and hyperinsulinemia (349), (365), (348). The above-mentioned physiological changes have also been observed in healthy humans exposed to intravenous AM (175). Wang *et al.* demonstrated the causative effects of AM in sepsis by studying rats after intravenous synthetic AM administration. Inducing circulating concentrations of AM that resemble those during clinical sepsis resulted in a hyperdynamic response characteristic of early sepsis. Moreover, administration of anti-AM neutralizing antibodies to animals after induction of sepsis by cecal ligation and puncture procedure prevented the development

of the hyperdynamic response (348). In addition, the AM-induced hyperdynamic state has been reported to maintain renal blood flow during the early endotoxic shock thereby protecting against kidney failure (228). Interestingly, it has been documented that despite the sustained elevation in AM levels throughout sepsis, a marked decrease in vascular responsiveness to AM takes place during the later stages characterized by multi-organ failure and circulatory collapse. This presumably facilitates the transition from the hyperdynamic to the hypodynamic state where marked increase in vascular resistance, decrease in cardiac output and peripheral oxygen delivery are concomitantly observed (350). It is hypothesized that the initial responsiveness to elevated AM levels prolongs the hyperdynamic state thus protecting against the cardiovascular failure while the later loss of AM reactivity culminates with multiple organ failure and impending mortality. The mechanism responsible for these changes is not currently well understood. However the process of AM receptor desensitization, which has been documented to occur in vascular smooth muscle (129) as well as mesangial cells (245), may potentially explain the observed shift in AM sensitivity during the progression of sepsis. Alternatively, changes in AM receptor affinity and/or receptor-adenylate cyclase uncoupling as well as the direct effect of LPS on AM receptor complex gene expression could also provide for the observed temporal changes in AM receptor activity. Despite the ambiguity regarding the exact mechanisms involved, researchers agree on the beneficial effects of AM activity during sepsis. The protective role of AM has been elegantly demonstrated by Shido *et al.* (302), who reported that transgenic mice overexpressing AM exhibited a remarkable resistance to the induction and progression of septic shock. Mice overexpressing AM in their vasculature showed greater vasomotor stability, diminution in the end-organ

damage, and consequently greater survival rates following LPS challenge equated to bacterial endovascular burden encountered in septicemia.

The production and cellular responsiveness of AM are clearly affected by both PDGF and LPS. In view of the wide spectrum of pathologies with PDGF and/or LPS involvement as well as the fact that AM appears to play an important protective role during the development and progress of these pathological states, further investigation of the interactions between AM and these regulatory molecules needs to be considered. In particular little is currently known about the effects of PDGF and LPS at the AM receptor level. PDGF and/or LPS can conceivably regulate the gene expression of the AM receptor components, thus leading to the observed alterations in AM responsiveness. Accordingly, this chapter will describe the experiments designed to inspect the possible effect(s) of PDGF and LPS on the mRNA and protein expression of the AM receptor complex (AM_1 and AM_2) present in mesangial cells.

4.2. Materials and methods.

4.2.1. Materials.

Adrenomedullin, adrenomedullin(22-52) fragment and PDGF-BB were purchased from Sigma® RBI® (St. Louis, MO). Lipopolysacccharide, *E.coli* 055:B5 was from Calbiochem (La Jolla, CA). RPMI-1640, fetal bovine serum, penicillin/streptomycin, trypsin-EDTA were from GibcoBRL® (Grand Island, NY). RAMP-2 and RAMP-3 polyclonal primary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz,

CA). Horseradish peroxidase-conjugated secondary Anti-rabbit IgG antibodies were from Sigma® RBI® (St. Louis, MO).

All other reagents were of highest quality available.

4.2.2. Cell Culture.

Rat mesangial cell (RMC) cultures were established from glomeruli as described in section 3.2.2. RMC were maintained in RPMI-1640 with 15% fetal bovine serum unless otherwise stated for particular experiments. Passages 15-24 were used for subsequent experiments with specific confluency determined by preliminary studies.

4.2.3. RAMP 1, 2, 3, and CRLR cloning and expression.

Full-length cDNA of human RAMP-1, 2, 3, and bovine CL receptor were cloned into Myc-tagged and pCDN vectors obtained from Clonetech (Palo Alto, CA). Multiple DNA preparation batches were used. Expression vectors were transfected into RMC using LipofectAMINE PLUS™ Reagent (GibcoBRL®, Grand Island, NY) following the suggested protocol. In brief, RMC were plated on P-100 tissue culture plates a day prior to the transfection to achieve an approximate 60-70% confluency. On the following day, cells were transfected with a total of 2μg of each vector-cDNA in a serum-free transfection medium for 4 hrs at 37°C/5% CO_2. The total amount of transfected cDNA was kept constant by adding empty Myc or pCDN vector. Afterwards, 4ml of serum-containing medium was added and cells were incubated for an additional 24-36 hrs. This transient transfection protocol resulted in an approximate 70% transfection efficiency as assessed by green fluorescent protein (GFP).

4.2.4. Membrane preparation and adenylate cyclase assay.

Cells were harvested from P150 plates and homogenized in Tris HCl, pH 7.4, (10mM)/EDTA (10mM) buffer. Membranes were prepared by homogenization in a Dounce ground glass homogenizer, centrifuged for 20 min at 12,000*g* at 4°C and washed in Tris HCl, pH 7.4, (50mM)/MgCl (10mM) buffer. Final concentration of 40μg of protein/assay tube was obtained and the membranes were immediately subjected to adenylate cyclase assay, as follows. Membrane associated adenylate cyclase activity was measured as the rate of conversion of [α^{32}P]ATP to [^{32}P]cAMP as described by Elshourbagy et al. (67). Accordingly, membranes were incubated for 20 min at 30°C with appropriate drugs and assay mix containing ATP regeneration system (50mM Tris-HCl, pH 7.4, 10mM $MgCl_2$, 1.2 mM ATP, 0.1 mM cAMP, 2.8 mM phosphoenolpyruvate, and 5.2 μg/ml myokinase) and 1.0 μCi of [α^{32}P]ATP. Total reaction volume was 100μL, drug as well as AM concentrations were as described for particular experiments. Reactions were stopped with 1 ml of stop solution containing 0.28mM "cold" cAMP, 0.33mM ATP, and 22,000dpm of ^{3}H-cAMP. Contents of the assay tubes were washed through a Dowex and subsequently alumina columns to separate the degradation products of ATP as previously described by Salmon *et al.* (276). Elution profiles were performed prior to experiments to determine the amount of water (for Dowex columns) and imidazole (for alumina columns) needed to wash and elute the products. Products eluted from alumina column were counted for the presence of ^{3}H-cAMP and α^{32}P-cAMP. Each experiment was done in triplicates, repeated at least 3 times, and expressed as percentage of AM-mediated adenylate cyclase activity compared to basal.

4.2.5. [^{3}H]thymidine incorporation.

Cells were plated in 24 well plates (30000 cells/well) and grown for 2 days with subsequent serum starving for 48 hrs. Then, they were treated with the compounds for a period of 16 hrs and pulsed with [^{3}H]thymidine for 4 hrs. The radioactivity was counted in Beckman LS counter, after washing the cells and stopping the reaction with 5% trichloro acetic acid and solubilizing the cells in 0.5 ml of 0.25 N sodium hydroxide. Each experiment was performed in quadruplicates and repeated at least three times.

4.2.6. Northern blot analysis.

Immediately after cell culture medium was aspirated from the tissue culture plates, 2-4 ml of TRIzol® was added, dispersed uniformly, and plates were stored at -80°C until further use. Following a quick thaw, cells were scraped with cell lifters and transferred to 15 ml centrifuge tubes. Total cellular RNA was isolated with TRIzol® according to manufacturer's specifications. RNA was precipitated by adding 3 M sodium acetate and absolute ethanol, washed with 75% ethanol, pelleted in microcentrifuge tubes, and dried prior to resuspension in RNAse-free water. Its purity was checked by measuring the ratio of $\text{absorbance}_{@260nm}/\text{absorbance}_{@280nm}$. All of the RNA used had the ratio equal or greater to 1.8. A standardized aliquot of RNA (30 μg) was separated by electrophoresis on a formaldehyde agarose denaturing gel and transferred to an Optitran® membrane (Schleicher & Schuell, Keene, NH) by capillary transfer. Subsequently, RNA samples were immobilized to the membrane by ultraviolet cross-linking. Membranes were successively hybridized at 42°C for 16-24 hrs with four parts (15 ml of Formamide, 0.6 ml Denhardt's solution, 1.5 ml of 1 M phosphate buffer, 7.5 ml of 20x SSC, 1.5 ml of

SDS, 2.4 ml of diethylpyrocarbonate water, and 1.5 ml of salmon sperm DNA)/blot and one part ^{32}P-dCTP-labeled cDNA probes (specific for RAMP-1, 2, 3 or 18S ribosomal subunit; RAMP probes were obtained as RT-PCR products using RAMP-specific primers designed from published sequences). The cDNA was radiolabeled using a random prime labeling kit. Following hybridization for 16-24 hrs, the membranes were washed and placed in an x-ray cassette for the requisite exposure time. Signals were quantitated by phosphoimager analyses and expressed relative to 18S levels.

4.2.7. Western blot analysis.

Western blot analysis was done as described before (247). Briefly, equal concentrations of protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were subsequently blocked with 5% non-fat dry milk in Tris buffered saline containing 0.05% Tween-20 (TBS-T) and incubated with RAMP-2 or RAMP-3 polyclonal primary antibodies at final concentration of 400 ng/ml followed by horse radish peroxidase-conjugated secondary Anti-rabbit IgG antibodies at final dilution of 1:10,000, according to manufacturer's instructions. Enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL) was used to visualize the blots.

4.2.8. Statistical analysis.

Data are presented as mean±S.E.M. Multiple group comparisons were made using a two-way analysis of variance (ANOVA). Single group comparisons exercised Student's t-test method. Statistical significance was set at $P<0.05$

4.3 Results.

4.3.1. Effect of PDGF on RAMP mRNA expression.

In concordance with findings from other cell systems, over-expression of RAMP-2 and RAMP-3 in mesangial cells also led to potentiated AM responsiveness (fig. 3). Considering the fact that altered RAMP expression has been reported in several disease states and that PDGF is a prime factor responsible for pathophysiological changes in glomerular biology, we hypothesized that PDGF may also modify RAMP expression. To test this hypothesis, we investigated the effects of PDGF-BB on mesangial cell RAMP mRNA abundance. Exposure of mesangial cells to exogenous PDGF (0.1-100 ng/ml) increased RAMP-3 mRNA expression in a concentration-dependent manner (Fig. 5A) while it had no effect on RAMP-2 mRNA abundance (Fig. 5B).

In order to evaluate the temporal character of the PDGF-induced RAMP-3 mRNA expression, we quantified the RAMP-3 mRNA abundance during the 48 hour period following introduction of 50 ng/ml PDGF into the mesangial cell culture medium. Significant increase in RAMP-3 mRNA levels was observed within 6 hours post PDGF exposure with a peak elevation noted at 24 hours (Fig. 6). Thus RAMP-3 gene upregulation in response to 50 ng/ml PDGF is time-dependent within the exposure period studied.

A-a

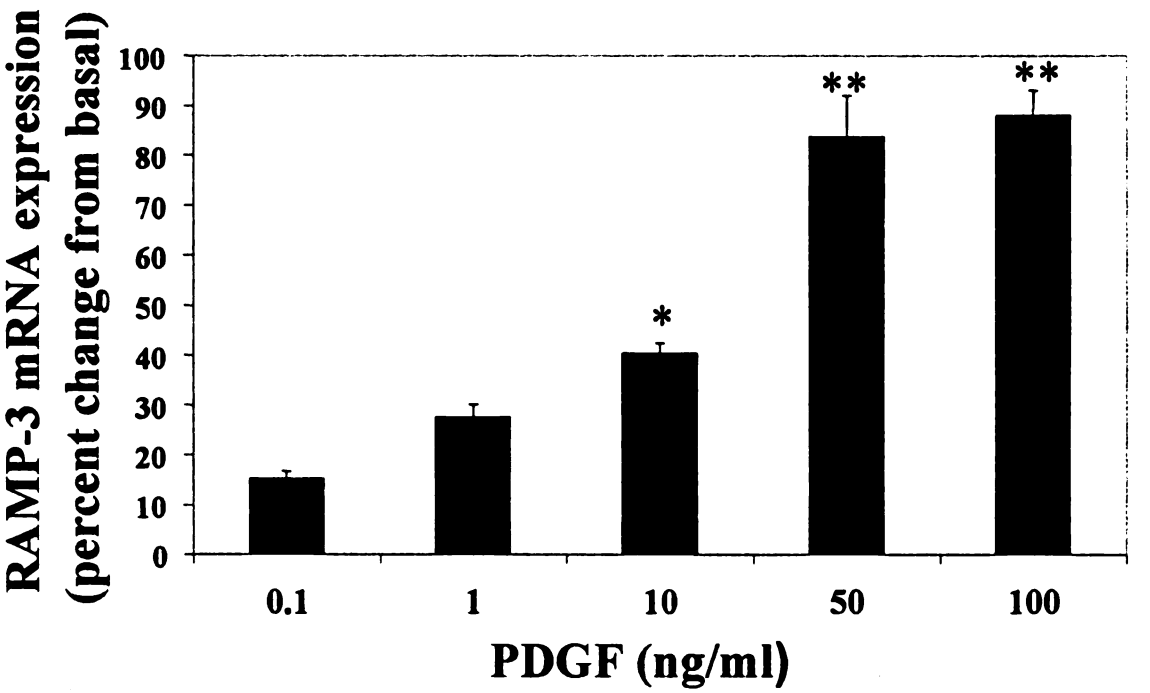


Figure 5A. Effect of platelet-derived growth factor (PDGF) on RAMP-3 mRNA expression in RMC. Cells were treated with vehicle (basal) or doses of PDGF, as indicated, for 24 hours. RNA was extracted and northern blot analysis performed as described in materials and methods. Blots were first probed for RAMP-3 and then stripped and reprobed for 18s RNA. Raw values were converted to ratios of RAMP-3 mRNA to 18s and then expressed as percent change from basal. A-a: PDGF increased RAMP-3 mRNA expression in a dose-dependent manner; *$p<0.01$, **$p<0.001$ as compared with 0.1ng/ml PDGF dose, n=5.

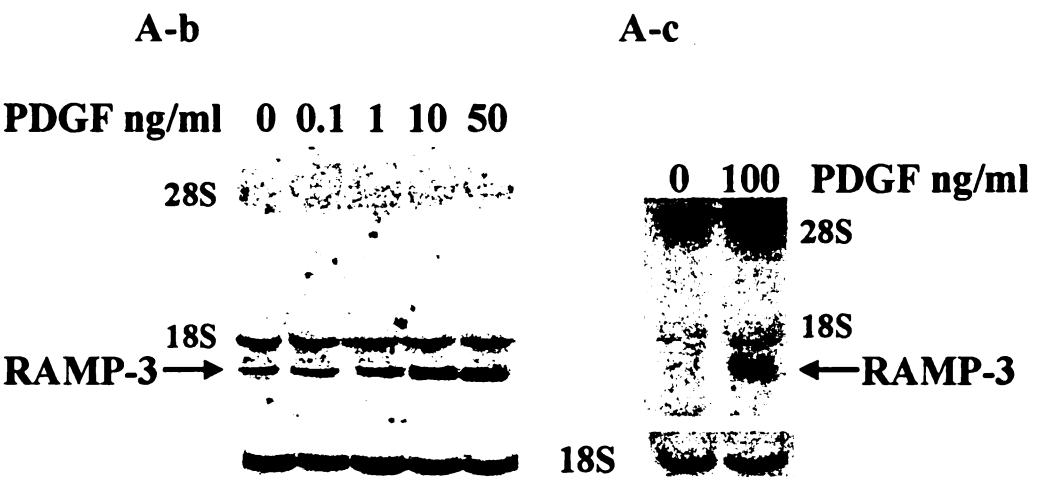


Figure 5A. Effect of platelet-derived growth factor (PDGF) on RAMP-3 mRNA expression in RMC. A-b and A-c: representative Northern blots of RAMP-3 expression in response to PDGF in RMC.

B

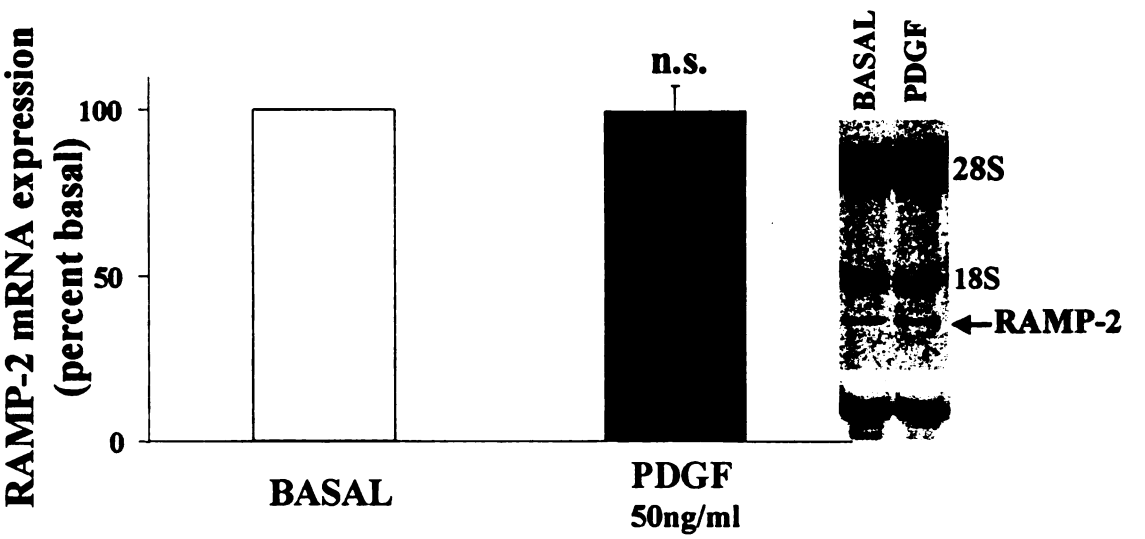


Figure 5B. Effect of platelet-derived growth factor (PDGF) on RAMP-2 mRNA expression in RMC. Cells were treated with vehicle (basal) or doses of PDGF, as indicated, for 24 hours. RNA was extracted and northern blot analysis performed as described in materials and methods. Blots were first probed for RAMP-2 and then stripped and reprobed for 18s RNA. Raw values were converted to ratios of RAMP-2 mRNA to 18s and then expressed as percent change from basal. B: PDGF had no significant effect on RAMP-2 expression as compared to basal; n.s.-statistically not significant, n=4. Insert shows a representative northern blot of RAMP-2 expression in response to PDGF in RMC.

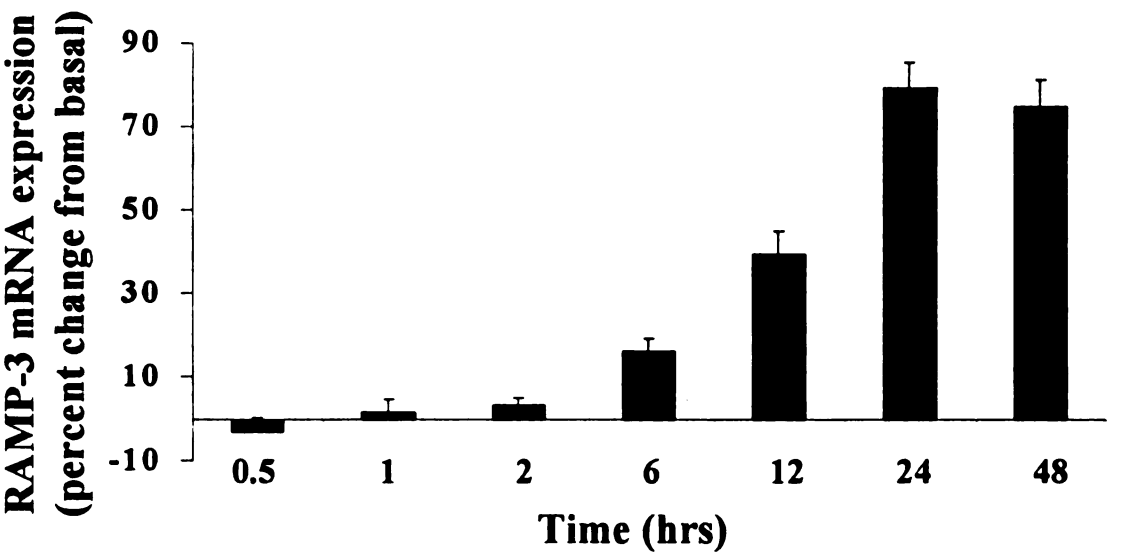


Figure 6. Effect of platelet-derived growth factor (PDGF) on RAMP-3 mRNA expression in RMC. Cells were treated with vehicle (basal) or 50 ng/ml PDGF for time periods as indicated. RNA was extracted and northern blot analysis performed as described in materials and methods. Blots were first probed for RAMP-3 and then stripped and reprobed for 18s RNA. Raw values were converted to ratios of RAMP-3 mRNA to 18s and then expressed as percent change from basal. PDGF increased RAMP-3 mRNA expression in a time-dependent manner.

4.3.2. Effect of PDGF on RAMP protein expression and AM-mediated adenylate cyclase activity.

To determine whether the PDGF-induced increase in RAMP-3 mRNA corresponds to an elevated expression of RAMP-3 protein, we examined RAMP-3 protein levels in the membrane-associated fraction of cells cultured in the presence or absence of PDGF. Western blot data revealed a 3.3 ±0.28 fold increase in the amount of RAMP-3 protein in cells exposed to PDGF as compared to controls (Fig. 7). As predicted, PDGF-induced elevation of RAMP-3 correlated to a functional increase in cell responsiveness to various concentrations of AM as measured by AM-mediated adenylate cyclase activity (Fig. 8A). Also, membranes of cells treated with various concentrations of PDGF (1-100 ng/ml) exhibited a concentration-dependent increase in AM-stimulated AC activity (Fig. 8B). This effect was inhibited by pre-treatment of membranes with AM(22-52), suggesting a direct involvement of AM-specific receptors (Fig. 8C).

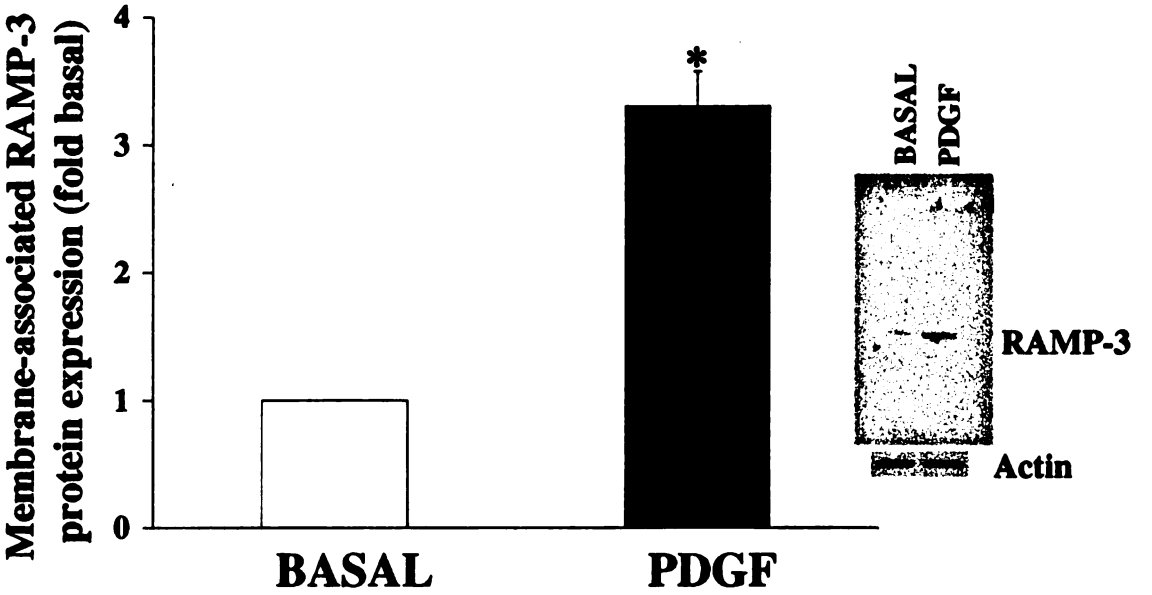


Figure 7. Effect of PDGF on membrane-associated RAMP-3 protein expression in RMC. Cells were incubated with PDGF (50 ng/ml) for 24 hours. Subsequently membranes were extracted as per membrane preparation protocol for AC assay (see materials and methods) and equal concentrations of protein were loaded onto a gradient polyacrylamide gel. Western blotting was performed as described in the materials and methods. Raw values were converted to ratios of RAMP-3 protein to actin and expressed as fold of basal (with basal expression arbitrarily set at 1). Right: insert represents an exemplary Western blot obtained in this set of experiments. PDGF significantly increased RAMP-3 protein expression in the membrane-associated fraction of RMC. *$p<0.001$; $n=4$.

A

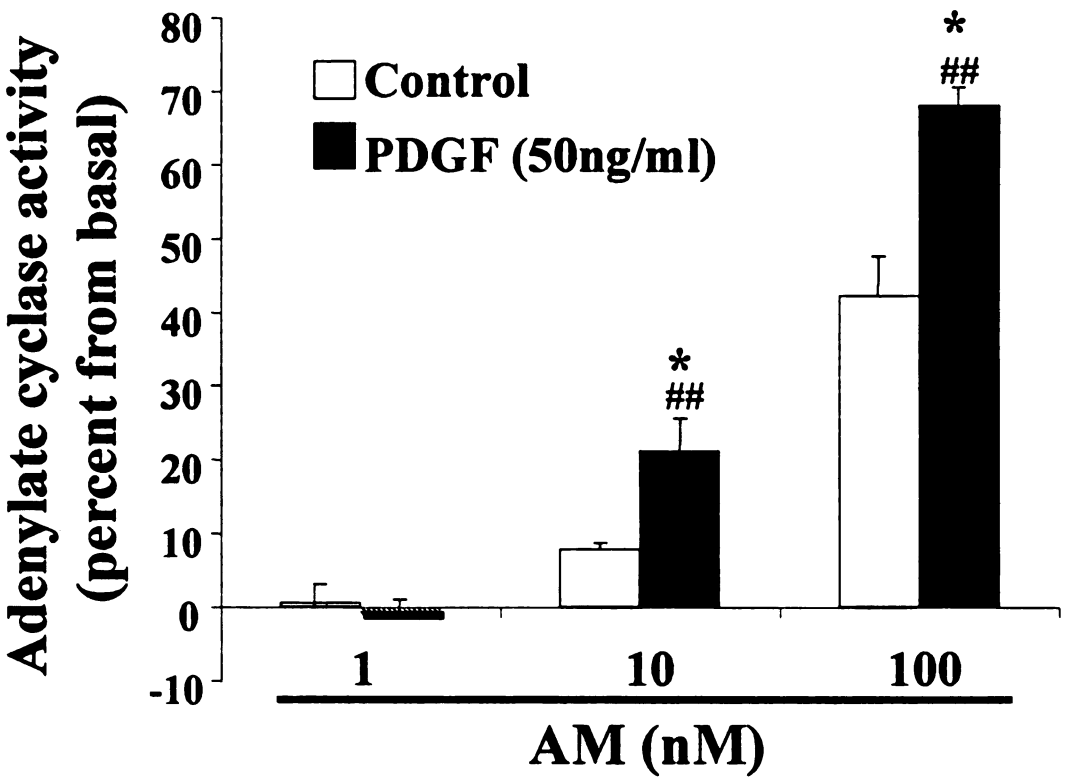


Figure 8. Effect of PDGF on AM-mediated adenylate cyclase activity in RMC. Cells were incubated in the presence or absence of PDGF (50 ng/ml) for 24 hours. Membranes were then extracted and AC activity assay in response to indicated concentrations of AM was performed. A: AC activity increased with increasing doses of AM in PDGF treated and controls. PDGF treated cells exhibited significantly higher AC activity as compared to corresponding basals (*$p<0.01$) and control treated cells ($^{\#\#}p<0.01$). Experiments were performed in triplicates, n=5.

B

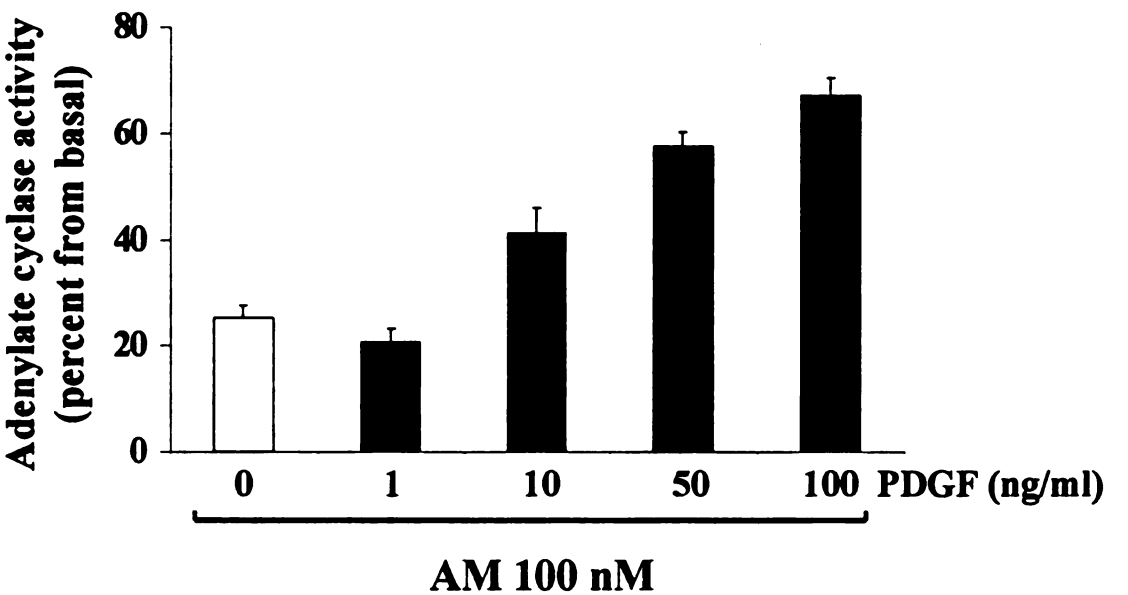


Figure 8. Effect of PDGF on AM-mediated adenylate cyclase activity in RMC. B: Cells were treated with different concentrations of PDGF, as indicated, for 24 hours. Membranes were then extracted and AC activity assay in response to 100 nM AM was performed. PDGF caused a concentration-dependent increase in AM-stimulated AC activity, which closely correlated with PDGF-stimulated RAMP-3 mRNA expression (Fig. 5A); experiments were done in triplicates, n=3.

C

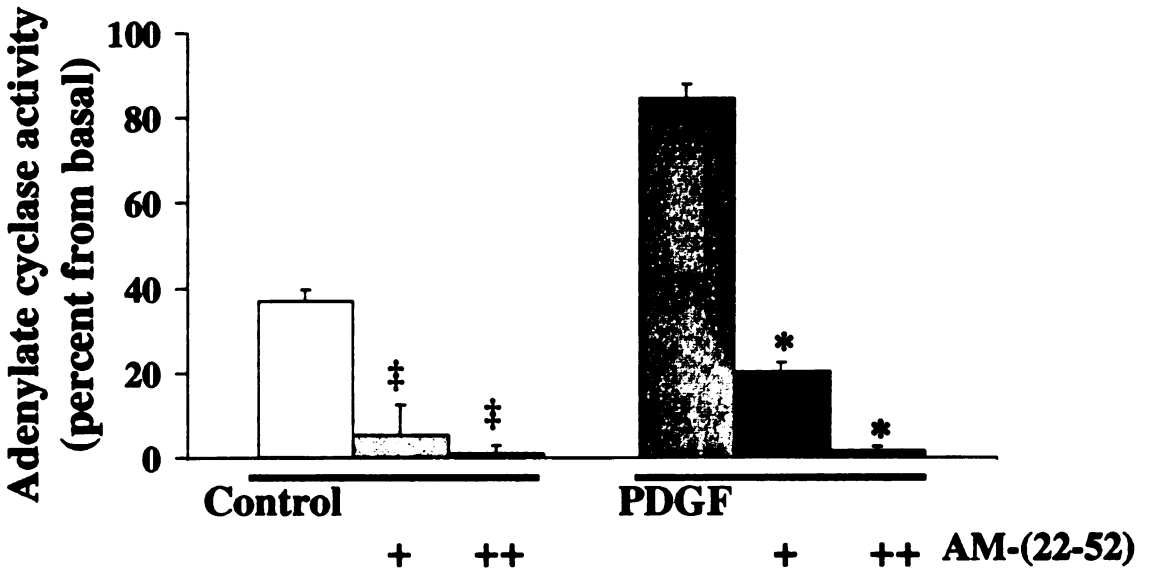


Figure 8. Effect of PDGF on AM-mediated adenylate cyclase activity in RMC. C: Effect of AM-(22-52), the AM receptor antagonist, on AM-mediated AC activity in rat mesangial cells exposed to PDGF. Cells were grown in the presence of PDGF (50 ng/ml) or its absence (control) for 24 hours. Next, membranes were extracted and pre-treated with AM-(22-52) at 100 nM (+) or 1μM (++) for 10 minutes prior to AC activity assay in response to 100 nM AM. PDGF-dependent increase in AM-mediated AC activity was significantly inhibited by AM receptor antagonist, AM-(22-52); *$p<0.01$ (as compared with PDGF treatment), $^{\ddagger}p<0.01$ (as compared with control); experiments performed in triplicates; n=3.

4.3.3. Effect of LPS on RAMP mRNA expression.

Similar to the PDGF findings, LPS treatment of mesangial cells also induced the mRNA expression of RAMP-3. The LPS-evoked increase in RAMP-3 mRNA was dose-dependent with the LPS concentrations of 1-30 μg/ml (Fig. 9). LPS concentrations above 30 μg/ml appeared to be highly toxic to mesangial cells, resulting in gross cell structure changes and loss of vital cell adhesion properties. RAMP-3 mRNA expression was significantly increased by 30 minutes following the introduction of 10 μg/ml LPS into the cell culture medium and remained elevated throughout the 24-hour period studied (Fig. 10).

Next, we investigated the effect of LPS on RAMP-2 gene expression by RT-PCR and northern blot analysis. Exposure of mesangial cells to various concentrations of LPS (1-30 μg/ml) did not affect the basal levels of RAMP-2 expression as assessed by RT-PCR (data not shown) and northern blot analysis (see Fig 11. for representative data).

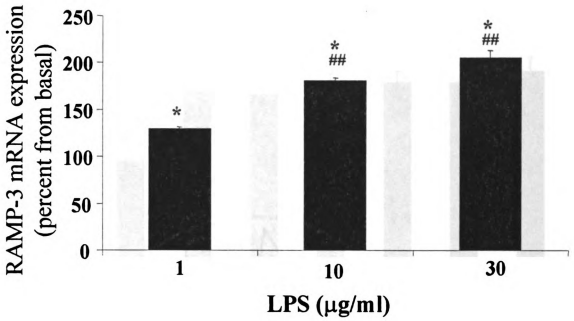


Figure 9. Effect of lipopolysaccharide (LPS) on RAMP-3 mRNA expression in RMC. Cells were incubated with vehicle (basal) or doses of LPS, as indicated, for 24 hours. RNA was extracted and northern blot analysis performed as described in materials and methods. Blots were first probed for RAMP-3 and then stripped and reprobed for 18s RNA. Raw values were converted to ratios of RAMP-3 mRNA to 18s and then expressed as percent change from basal. LPS increased RAMP-3 mRNA expression in a dose-dependent manner; *$p<0.001$ as compared with basal, ##$p<0.001$ as compared with 1 μg/ml LPS dose; n=3.

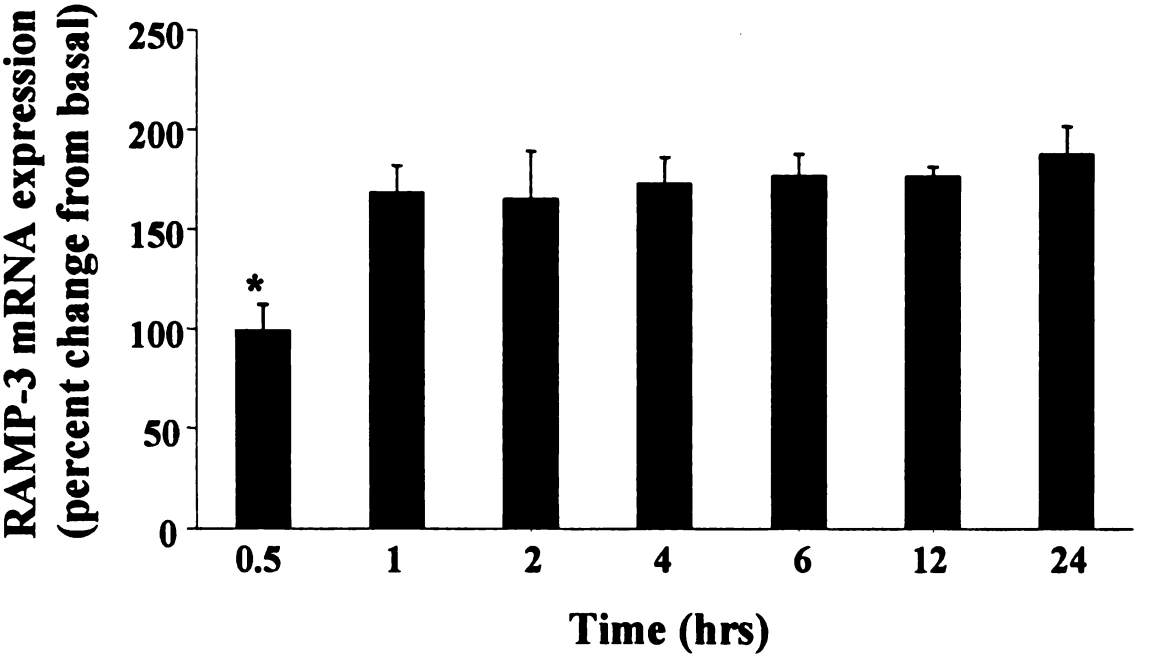


Figure 10. Effect of lipopolysaccharide (LPS) on RAMP-3 mRNA expression in RMC; temporal effect. Cells were treated with vehicle (basal) or 10 μg/ml LPS for time periods as indicated. RNA was extracted and northern blot analysis performed as described in materials and methods. Blots were first probed for RAMP-3 and then stripped and reprobed for 18s RNA. Raw values were converted to ratios of RAMP-3 mRNA to 18s and then expressed as percent change from basal. LPS significantly increased RAMP-3 mRNA expression by half an hour of exposure. Peak elevation of RAMP-3 mRNA expression is observed within one hour of LPS exposure and it is sustained during the 24-hour period; *$p<0.001$ as compared with basal; $n=3$.

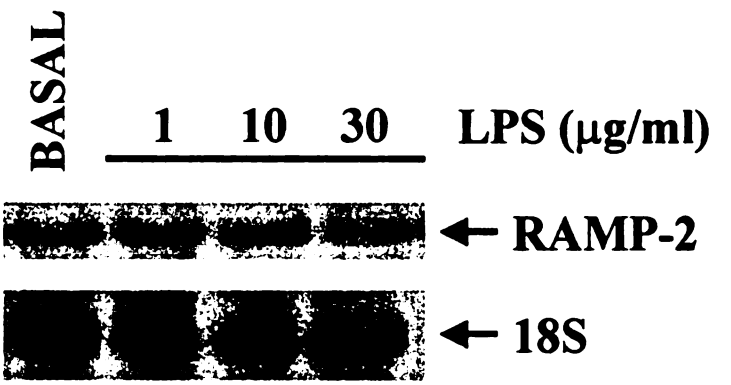


Figure 11. Effect of lipopolysaccharide (LPS) on RAMP-2 mRNA expression in RMC. Cells were treated with vehicle (basal) or doses of LPS, as indicated, for 24 hours. RNA was extracted and northern blot analysis performed as described in materials and methods. Blots were first probed for RAMP-2 and then stripped and reprobed for 18s RNA. Depicted above is a northern blot of RAMP-2 expression representative of three independent experiments. LPS, at concentrations examined, has no significant effect on RAMP-2 mRNA expression as compared to basal.

4.3.4. Effect of LPS on RAMP protein expression and AM-mediated adenylate cyclase activity.

Parallel to the LPS-mediated increase in RAMP-3 mRNA, we also noted an elevation in RAMP-3 protein expression in the cell membrane fraction of mesangial cells treated with 10 μg/ml LPS for 24 hours. Specifically, a 2.94±0.23 fold increase in RAMP-3 membrane-associated protein was observed (Fig. 12).

Since previously observed elevation in RAMP-3 protein expression resulted in a concomitant increase in mesangial cell responsiveness to AM, we tested if this was also true for the LPS-induced increase in RAMP-3 protein. Our data indicate, that exposure of mesangial cells to LPS also increases AM-mediated adenylate cyclase activity. As predicted, this effect is inhibited by AM_{22-52} in a concentration dependent manner, hence it appears to be AM receptor specific (Fig. 13). Furthermore, the significant increase in AM responsiveness following LPS treatment is observed in membranes stimulated with varied AM concentrations, thus augmenting a basal, AM dose-dependent trend in adenylate cyclase activity (Fig. 14).

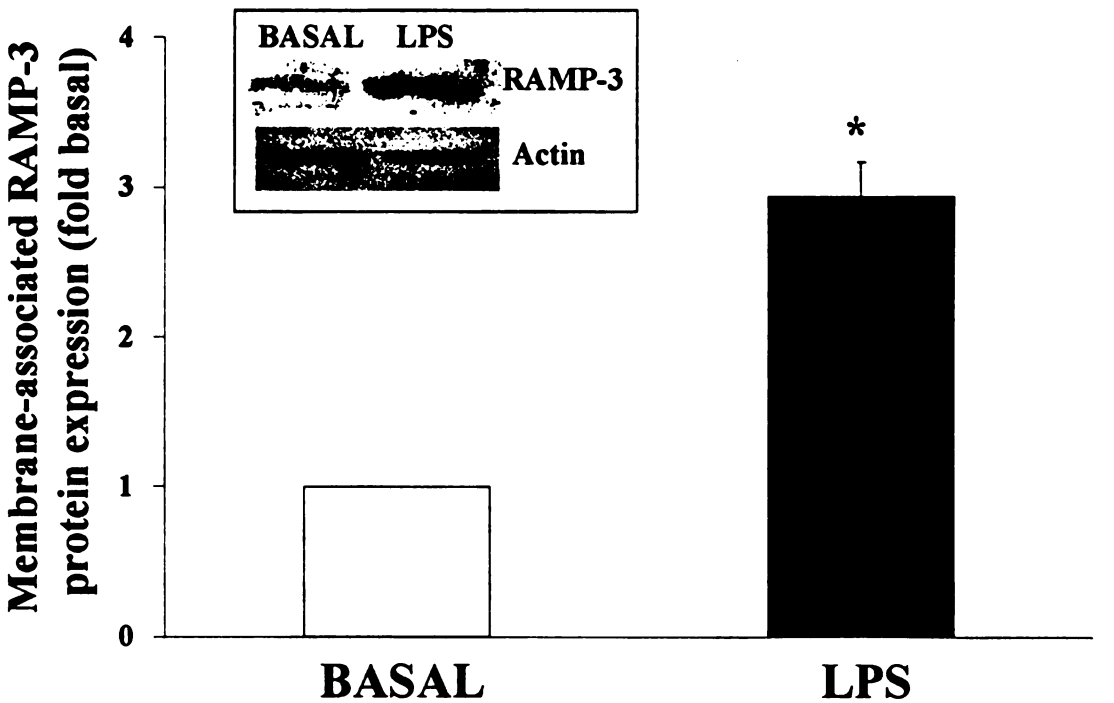


Figure 12. Effect of lipopolysaccharide (LPS) on membrane-associated RAMP-3 protein expression in RMC. Cells were incubated with LPS (10 μg/ml) for 24 hours. Subsequently membranes were extracted as per membrane preparation protocol for AC assay (see materials and methods) and equal concentrations of protein were loaded onto a gradient polyacrylamide gel. Western blotting was performed as described in the materials and methods. Raw values were converted to ratios of RAMP-3 protein to actin and expressed as fold of basal (with basal expression arbitrarily set at 1). Insert represents an exemplary Western blot obtained in this set of experiments. LPS significantly increased RAMP-3 protein expression in the membrane-associated fraction of RMC. *$p<0.01$; $n=4$.

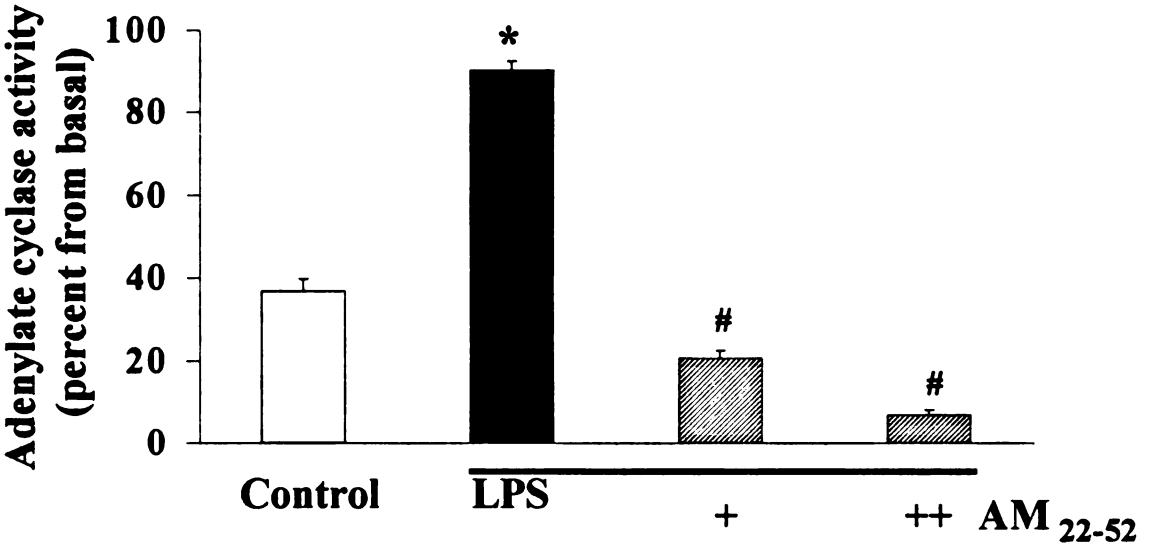


Figure 13. Effect of lipopolysaccharide (LPS) on AM-mediated adenylate cyclase activity in RMC; response to pre-treatment with AM_{22-52}. Cells were incubated in the presence of LPS (10 μg/ml) or its absence (control) for 24 hours. Membranes were then extracted and AC activity assay in response to 100 nM AM was performed. LPS treated cells exhibited significantly higher AC activity as compared to control treated cells; *$p<0.01$. Membranes pre-treated with AM_{22-52} (AM receptor antagonist) at 100 nM (+) or 1μM (++) for 10 minutes prior to AC activity assay exhibited significantly attenuated response to 100 nM AM. #$p<0.01$ (as compared with LPS treatment; experiments performed in triplicates; n=3.

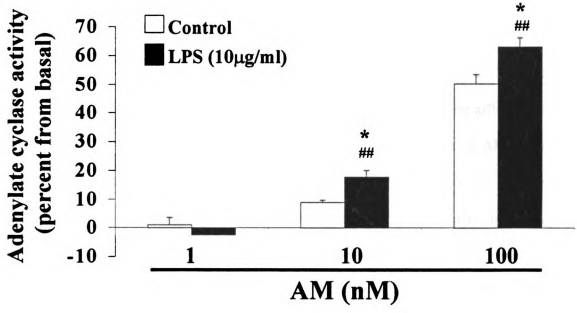


Figure 14. Effect of lipopolysaccharide (LPS) on AM-mediated adenylate cyclase activity in RMC; response to varied concentrations of AM. Cells were incubated in the presence or absence of LPS (10 μg/ml) for 24 hours. Membranes were then extracted and AC activity assay in response to indicated concentrations of AM was performed. AC activity increased with increasing doses of AM in LPS treated cells and controls. LPS treated cells exhibited significantly higher AC activity as compared to corresponding basals (*$p<0.01$) and control treated cells ($^{\#\#}p<0.01$). Experiments were performed in triplicates, n=5.

4.4. Discussion.

Mesangial cells express a functional AM receptor complex (AM_1 and AM_2) in culture. As observed by several investigations of various cell lines, over-expression of RAMP-2 or RAMP-3 also increases RMC responsiveness to AM as measured by AM-induced adenylate cyclase activity ((83), (121) and Fig. 3). Fraser *et al.* (83) and Husmann *et al.* (121) have reported that co-transfection of HEK-293 and COS-7 cells with CL receptor and RAMP2/3 resulted in an increased ^{125}I-AM binding affinity and an augmented cAMP accumulation in response to various concentrations of AM. Clearly, modification of RAMP expression can serve as a major mechanism for effectively altering cell's responsiveness to AM not only in other cell types, but also in mesangial cells. Here, we propose that two factors that are known to influence the adrenomedullin signaling system in mesangial cells: PDGF and LPS, may modulate this system through their action(s) on RAMP expression.

PDGF is a well-known, important autocrine/paracrine growth factor in mesangial cells and has been shown to interact with adrenomedullin anti-proliferative pathway. Specifically, PDGF-induced mitogenesis is blocked by adrenomedullin in rat mesangial cells in culture (39), (292). Indeed, if PDGF enhances adrenomedullin signaling in mesangial cells, it may serve as a negative feedback mechanism in regulating normal turnover of mesangial cells. Thus, we investigated the effects of PDGF on adrenomedullin receptor system, including its effects on CL receptor, RAMP2 and RAMP3 in rat mesangial cells. We report here that PDGF regulates adrenomedullin receptor signaling through modulation of RAMP3 mRNA expression. PDGF did not have any effect on CL receptor (data not shown) or RAMP2 mRNA expression. Others have

found similar results in both animal and cell culture models. For example, Totsune *et al.* presented findings from a rat model of 5/6 nephrectomy where RAMP-2 mRNA levels were unchanged while a significant decrease in RAMP-3 mRNA was found in the remnant kidney (330). In general, ample evidence exists for differential expression of RAMP mRNA in tissues from animal disease models, including those with renal pathology (217), (237), (331), (236), (47), (263), (369). Frayon *et al.* have reported that glucocorticoid treatment in VSMC in culture causes a transient increase in RAMP-1 mRNA expression without any change in RAMP-2 expression (84). This glucocorticoid-directed RAMP expression results in a CGRP responsive receptor ($CGRP_1$: CL receptor+RAMP-1). These results also suggest that in vivo, glucocorticoid regulation of RAMP1 mRNA expression may lead to a subsequent consequence on ligand recognition. Kitamuro and colleagues (159) observed suppression of RAMP-2 (and no change in RAMP-1) mRNA expression in two neuroblastoma cell lines following a pharmacological induction of hypoxic state with cobalt chloride and desferrioxamine mesylate. They hypothesized that the differential expression of RAMP-2 may in fact constitute a viable mechanism for cellular adaptation to hypoxic stress by modifying AM receptor complex expression. Similarly, Robert-Nicoud *et al.* reported vasopressin-induced RAMP-3 mRNA levels in mouse clonal cortical collecting duct (CCD) principal cell line (268). To date, it is not known if the well-established role for RAMP-3 in AM receptor pharmacology can be extended to other, unrelated systems such as that of vasopressin. However, ample evidence from related studies supports the hypothesis that PDGF-mediated changes in RAMP-3 expression in mesangial cells may have functional consequence in terms of adrenomedullin signaling. In fact, our results suggest that

PDGF-induced RAMP-3 expression leads to an increase in AM-stimulated adenylate cyclase activity. This effect, if present in vivo, may augment adrenomedullin signaling and hence increase the anti-proliferative effect of AM, opposing the proliferative effect of PDGF. This negative feedback mechanism may be present to keep the mesangial cell growth in check. Alternatively, this system of PDGF-induced RAMP-3 expression may be altered or absent under disease conditions thus resulting in an aberrant negative feedback and consequently leading to uncontrolled mesangial growth. While this hypothesis is attractive, clearly further studies are essential. It is worth noting that PDGF had no significant effect on RAMP-2 expression suggesting a differential regulation between closely related RAMP-2 and RAMP-3.

As alluded to previously, LPS exerts direct effect on AM secretion resulting in a large increase in the circulating levels of this polypeptide during sepsis. In turn, the high levels of AM during sepsis bear a great importance on the overall outcomes following the septic insult. Studies using transgenic mice overexpressing AM in their vasculature showed a marked protective role of AM during LPS induced sepsis. Transgenic mice responded to LPS administration with a smaller decrease in blood pressure, less severe end-organ damage, and overall significantly greater 24-hour survival rate as compared to the control animals (302). Recent studies suggest that LPS may also influence the AM receptor system. Ono *et al.* reported that the high basal expression of CL receptor and RAMP-2 mRNA in lungs of normal mice was markedly decreased in endotoxemic mice treated with LPS. Furthermore, they noted that under these conditions RAMP-3 gene expression increased significantly in lungs, spleen, and thymus (237). Similarly, research from another laboratory also reported a marked upregulation in pulmonary RAMP-3 gene

expression following an induction of polymicrobial sepsis by cecal ligation and puncture (CLP) in male rats (238). Interestingly, this effect was seen only during the early stage of sepsis and was absent 20 hours following CLP procedure. Also, in contrast to the findings reported by Ono *et al.*, they found no alterations in the expression of CL receptor or RAMP-2 in lung tissues at 5 or 20 hours post CLP. Altogether, these reports present a strong evidence for LPS-directed AM receptor complex regulation. In addition, they document a differential response of the individual AM receptor components during the state of sepsis.

The current investigation identifies LPS as an extracellular agent capable of potent induction of RAMP-3 gene expression and RAMP-3 protein production in cultured rat mesangial cells. This effect may provide at least a partial mechanism responsible for LPS interactions with the AM system discussed in the previous sections. To that extent, our experimental findings clearly demonstrate that the LPS-mediated elevation in RAMP-3 protein levels is accompanied by a significant increase in mesangial responsiveness to AM. Specifically, cells exposed to LPS concentrations similar to those observed in fulminant septicemia showed a greater than 90 percent increase in cAMP production in response to AM stimulation (Fig. 13). Considering that the decline in vascular reactivity of the end-organs to AM and the consequent transition to the hypodynamic phase of sepsis hallmark the impending morbidity and mortality of endotoxic shock ((347), (302)), the effect of LPS on the AM activity in RMC observed here may indeed provide for a protective mechanism.

As already noted for PDGF, LPS also did not exhibit any effects on RAMP-2 gene expression in RMC. This finding correlates with the data from the animal sepsis

model, where rats subjected to the CLP procedure showed no evidence of alteration in pulmonary tissue RAMP-2 gene expression (238).

This part of the study identifies PDGF, a pleotrophic cytokine well established to influence mesangial cell biology, and LPS, a potent causative agent of sepsis as novel factors capable of regulating RAMP-3 mRNA and membrane-associated RAMP-3 protein expression in RMC. Both PDGF and LPS selectively increase RAMP-3 (but not RAMP-2) mRNA expression in a dose-dependent manner (Fig. 5A and Fig. 9). These effects correlate with an elevation in cellular responsiveness to AM since membranes of RMC exposed to PDGF or LPS exhibit a significant increase in AM-stimulated adenylate cyclase activity (Fig. 8 and Fig. 13).

5. Mechanism of PDGF and LPS-dependent RAMP-3 up-regulation.

5.1. Introduction.

The present section of the study was designed to evaluate possible signal trasduction pathways responsible for PDGF and LPS effects on RAMP-3 gene and protein expression. Current literature has extensively characterized the molecular pathways affected by both of these agents (138), (71), (78), (109), (359), (357), thus clearly identifying possible target mechanisms involved. Considering the fact that both PDGF and LPS cause similar upregulation of RAMP-3 gene and protein expression in mesangial cells, it is reasonable to suggest that both exercise the same signal transduction pathway(s) to achieve their molecular effects. Accordingly, we have investigated the role of mitogen-activated protein kinase (MAPK) pathways as these are well-established primary intracellular enzymatic cascades responsible for the majority of cellular actions for PDGF and LPS alike. In general, mitogen-activated protein kinases belong to a group of intracellular proteins forming discrete signaling cascades, which in turn serve as a focal point for a multitude of diverse extracellular stimuli capable of regulating fundamental cellular processes.

PDGF activates its receptor, PDGFR, by binding it with a one-to-one stoichiometry and initiating receptor autodimerization followed by intrinsic tyrosine kinase activitation. This in turn results in phosphorylation of several cytoplasmic SH2 domains-containing proteins. These proteins (phospholipase C, GTPase activating protein, and tyrosine phosphatase SH-PTP), now in active form, proceed with specific intracellular enzymatic reactions interacting with a variety of other downstream proteins,

including those of the MAPK cascade (2), (192). Alternatively, activated PDGFR can directly stimulate Ras protein by recruiting Grb-2/SOS complex to the plasma membrane location of Ras. This also initiates an enzymatic cascade leading to stimulation of MAPK, translocation to the nucleus, and phosphorylation of specific transcription factors responsible for regulation of gene expression originally shown to be induced by PDGF (192), (176).

The intracellular signaling for LPS has only recently been actively studied with an appreciable success in identifying the precise molecular machinery involved. Unlike the well-established cell surface events for PDGF, the precise nature of the LPS receptor and its function are still under active investigation. Current data indicate that LPS present in plasma binds to LPS-binding protein and is delivered to the cell membrane where it is capable of interacting with cell surface receptors, including CD14 and CD11b/18 (74). Since CD14 lacks a transmembrane domain it is believed that LPS/CD14 interaction with surface Toll-like receptors (TLR), in particular TLR-4 and its accessory protein MD-2, confers upon the LPS receptor complex the ability to activate the intracellular enzymatic machinery (20), (12), (22), (181), (298), (54). Several signal transduction pathways have been reported to respond to LPS exposure (53), (52). However, activation of MAPK cascade, especially the extracellular signal-regulated kinases ERK-1/ERK-2 (p42/p44 MAPK) and p38 MAPK, is regarded as a hallmark of LPS-induced intracellular signaling in variety of cell types including mesangial cells (281), (291), (60), (352), (58), (290), (262), (265). Interestingly, p38 MAPK was initially isolated as 38-kDa protein which was rapidly tyrosine phosphorylated in response to exogenous LPS stimulation (98), (97).

Activation of signaling pathways, like the MAPK cascade, represents a common mechanism by which variety of extracellular effector molecules regulate gene transcription. However, post-transcriptional events also play a significant role in the overall control of gene expression. Our current knowledge emphasizes the importance of mRNA molecules not only as coding for appropriate proteins but also as dynamic self-regulatory molecules capable of controlling their own processing, transport, localization, proper translation, and life span (340), (89), (202). Through the cis-acting signals, directly encoded by the mRNA, a host of protein factors ("end-effectors" of various signaling cascades) are capable of interacting with the message thus regulating its function (207). Accordingly, in order to elucidate the effects of extracellular agents on gene regulation, one must consider their potential influence on these post-translational events. In particular, understanding the cellular events that control the half-life of an mRNA is critical during an investigation of gene expression regulation. The half-lives of different mRNAs vary considerably from as short as 10 minutes to greater than 24 hours and are largely dependent on the cell type, mRNA sequence characteristics, and a significant influence of numerous signal transduction events (257), (272). Modulation of mRNA stability is governed by at least two independent processes: the unique decay rates characteristic of different mRNA species and the apperent stability of mRNA dynamically regulated by a host of extracellular signals. Since the stability of an mRNA is a key determinant of its steady state level and subsequently the translated protein level (272), (27), (131), we have also investigated the effects of PDGF and LPS on the half-life of RAMP-3 message.

5.2. Materials and methods.

5.2.1. Materials.

Adrenomedullin, adrenomedullin(22-52) fragment and PDGF-BB, Actimonycin D, α-amanitin were purchased from Sigma® RBI® (St. Louis, MO). Lipopolysacccharide, *E.coli* 055:B5, AG 1296, PD 98059, PD 153035, PD168393, SB203580, and cycloheximide (CHX) were from Calbiochem (La Jolla, CA). RPMI-1640, fetal bovine serum, penicillin/streptomycin, trypsin-EDTA were from GibcoBRL® (Grand Island, NY). RAMP-2 and RAMP-3 polyclonal primary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary Anti-rabbit IgG antibodies were from Sigma® RBI® (St. Louis, MO).

All other reagents were of highest quality available.

5.2.2. Cell Culture.

Rat mesangial cell (RMC) cultures were established from glomeruli as described in section 3.2.2. RMC were maintained in RPMI-1640 with 15% fetal bovine serum unless otherwise stated for particular experiments. Passages 15-24 were used for subsequent experiments with specific confluency determined by preliminary studies.

5.2.3. Northern blot analysis.

Immediately after cell culture medium was aspirated from the tissue culture plates, 2-4 ml of TRIzol® was added, dispersed uniformly, and plates were stored at -80°C until further use. Following a quick thaw, cells were scraped with cell lifters and transferred to 15 ml centrifuge tubes. Total cellular RNA was isolated with TRIzol®according to manufacturer's specifications. RNA was precipitated by adding 3 M sodium acetate and absolute ethanol, washed with 75% ethanol, pelleted in microcentrifuge tubes, and dried prior to resuspension in RNAse-free water. Its purity was checked by measuring the ratio of $absorbance_{@260nm}/absorbance_{@280nm}$. All of the RNA used had the ratio equal or greater to 1.8. A standardized aliquot of RNA (30 μg) was separated by electrophoresis on a formaldehyde agarose denaturing gel and transferred to an Optitran® membrane (Schleicher & Schuell, Keene, NH) by capillary transfer. Subsequently, RNA samples were immobilized to the membrane by ultraviolet cross-linking. Membranes were successively hybridized at 42°C for 16-24 hrs with four parts (15 ml of Formamide, 0.6 ml Denhardt's solution, 1.5 ml of 1 M phosphate buffer, 7.5 ml of 20x SSC, 1.5 ml of SDS, 2.4 ml of diethylpyrocarbonate water, and 1.5 ml of salmon sperm DNA)/blot and one part ^{32}P-dCTP-labeled cDNA probes (specific for RAMP-1, 2, 3 or 18S ribosomal subunit; RAMP probes were obtained as RT-PCR products using RAMP-specific primers designed from published sequences). The cDNA was radiolabeled using a random prime labeling kit. Following hybridization for 16-24 hrs, the membranes were washed and placed in an x-ray cassette for the requisite exposure time. Signals were quantitated by phosphoimager analyses and expressed relative to 18S levels.

5.2.4. Analysis of RNA stability.

Rat mesangial cells were grown on P-150 tissue culture plates to approximately 80% confluency (as described above) and subsequently rendered quiescent by serum-starving for 24 hours. Next, cells were incubated with PDGF-BB (50 ng/ml) or LPS (10μg/ml) for 16 hours, washed and placed in serum-free medium containing Actinomycin D, an inhibitor of gene transcription, at final concentration of 10μg/ml and in the presence or absence of PDGF-BB (50 ng/ml) for 0, 1, 2, 4, and 8 hours. Total RNA was isolated and analyzed by Northern blotting as described above. RNA degradation curves were obtained by setting the 100% value to the amount of RAMP mRNA present immediately prior to Actinomycin D exposure [maximum value at *time 0* (t_0)]. mRNA levels remaining at indicated times following t_0 were compared as per-cent of the maximum value. A one-phase exponential decay curve was fitted including the maximum value at t_0 and decay rate constant, K, calculated for each nonlinear regression curve. The half-life of RAMP message was calculated as equal to ln (2/K).

5.3.Results.

5.3.1. Involvement of signal transduction pathway(s) in PDGF and LPS-dependent RAMP-3 up-regulation.

PDGF has been show to exert a myriad of biological effects principally by acting through its receptor, PDGFR, known to have an intrinsic tyrosine kinase activity (110). To investigate whether PDGF-induced RAMP-3 mRNA expression is PDGFR specific, we utilized several pharmacological tyrosine kinase inhibitors. AG 1296, a specific and selective inhibitor of PDGFR tyrosine kinase activity (164), abrogated PDGF-induced response (Fig. 15). PD153035 and PD168393, selective inhibitors of epidermal growth factor receptor-associated tyrosine kinase (23), (85), did not, however, have any effect on PDGF- stimulated RAMP-3 mRNA expression (Fig. 15). Accordingly, the observed increase in RAMP-3 mRNA abundance after exposure to PDGF is PDGFR mediated and receptor-associated tyrosine kinase dependent.

Because initiation of intracellular tyrosine kinase activity often triggers a cascade of events mediated via mitogen-activated protein kinases (MAPK-s), we investigated the effects of PD 98059 (10μM) and SB 203580 (10μM) (selective inhibitors of MAP kinase kinase (MEK) and p38 MAP kinase, respectively) on PDGF-induced RAMP-3 expression. Both inhibitors significantly attenuated the PDGF-induced response (Fig. 16), indicating that RAMP-3 expression in RMC is, at least in part, regulated by MAPK pathway.

Similarly, considering the fact that MAPK-s (especially MEK, p38 kinase, and PI3-kinase) often serve as an enzymatic relay for LPS mediated intracellular responses,

we have examined the effects of SB 203580, PD 98059, and Wortmannin on LPS-induced RAMP-3 mRNA expression. Pretreatment of RMC with pharmacological doses of SB 203580 and PD 98059 ablated the LPS-evoked elevation in RAMP-3 message by over 75 and 90 percent, respectively. Conversely, inhibition of PI3-K with Wortmannin had no statistically significant outcome. None of the blockers showed any appreciable effect on RAMP-3 expression when used alone (Fig. 17).

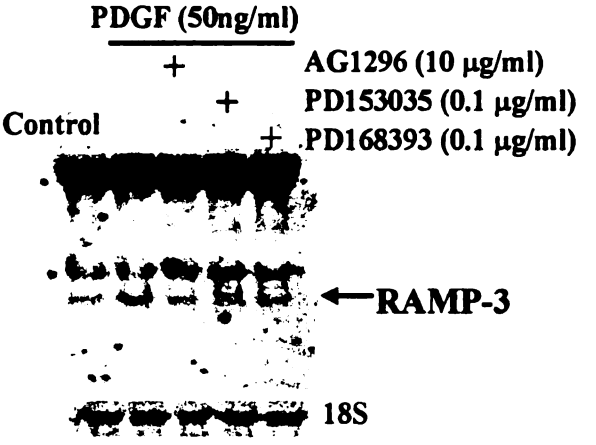


Figure 15. Effects of AG1296, PD153035, and PD168393 on PDGF-stimulated RAMP-3 mRNA expression in rat mesangial cells. Cells were pre-treated with inhibitors and subsequently exposed to PDGF (50 ng/ml) for 24 hours. Representative northern blot showing the selective dependence of PDGF-induced response on the activation of PDGF receptor but not EGF receptor-associated tyrosine kinase activity.

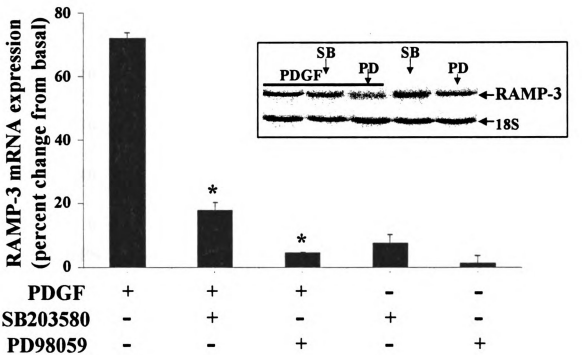


Figure 16. Effects of SB203580 (10μM) and PD98059 (10μM) on PDGF-induced RAMP-3 expression in RMC. Cells were pre-treated with inhibitors and subsequently exposed to PDGF (50 ng/ml) for 24 hours. RNA was collected as in previous experiments and analyzed by Northern blot hybridization with RAMP-3 probes. To correct for loading variability, blots were stripped and reprobed for 18s RNA. Raw values were converted to ratios of RAMP-3 mRNA to 18s and then expressed as percent change from basal. Insert: representative Northern blot of RAMP-3 expression in response to pre-treatment of RMC with SB203580 and PD98059 followed by PDGF exposure. Both SB203580 and PD98059 significantly attenuated PDGF-induced RAMP-3 expression; *$p<0.001$, n=5.

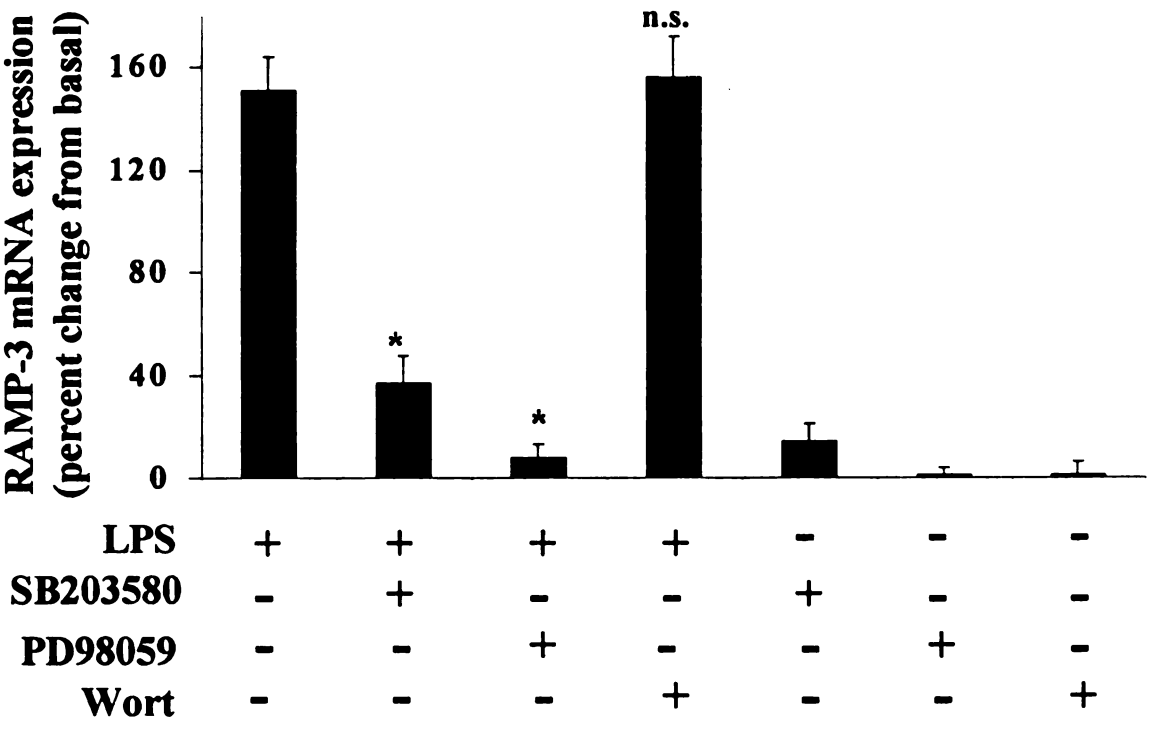


Figure 17. Effects of SB203580, PD98059, and Wortmannin (Wort) on LPS-induced RAMP-3 mRNA expression in RMC. Cells were pre-treated with inhibitors and subsequently exposed to LPS (10 μg/ml) for 24 hours. RNA was collected as in previous experiments and analyzed by Northern blot hybridization with RAMP-3 probes. To correct for loading variability, blots were stripped and reprobed for 18s RNA. Raw values were converted to ratios of RAMP-3 mRNA to 18s and then expressed as percent change from basal. SB203580 (10 μM) and PD98059 (10 μM) significantly attenuated LPS-induced RAMP-3 expression, *$p<0.001$ as compared to LPS treated cells ($n\geq3$); while Wortmannin (0.1 μM) had no effect (n=5), n.s.- no statistical significance as compared to LPS treated cells.

5.3.2. Mechanism of PDGF and LPS-stimulated RAMP-3 expression.

To determine whether PDGF and LPS affect transcriptional events leading to increased abundance of RAMP-3, we tested Actinomycin D and α-amanitin (inhibitors of DNA-dependent RNA synthesis) for their ability to influence the PDGF and LPS-dependent effect. Preincubation of mesangial cells with Actinomycin D (5 μg/ml) did not alter PDGF or LPS-induced RAMP-3 expression (Fig. 18A) suggesting the lack of regulation at a transcriptional level. To verify the effectiveness of transcriptional inhibition by Actinomycin D, Northern blots were re-probed with mitogen-activated protein kinase (MAPK) kinase, MEK-1, cDNA and quantification of MEK-1 RNA was performed. As previously reported by Schramek *et al.* (288), PDGF induced MEK-1 mRNA and this effect was inhibited by Actinomycin D, indicating a good transcriptional inhibition by Actinomycin D. Interestingly, LPS also elevated MEK-1 mRNA expression in an Actinomycin D-sensitive manner. To our knowledge, this incidental finding albeit previously reported for other cell types, appears to be novel for RMC (Fig. 18B). Similarly, pre-incubation of the cells with α-amanitin (1 μg/ml) for 5-6 hrs did not have any effect on PDGF-induced or LPS-induced RAMP-3 mRNA elevation (Table 4).

Next, a requirement for new protein synthesis was examined by pretreatment of PDGF or LPS exposed cells to cycloheximide (CHX), a potent eukaryotic translational inhibitor. At 10 μg/ml, CHX significantly inhibited PDGF and LPS-mediated RAMP-3 mRNA expression, identifying a requisite step of *de novo* protein synthesis (Fig. 19).

Given that neither PDGF nor LPS-induced increase in RAMP-3 mRNA expression were mediated through transcriptional events, we further hypothesized that these agents enhance RAMP-3 mRNA stability, thus leading to increased abundance of

RAMP-3 message. Accordingly, we analyzed the effect of PDGF and LPS on the half-life of RAMP-3 mRNA as described in materials and methods (5.2.4.). The baseline half-life of RAMP-3 mRNA in mesangial cells is approximately 73 minutes. PDGF (50 ng/ml) treatment raised the apparent RAMP-3 mRNA half-life to 331.6 minutes, resulting in a 4.99 fold increase (Fig. 20) while exposure to LPS (10μg/ml) attained a 2.59 fold increase (Fig. 21). This indicates that the mechanism of PDGF-induced and LPS-induced RAMP-3 expression is mediated by a posttranscriptional event of mRNA stability enhancement.

A

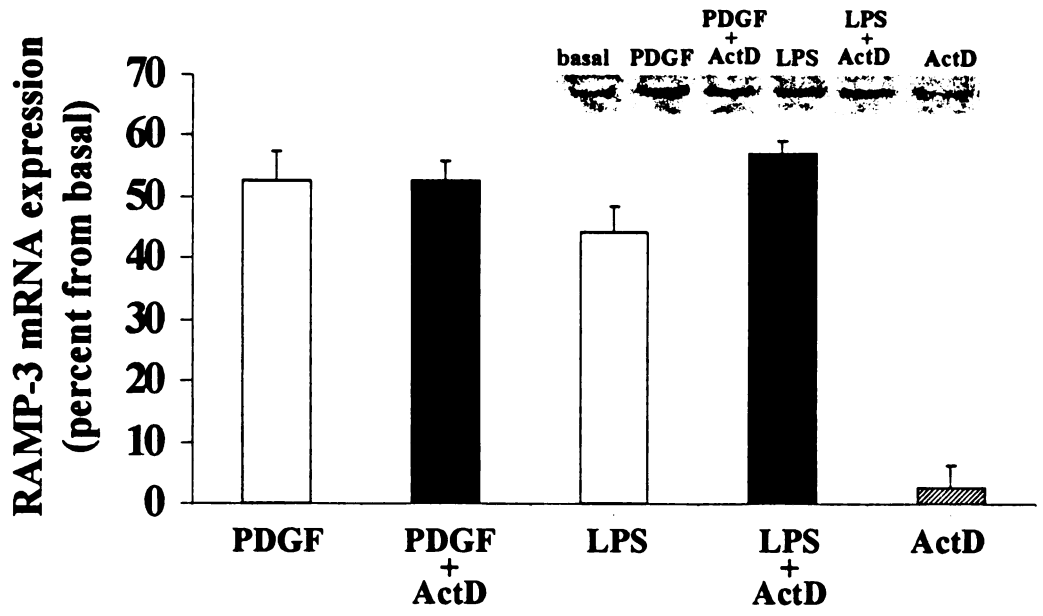


Figure 18. Effect of Actinomycin D (ActD) on PDGF and LPS-stimulated RAMP-3 expression in RMC. RMC were serum starved overnight and treated with PDGF (50 ng/ml) or LPS (10 μg/ml) or vehicle (basal) in the absence or presence of 5 μg/ml ActD for 24 hours, as indicated. Northern blot analysis was performed as described in materials and methods and the blots were probed for RAMP-3. A: ActD did not inhibit PDGF or LPS-induced RAMP-3 mRNA expression suggesting that RAMP-3 expression in RMC may not be regulated by increase in transcription. Insert: representative northern blot for this experiment; n≥3.

B

Figure 18. Effect of Actinomycin D (ActD) on PDGF and LPS-stimulated RAMP-3 expression in RMC. B: control experiment verifying that PDGF and LPS-increased transcription of MEK-1 is inhibited by ActD. This experiment is shown to eliminate the possibility that ActD is inactive. The representative northern blot shown here for MEK-1 expression is the same one that was used for probing RAMP-3.

	PDGF (50ng/ml)	PDGF + α-amanitin (1 μg/ml)	LPS (10μg/ml)	LPS + α-amanitin (1 μg/ml)
RAMP-3 mRNA expression (percent from basal ± SE)	58.49 ±2.56	55.46 ±3.47	67.28 ±9.67	70.39 ±5.88
	p=0.67 n=3		p=0.79 n=4	

Table 4. Effect of α-amanitin pretreatment on PDGF and LPS- induced RAMP-3 mRNA expression. RMC were incunated for 5 to 6 hrs in pretreatment medium contating 1 μg/ml α-amanitin. Cells were subsequently washed and exposed to PDGF or LPS for an additional 24 hrs. RAMP-3 mRNA was examined in the usual manner, as described previously. α-Amanitin did not inhibit PDGF or LPS-induced RAMP-3 mRNA expression. This correlates with findings from the ActD pretreatment experiments and suggests an involvement of a transcriptionally independent mechanism for RAMP-3 expression in RMC.

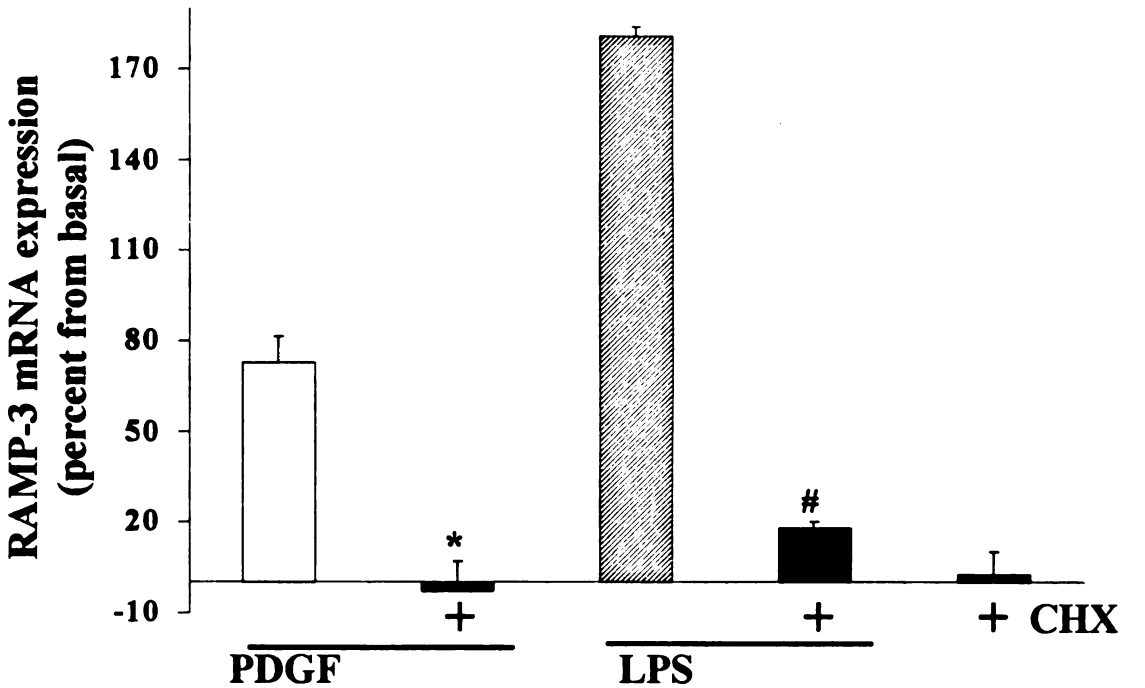


Figure 19. Effect of Cycloheximide (CHX) on PDGF and LPS-induced RAMP-3 mRNA expression in RMC. RMC were serum starved overnight and treated with vehicle (basal), PDGF (50 ng/ml), or LPS (10μg/ml) in the presence or absence of CHX (10μg/ml) for 24 hours. RNA was extracted and northern blot analysis performed as indicated in materials and methods. Blots were first probed for RAMP-3 and then stripped and reprobed for 18s RNA. Raw values were converted to ratios of RAMP-3 mRNA to 18s and then expressed as percent change from basal. CHX significantly attenuated PDGF-induced and LPS-induced RAMP-3 mRNA expression; $^{*}p \leq 0.001$ as compared to PDGF treated cells; $^{\#}p \leq 0.001$ as compared to LPS treated cells; n=3.

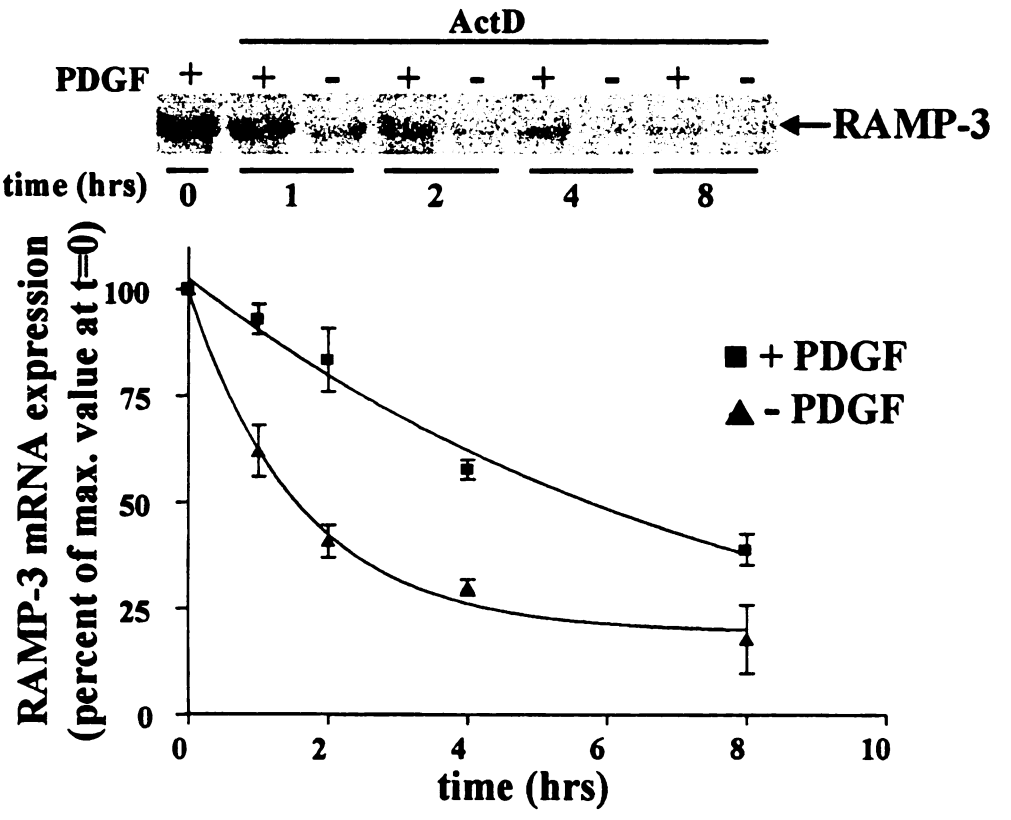


Figure 20. Effect of PDGF on RAMP-3 mRNA rate of decay in RMC. RNA stability assay was performed on RMC as described in materials and methods. RNA from each time point was analyzed by Northern hybridization and relative RAMP-3 mRNA abundance expressed as percent of that present at time=0 (percent of maximum value). RAMP-3 mRNA half-lives were calculated using the decay constants (*K*) obtained from one-phase exponential decay curves. Insert: representative Northern blot obtained following the RNA stability assay protocol. PDGF significantly increased RAMP-3 mRNA half-life from 1.1 to 5.5 hours. Regression lines (half-lives) for RNA decay from PDGF (50ng/ml) treated (■) and no PDGF (▲) RMC were compared by analysis of covariance. Regressions were regarded significantly different with a $p \leq 0.01$; n=3.

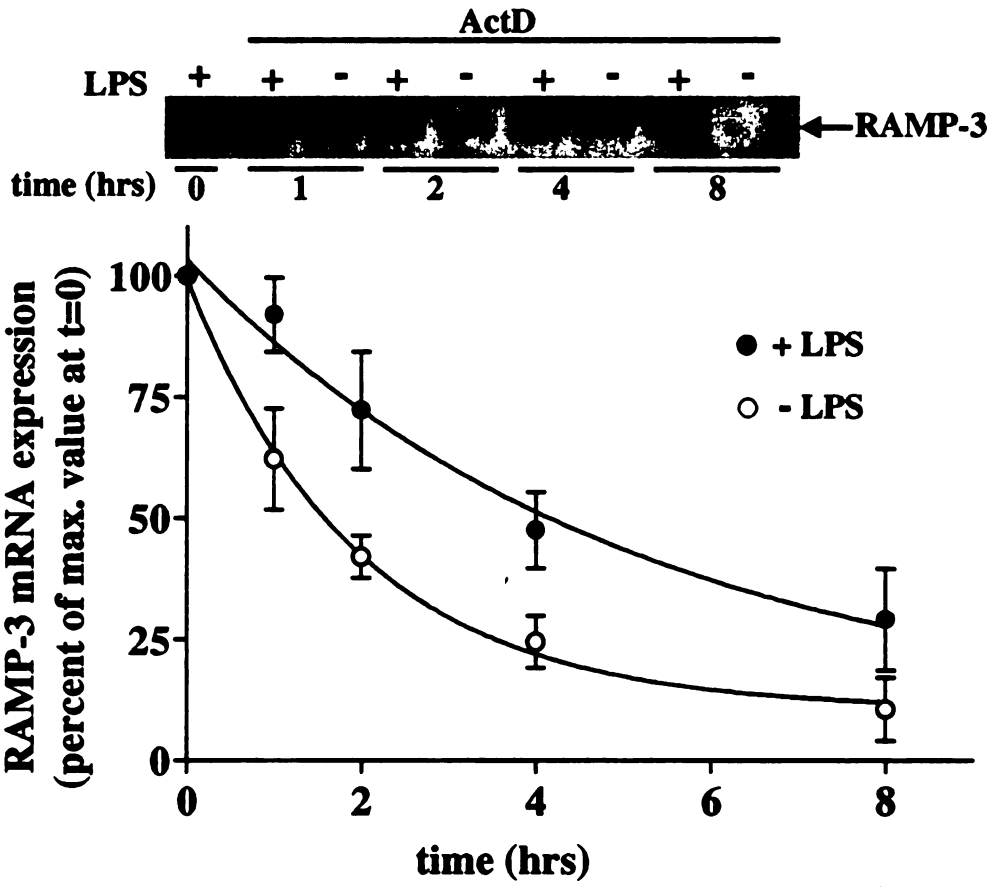


Figure 21. Effect of LPS on RAMP-3 mRNA rate of decay in RMC. RNA stability assay was performed on RMC as described in materials and methods. RNA from each time point was analyzed by Northern hybridization and relative RAMP-3 mRNA abundance expressed as percent of that present at time=0 (percent of maximum value). RAMP-3 mRNA half-lives were calculated using the decay constants (*K*) obtained from one-phase exponential decay curves. Insert: representative Northern blot obtained following the RNA stability assay protocol. LPS significantly increased RAMP-3 mRNA half-life from 1.3 to 3.5 hours. Regression lines (half-lives) for RNA decay from LPS (10 μg/ml) treated (•) and no LPS (o) RMC were compared by analysis of covariance. Regressions were regarded significantly different with a $p \leq 0.01$; n=3.

5.4. Discussion.

One of the aims in this study was to investigate the mechanism of PDGF and LPS-dependent induction of RAMP-3 expression at the signaling level. In particular, we examined the role of mitogen-activated protein kinases (MAPKs), a highly conserved family of protein serine/threonine kinases that are fundamental to multiple signaling cascades relaying extracellular signals to the nucleus. The MAPK family of proteins includes the extracellular signal-regulated kinases (ERKs) and the stress activated protein kinases (SAPKs): c-Jun N-terminal kinase (JNK) and p38 kinase (293), (43). Upon phosphorylation, activated MAPKs translocate into nucleus where they activate transcription factors thus regulating expression of a wide variety of target genes (244). MAPKs are also implicated in direct regulation of target mRNA stability, thus influencing the relative mRNA abundance, which is independent of their effects on the transcriptional machinery. This action is presumably mediated by specific recruitment of proteins, like AUF1 proteins, to various parts of the 3'-untranslated regions (UTRs) of mRNA molecules. A number of instability elements (for example the AU-rich element, ARE) have been identified in the UTRs and are thought to constitute the target for the regulatory proteins like AUF1. Recently, several laboratories reported that the binding of AUF1 and similar proteins to UTRs is dependent on protein kinase directed phosphorylation. For example, JNK and p38 MAPK signaling pathways have been shown to directly stabilize mRNAs of VEGF, interleukin-2, -3, -6, and –8 (207), (257), 272).

Cellular actions of PDGF have been shown to involve both its receptor tyrosine kinase activity and MAPK pathways (110), (192), (261). MAPKs are also activated in

many cell types upon LPS exposure (52), (60), (352). To examine the role of MAPKs, we utilized specific pharmacological inhibitors to block these signaling molecules.

Our observation that AG1296 (PDGF receptor tyrosine kinase blocker) but not PD153035 and PD168393 (selective inhibitors of epidermal growth factor receptor-associated tyrosine kinase) blocked the PDGF-stimulated RAMP-3 mRNA expression suggests that PDGF actions in mesangial cells are indeed mediated through PDGF receptor tyrosine kinase activity. Furthermore, we showed that pharmacological inhibition of MEK (kinase upstream of ERK) and p38 MAPK, reversed the PDGF-dependent elevation of RAMP-3 mRNA abundance, indicating that activation of these kinases by PDGF may be important in the regulation of RAMP-3 expression.

Similarly, we showed that LPS-induced RAMP-3 gene expression is almost completely abolished by inhibition of MAPK kinase (MEK) with PD 98059. This finding also implicates MAPK signaling in LPS-directed regulation of RAMP-3 gene in mesangial cells. Since LPS-dependent regulation of gene expression is known to involve a series of positive and negative signal transduction pathways, we also investigated the role of phosphatidyl inositol 3-kinase (PI3K) in LPS-induced RAMP-3 gene expression. LPS activates PI3K consequently leading to a potent inhibition of MAPK pathways (ERK1/2, p38, and JNK), thus imposing a functional "braking mechanism" on the LPS-induced gene activation (180), (243), (252). For example, inhibition of PI3K enhanced LPS-dependent nitric oxide (NO) production in murine macrophages (252), while LPS-dependent induction of NO synthase in astrocytes and lipoprotein lipase expression in macrophages were inhibited by direct activation of PI3K (243), (324). In contrast to the above studies showing the negative involvement of PI3K pathway in LPS signaling, our

findings suggest that PI3K is not engaged in LPS-dependent RAMP-3 gene regulation. Explicitly, we have observed that mesangial cells pretreated with pharmacological doses of Wortmannin, a specific inhibitor of PI3K, exhibited no significant change in RAMP-3 mRNA expression following LPS exposure as compared to non-pretreated cells.

To our knowledge this is the first report of an involvement of MAPK pathways in regulating RAMP-3 expression in mesangial cells. Considering that MAPK pathways serve as a common denominator for a variety of divergent intracellular signals, this finding may bear an important implication for the complexity of RAMP gene expression regulation.

While both Frayon *et al.* and Robert-Nicoud *et al.* demonstrated that exogenous addition of factors can alter RAMP expression, they did not address the underlying mechanisms, which may be responsible for these effects. Here, we present evidence that PDGF and LPS-induced RAMP-3 expression is transcriptionally independent, as it was not inhibited by Actinomycin D or α-amanitin. Furthermore, our data indicate that PDGF and LPS act via stabilizing RAMP-3 mRNA, consequently increasing the apparent half-life of the message by nearly 5 and 2.6 fold, respectively. While we established the requirement of *de novo* protein synthesis for this effect, future studies are necessary to further characterize the exact mechanism(s) by which PDGF and LPS influence the stability of RAMP-3 message. Recently, Kitamuro *et al.* (159) also proposed that the total abundance of RAMP message may be regulated specifically by changes in RAMP mRNA stability. They estimated the basal half-life of RAMP-2 mRNA for two human neuroblastoma cell lines, IMR-32 and NB69, to exceed 6 hours. In addition, they reported an increase in RAMP-2 mRNA stability in IMR-32 cells under hypoxic conditions.

Interestingly, exposure of IMR-32 cells to a hypoxia-mimetic agent, $CoCl_2$ (cobalt chloride), decreased RAMP-2 mRNA half-life while NB69 cells did not exhibit any change in response to hypoxia or $CoCl_2$. It is worth noting that neither PDGF nor LPS had a significant effect on RAMP-2 expression in mesangial cells, suggesting a differential gene regulation between closely related RAMP-2 and RAMP-3.

In conclusion, this chapter presents evidence for the mechanism(s) which may be involved in the observed PDGF and LPS-induced increase in RAMP-3 mRNA and RAMP-3 membrane protein expression. Our results indicate that PDGF-induced RAMP-3 mRNA elevation is MEK and p38 MAPK dependent (Fig. 16). Moreover, as predicted, this effect relies on the activation of the PDGF receptor and its inherent tyrosine kinase activity (Fig. 15). LPS also mediates RAMP-3 expression via MEK/p38 MAPK pathway but appears to be independent of PI3K signaling (Fig. 17). In addition, both PDGF and LPS regulate RAMP-3 gene expression in a post-transcriptional manner as pharmacological inhibition of transcription by actinomycin D and α-amanitin appears to have no effect on the original observations (Fig. 18 and Table 4). The dependence on *de novo* protein synthesis was however evidenced by significant attenuation of PDGF and LPS-induced RAMP-3 mRNA expression following preexposure to cycloheximide (Fig. 19). Furthermore, our results demonstrate for the first time that PDGF and LPS augment the abundance of RAMP-3 mRNA via stabilization of the message and a consequent increase in RAMP-3 mRNA half-life (Fig. 20 and Fig. 21). As alluded to previously, this effect may result from the activation of MEK/p38 MAPK pathway(s) leading to subsequent direct targeting of cis-acting signals encoded by RAMP-3 mRNA and/or regulation of mRNA interacting proteins. This hypothesis however awaits further testing.

6. Summary and conclusions.

6.1. Major hypothesis and results of the study.

In general, we hypothesized that the regulation of RAMPs expression provides a mechanism for modulation of AM activity in mesangial cells.

The major aims of this study were to investigate the effects of two extracellular signaling agents, PDGF and LPS, on the regulation of RAMP expression and the consequent changes in mesangial cell responsiveness to AM. After documenting that both PDGF and LPS induce RAMP-3 mRNA and protein expression, we also scrutinized the molecular mechanisms responsible for this observation. Accordingly, as summarized below, the following major hypothesis were tested and the corresponding experimental results obtained:

Hypothesis #1:

Over-expression of RAMP-1, 2, and 3 results in an increase in functional responsiveness of RMC to AM as measured by AM-induced cAMP production and [^{3}H]thymidine incorporation.

Results:

1. At basal cell culture conditions RMC express all of the essential components of the AM receptor system, namely CL receptor (formerly CRLR), RAMP-1, 2, and 3, as examined by RT-PCR method.
2. Over-expression of RAMP-2 and RAMP-3 cDNA in RMC results in a significant increase of AM-induced adenylate cyclase activity as measured by AM-induced cAMP production.

3. Over-expression of RAMP-1 in RMC does not effect AM-induced cAMP production.
4. Over-expression of RAMP-2 and RAMP-3 cDNA in RMC results in marked enhancement of AM-mediated decrease in mesangial cell proliferation as measured by [^{3}H]thymidine incorporation.
5. Over-expression of RAMP-1 cDNA in RMC does not have any significant effect on basal level of AM-mediated decrease in [^{3}H]thymidine incorporation.

Conclusion:

Accept hypothesis #1 for RAMP-2 and RAMP-3.

Reject hypothesis #1 for RAMP-1.

Hypothesis #2:

PDGF and LPS up-regulate RAMP-2 and RAMP-3 expression and increase functional responsiveness of RMC to AM.

Results:

1. Exposure of cultured RMC to PDGF increases RAMP-3 mRNA expression and RAMP-3 protein content in the cell membrane-associated fraction of RMC cell lysate.
2. RMC treated with PDGF exhibit a significant increase in AM-mediated adenylate cyclase activity as measured by AM-induced cAMP production.

3. Treatment of RMC with LPS in culture results in a significant increase of RAMP-3 mRNA expression and a concordant elevation of RAMP-3 protein levels in the cell membrane-associated fraction of RMC lysate.
4. RMC treated with LPS show significant increase in AM-induced cAMP production.
5. Exposure of cultured RMC to PDGF or LPS does not affect the basal level of RAMP-2 expression.

Conclusion:

Accept hypothesis #2 for RAMP-3.

Reject hypothesis #2 for RAMP-2.

Hypothesis #3:

A. PDGF and LPS-mediated elevation in RAMP-3 mRNA expression is MAPK-dependent.
B. PDGF-mediated change in RAMP-3 mRNA expression requires PDGF receptor activation.
C. LPS-mediated elevation in RAMP-3 mRNA expression is independent of PI3K.

Results:

1. Pharmacological inhibition of MAPK kinase (MEK) and p38 MAPK abrogated the PDGF-induced RAMP-3 mRNA elevation. LPS effect on RAMP-3 expression was also arrested by MEK and p38 MAPK inhibition.

2. Pre-treatment of cultured RMC with AG 1296, a specific and selective inhibitor of PDGF receptor tyrosine kinase activity, abrogated PDGF-induced RAMP-3 mRNA elevation. Pre-treatment with PD153035 and PD168393, selective inhibitors of epidermal growth factor receptor-associated tyrosine kinase, exhibited no effect.
3. Pre-treatment of cultured mesangial cells with wortmannin, a potent PI3K inhibitor, did not have any significant effect on LPS-induced RAMP-3 mRNA expression.

Conclusion:

Accept Hypothesis #3, A-C.

Hypothesis #4:

PDGF and LPS increase RAMP-3 mRNA abundance by stabilizing the message thus increasing the apparent half-life of RAMP-3 mRNA.

Results:

1. PDGF and LPS-induced elevation of RAMP-3 mRNA abundance is transcriptionally independent in RMC, since inhibition of transcription by Actinomycin D or α-amanitin did not alter PDGF and LPS-mediated effects.
2. PDGF and LPS-stimulated RAMP-3 expression requires *de novo* protein synthesis as it was inhibited by pre-treatment of RMC with cycloheximide.

3. Treatment of RMC with PDGF and LPS results in a significant increase of RAMP-3 mRNA half-life and an apparent abundance of the RAMP-3 message.

6.2. Limitations of this study.

The main limitation of this study stems from the inherent constraints of a cell culture system. All of the experimental data obtained in this work are based on biological responses observed in rat mesangial cells cultured in a homogeneous, controlled milieu. Clearly, mesangial cells *in vivo* experience a much more dynamic environment, where interactions with other cell types, paracrine and endocrine influences, as well as hemodynamic alterations within the glomerular apparatus unquestionably contribute to the final cellular response. In addition, as previously discussed, cellular responses are greatly varied among cells derived from even closely related species; hence one should avoid a mere extrapolation of the observations from the current study to those expected for human cell lines. For these reasons, the experimental data presented and conclusions drawn from this work should be interpreted with caution, bearing in mind their innate limitations. While our choice of rat mesangial cell culture model presents the above-discussed limitations, it nevertheless lends itself nicely to experimental manipulations. It is well characterized in the scientific literature, relatively inexpensive, non-laborious to maintain, and easily reproducible.

Another limitation of this work is the use of pharmacological inhibitors to investigate the involvement of various signaling pathways and post-transcriptional mechanisms. Since the specificity and effectiveness of inhibitory activity of these agents

cannot be unequivocally determined, one must always consider their use carefully. In the present study, only well characterized and widely used pharmacological inhibitors were utilized. In addition, previously published inhibitory concentrations of these agents were strictly observed.

Methodology for the analysis of mRNA turnover and mRNA half-life determination is currently under active investigation. The existence of numerous technical problems and shortcomings is perhaps the single most reason why so many different methods have been developed over the years (272), (19). In this study we used a relatively convenient way of analyzing mRNA kinetics. We utilized a pharmacological inhibition of transcription followed by serial determination of remaining mRNA of interest. While this is the most frequently used method in the scientific literature, it too has some limitations. As an indirect study of mRNA stability it examines the steady-state level of mRNA assuming that changes in mRNA abundance affect directly the stability of a given message. In addition, the mathematical derivation of the half-life presumes that mRNA decays according to the first-order kinetics. Recent data suggests however, that such assumptions while true for prokaryotic mRNA processing events do not always hold in case of mammalian transcriptional machinery (18). On the other hand, this approach allows for minimal investigational error due to an uncomplicated, easily reproducible experimental setup. It is compatible with analysis of relatively scarce and unstable mRNAs and bypasses often-troublesome genetic manipulations of the message required by alternative approaches. Thus, for practical reasons this method is widely used and has been elected in the current investigation.

6.3. Positive outcomes of this study.

The original publication of McLatchie *et al.* implicated RAMPs in regulation of CL receptor at the level of receptor trafficking, glycosylation, and a direct RAMP-receptor interaction (199). Accumulating data suggests that the dynamic alteration of RAMP expression levels may provide for yet another mode by which these proteins regulate the function of CL receptor and possibly other receptors. A number of previously published observations reported significant changes in RAMP gene expression in response to pathophysiological states or direct administration of an exogenous substance; they however do not propose a mechanism underlining these changes. The experimental outcomes of this work identify a novel mechanism by which AM receptor complex is regulated in response to exogenous agents. Here, we have demonstrated for the first time that regulation of RAMP-3 message stability by PDGF and LPS leads to an alteration of cell's functional responsiveness to an endogenous AM. We have also shown the involvement of MEK and p38 MAPK pathway in the regulation of RAMP-3 mRNA expression in mesangial cells. Considering the reno-protective effects of AM (specifically, the AM-mediated decrease in mesangial cell proliferation and increase in cell apoptosis), the regulation of RAMP-3, a well established component of AM receptor system, by PDGF and LPS may constitute one mechanism of cellular adaptation during the development and progress of kidney disease. In addition, the molecular mechanisms identified in this thesis may provide for plausible pharmacological targets aiming at alleviation of such pathophysiological processes as glomerulonephritis and renal consequences of septic shock.

Overall, the current study identified glomerular mesangial cells as yet another cell type in which RAMPs play an important role for AM receptor phenotype determination. Presumably by the mechanisms originally described by McLatchie *et al.* (199) and the direct regulation of RAMP gene stability outlined in the current work, RAMPs modulate AM activity in mesangial cells. In general, RAMPs unique ability to change receptor ligand specificity as described for RAMP-CL receptor and RAMP-CTR interactions as well as RAMPs potential to act as possible partners for hundreds of GPCRs identified in the Human Genome Project (177) hint at their distinct role with an evolutionary importance. Namely, RAMP-GPCRs interactions afford a combinatorial power for large phenotypic receptor diversity within a relatively small gene pool. Future research will unquestionably pursue the identification of novel RAMP-receptor interactions as well as the precise nature of their regulation.

In summary, we report that mesangial cells express CL receptor, RAMP-1, 2, and 3 at basal conditions. PDGF, a pleotrophic cytokine well established to influence mesangial cell biology, and LPS, a potent causative agent of sepsis derived from the outer membrane of Gram-negative bacteria, increase RAMP-3 mRNA and membrane-associated RAMP-3 protein expression. This effect correlates with an elevation in cellular responsiveness to AM as measured by AM-stimulated adenylate cyclase activity. Moreover, our data show the PDGF-induced RAMP-3 mRNA elevation to be MEK and p38 MAPK dependent. LPS also mediates RAMP-3 expression via MEK pathway but appears to be independent of PI3K signaling. Both agents, PDGF and LPS, regulate RAMP-3 gene expression in a post-transcriptional manner. They augment the abundance of RAMP-3 mRNA via stabilization of the message and a consequent increase in RAMP-

3 mRNA half-life. Taken together, these data suggest an important and novel mechanism of regulation of RAMP-3 in rat mesangial cells.

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