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ROLE FOR RECEPTOR ACTIVITY-MODIFYING PROTEINS (RAMPs) IN THE TRAFFICKING OF THE ADRENOMEDULLIN RECEPTOR

By

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ABSTRACT

ROLE FOR RECEPTOR ACTIVITY MODIFYING PROTEINS IN THE TRAFFICKING OF THE ADRENOMEDULLIN RECEPTOR

By

Jennifer Melinda Bomberger

Adrenomedullin (AM) is a vasodilatory peptide with effects in numerous physiological systems. AM exerts its effects through binding to a heterotrimeric G-protein-coupled receptor complex (calcitonin receptor-like receptor (CL-R) and single-transmembrane accessory protein, receptor activity modifying protein (RAMP)). CL-R is unique in that it requires association with the accessory protein, RAMP, for efficient receptor expression at the plasma membrane and for determination of receptor phenotype. A heterodimer of CL-R and RAMP1 yields a calcitonin gene-related peptide (CGRP1) receptor, whereas complexing of CL-R with the RAMP2 or RAMP3 isoforms characterizes a fully functional AM receptor (AM1 and AM2 receptors, respectively). Curiously, although RAMP expression differs from tissue to tissue, the receptor complex is coupled to $G\alpha_s$ in all systems studied to date, regardless of the RAMP isoform associated or ligand stimulation (AM or CGRP). Thus, it is the overall hypothesis of this thesis that although RAMP isoforms do not regulate the type of G-protein coupled to the receptor complex, the isoform of RAMP associated with CL-R does regulate the life cycle of the receptor complex.

Thus, this thesis focuses on the role of the RAMPs in regulation of the desensitization, internalization, recycling and degradation of the CL-R/RAMP receptor complex. In particular, this study focuses on the differential trafficking of the AM1 and AM2 receptor subtypes by RAMP2 and RAMP3, respectively. Renal cell culture systems, both heterologous expression and native, are the model systems utilized to study the differential receptor trafficking in this project.

Using mutagenesis approaches, data from this investigation demonstrates that association of CL-R with RAMP2 or RAMP3 isoforms could result in differential receptor phosphorylation

patterns with prolonged agonist stimulation. The Serine 421 residue on CL-R is critical to the desensitization and internalization of the CL-R/RAMP2 heterodimer, while Threonine 423 on CL-R is required for efficient CL-R/RAMP3 receptor complex desensitization and internalization. While Serine 421 is a putative phosphorylation site for PKA, Threonine 423 is a putative phosphorylation site for PKC. Selective inhibitors against these kinases also inhibited CL-R/RAMP2 (by H-89) and CL-R/RAMP3 (by Ro 32-0432) desensitization and internalization.

Differences were also observed in the interaction of AM receptor subtypes with Na^+/H^+ Exchanger Regulatory Factor-1 (NHERF-1). Upon prolonged AM exposure, NHERF-1 was found to inhibit the internalization, but not desensitization, of the CL-R/RAMP3 receptor complex. The effect of NHERF-1 was specific for the AM2 receptor. This inhibition of internalization was dependent on PDZ interaction of RAMP3 with NHERF-1 and NHERF-1 interaction with MERM proteins in the actin cytoskeleton. Finally, a model for differential post-endocytic targeting of the AM receptor subtypes was proposed from studies in HEK 293, Rat2 fibroblast, and rat mesangial cells. Through adenylate cyclase and cAMP accumulation assays, whole-cell competition binding, and immunofluorescence microscopy experiments it was demonstrated that RAMP3 interacts with NSF via a PDZ recognition sequence at the C-terminus of RAMP3. This was an effect specific for the CL-R/RAMP3 receptor complex. This RAMP3-NSF interaction is proposed to target the AM2 receptor complex for a recycling pathway after agonist-induced internalization, whereas expression of the AM1 receptor complex, unable to interact with NSF, will be targeted for degradation after agonist-induced endocytosis.

These findings report a novel function for the RAMPs in post-endocytic receptor trafficking and provide the first difference between the RAMP2 and RAMP3 isoforms in the trafficking of the AM1R and AM2R. In addition, this data demonstrates a functional difference between the AM1R (CL-R+RAMP2) and AM2R (CL-R+RAMP3) receptor complexes, in spite of very similar second messenger systems and the physiological responses thus far identified.

To Mike and my parents,

for your unwavering support and encouragement,

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KEY TO ABBREVIATIONS

AM	Adrenomedullin
AM1-R	Adrenomedullin receptor (composed of CL-R+RAMP2)*
AM2-R	Adrenomedullin receptor (composed of CL-R+RAMP3)*
AMPA	α-amino-3-hydroxy-5-methyl-isoxazolepropionate
AM-R	Adrenomedullin receptor (generic)
AMY	Amylin
AMY1-R	Amylin receptor (composed of CTR+RAMP1)*
AMY3-R	Amylin receptor (composed of CTR+RAMP3)*
ANG II	Angiotensin II
AVP	Arginine-vasopressin
β_2-AR	β_2-adrenergic receptor
cAMP	Adenosine 3',5' cyclic monophosphate
CGRP	Calcitonin gene-related peptide
CGRP1-R	CGRP receptor (composed of CL-R+RAMP1)*
CFTR	Cystic fibrosis transmembrane conductance regulator
CHX	Cycloheximide
CL-R	Calcitonin receptor-like receptor *
CT	Calcitonin
CTR	Calcitonin receptor
Cyto D	Cytochalasin D
DOCA	Deoxycorticosterone acetate
DOR	δ-opioid receptor
ERM	Ezrin/moesin/radixin domain
ET-1	Endothelin-1
EVH1	Drosophila enabled/vasodilator-stimulated phosphoprotein homology 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GASP	G protein accessory sorting protein
GFR	Glomerular filtration rate
GPCR	G protein-coupled receptor
GRK	GPCR-regulated kinase
HAoEC	Human aortic endothelial cells
HEK 293	Human embryonic kidney 293 cells
HIF	Hypoxia inducible factor
HRE	Hypoxia response element

* 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.

HUVEC	Human umbilical vein cells
KOR	κ-opioid receptor
L-NAME	Nω-nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
LV	Left ventricle
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
NEM	N-ethylmaleimide
NHE	Na+/H+ exchanger
NHERF	Na+/H+ exchanger regulatory factor
NSF	N-ethylmaleimide sensitive factor
NO	Nitric oxide
PAMP	Proadrenomedullin N-terminal 20 peptide
PDGF	Platelet-derived growth factor
PDGF-R	PDGF receptor
PDZ	PSD-95/Discs Large/Zona occludens-1
PG	Prostaglandin
PI	Phosphotidyl inositol
PI3K	Phosphotidyl inositol 3-kinase
PKA	Protein kinase A (cAMP protein kinase)
PKC	Protein kinase C
PLCβ	Phospholipase Cβ
PP2A	Protein phosphatase 2A
PSD-95	Post-synaptic density-95 protein
PTH	Parathyroid hormone
PTH-R	Parathyroid hormone receptor
Q-PCR	Quantitative-polymerase chain reaction
RAEC	Rabbit aortic endothelial cells
RAMP	Receptor activity-modifying protein
RBF	Renal blood flow
RCP	Receptor component protein
RMC	Rat mesangial cells
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase- polymerase chain reaction
SH2/3	Src Homology domain 2/3
SHR	Spontaneously hypertensive rat
SNAP	Soluble N-ethylmaleimide-sensitive factor attachment protein
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
UTRs	Untranslated regions
VSMC	Vascular smooth muscle cells

1. Introduction

Recent discovery of receptor activity modifying proteins (RAMPs) has enhanced our understanding of the mechanisms known to regulate the signaling of the G protein-coupled receptor (GPCR) superfamily. RAMPs (1-3) have been demonstrated to associate with the calcitonin (CTR) and calcitonin receptor-like (CL-R) receptors and differentially regulate the ligand selectivity of the receptors. Although RAMPs enable differential receptor phenotypes, the signaling elicited through receptor activation is quite similar, namely $G\alpha_s$ activation to activation adenylate cyclase enzymes, leading to cAMP accumulation and protein kinase A activation. Similar biological responses are even seen, regardless of ligand stimulation. For this reason, it has been hypothesized that the RAMP isoforms have additional regulatory roles, thus far unidentified. This thesis research investigates the role of RAMPs in the regulation of the CL-R/RAMP receptor complex after agonist exposure and receptor activation, in particular, during the processes of desensitization, internalization, and receptor trafficking from the endosomes.

This brief introductory chapter is followed by a comprehensive literature review in the second chapter. The literature review provides an in-depth discussion of the past and current scientific literature surrounding the topic of this thesis project, including the biology of adrenomedullin, the characterization of the CL-R/RAMP receptor complex, the protein-protein interactions involved in GPCR signaling, and the regulation of the GPCR life-cycle. Chapters three through five are organized according to the proposed specific aims of this study, and each consist of an introduction, experimental data, and specific discussion relevant to the individual aim. Chapter three investigates if phosphorylation of the receptor complex regulates the process of agonist-stimulated

desensitization and internalization of the receptor complex. An emphasis is placed on whether the desensitization and internalization are differentially regulated by RAMP2 vs. RAMP3 in complex with the receptor. The fourth chapter examines protein-protein interactions that regulate the internalization of the CL-R/RAMP receptor complex. The role of NHERF-1 in regulation of the CL-R/RAMP complex internalization and the mechanism for this regulation are investigated. The fifth chapter describes protein-protein interactions that regulate the trafficking of the CL-R/RAMP complex after agonist-stimulated receptor endocytosis. This chapter illustrates differential receptor complex trafficking by the RAMP2 and RAMP3 isoforms, suggesting a mechanism for the differential trafficking of the CL-R/RAMP complex observed in different cell lines. A model for RAMP expression determining receptor fate after agonist-induced internalization is proposed. Concluding this thesis, chapter six, offers a list of the major hypotheses tested and the experimental results obtained in each study. Also discussed are the limitations of this study and the positive outcomes of the thesis work, focusing on the contributions of these findings to the field of receptor biology and future directions.

2. LITERATURE REVIEW:

2.1. Adrenomedullin.

2.1.1. Gene and protein structure.

Adrenomedullin (AM) was initially isolated and characterized by Kitamura and colleagues from a human pheochromocytoma [1]. It was found to have the ability to increase cyclic AMP levels in rat platelets and exerted strong hypotensive effects through vasodilatory activity in the resistance vessels. AM levels were also measured in the circulation with radioimmunoassays. Later, the same laboratory published the sequence of the AM gene [2]. The AM gene is located in a single locus of chromosome 11, is comprised of 4 exons and 3 introns, and is flanked at the 5' end by RNA polymerase II responsive TATA, CAAT, and GC boxes. The AM gene also encodes binding sites for activator protein-2 (AP-2) [3], a cyclic AMP-regulated enhancer [4], nuclear factor- κB [5], hypoxia-inducible factor-1 (HIF-1) [6], hypoxia response elements (HREs) [7], and a binding site for steroidogenic factor-1 (SF-1) [8]. This multitude of binding sites for regulatory factors indicates a complex participation of the factors in the regulation of the AM gene. The AM gene encodes a 185 amino acid precursor protein termed preproadrenomedullin (preproAM), which is processed to yield the AM polypeptide and another bioactive molecule, proadrenomedullin N-terminal 20 peptide (PAMP) [9].

AM is a member of the CGRP family of peptides because of its structural homology to calcitonin gene-related peptide (CGRP), a potent vasodilator in the central nervous system and peripherally acting neurotransmitter. AM has low sequence homology with CGRP, but strong structural homology. The 52 amino acid AM peptide

shares an N-terminal ring structure, formed by one intramolecular disulfide bond (between residues 16 and 21), and an amidated carboxy-terminal end with the additional members of the CGRP family of peptides: calcitonin (CT), CGRP, amylin (AMY), and newly discovered intermedin [10, 11]. The structural integrity of the members of this family of peptides is crucial for their biological activity [12, 13]. For example, the carboxy-terminal portion of the peptides, lacking the N-terminal ring structure, serves as selective peptidyl competitive antagonists to the respective full-length counterparts. Peptide fragment AM_{22-52} serves as a competitive blocker for AM, while α-$CGRP_{8-37}$ and β- $CGRP_{8-37}$ are receptor blockers for CGRP receptors [13-15]. Given that these antagonists are peptide fragments and the limitations peptide inhibitors can impose with experimental manipulations, much effort is being placed in the development of non-peptide, selective antagonists. Thus far, BIBN4096BS, WO98/11128 (Compound 1), and SB-273779 have been characterized as highly selective, non-peptide antagonists for subtypes of the CGRP receptor [16-18]. Similar antagonists have yet to be developed for the AM receptor.

2.1.2. Location of adrenomedullin gene and protein expression

In the current literature, AM expression has been established to be ubiquitous in a wide diversity of tissues. Demonstrated with high-sensitivity radioimmunoassays and/or by immunohistochemical studies, AM expression has been reported in the adrenal medulla, heart, aorta, kidney, lung, brain, pancreas, skin, and additional tissues [1, 19-24]. AM protein and/or mRNA expression has been detected in a multitude of cell types, as well. Some examples are cardiac myocytes [6], vascular smooth muscle cells [25, 26],

endothelial cells [27], renal mesangial cells [28-30], renal proximal, distal, and collecting tubular cells [31-33], pulmonary cells [34], and various human tumor cell lines [7, 35-37]. Expression of AM in such diverse tissues suggests the multifunctional role for AM.

2.1.3. Biological actions of adrenomedullin

To date, the best-described biological actions of adrenomedullin are in the cardiovascular system. Most well-characterized is surely the hypotensive effect of AM, which is both very potent and long-lasting. However, AM's additional biological actions are very diverse, acting in almost any system tested, and therefore extend beyond the scope of discussion in this literature review. I will focus on the actions of AM in the cardiovascular and renal systems, the systems of particular interest to the laboratory of my thesis research. Following this discussion can be found a chart with a brief description of AM's wide assortment of actions in additional systems (Table1).

In the vascular beds of humans, rats, cats, dogs, and sheep, AM studies have shown a relaxation of the resistance vessels to attain a long-term drop in the mean arterial blood pressure (MAP) [38-43]. In most instances, this vasodilation is capable of attenuation by L-NAME, suggesting an involvement of a nitric oxide (NO)-dependent pathway for the effect [44-46]. Some variations have been reported in cases of different species or localization of AM stimulation [47-49]. In addition to decreasing blood pressure, AM administration will increase heart rate. NO-dependent mechanisms have also been found to be utilized by AM to dilate the renal vasculature and to mediate diuretic and natriuretic responses in the kidney [50]. In terms of the microvasculature, AM's effects are very similar to those in the larger resistance vessels, with AM acting on

CGRP or AM receptors to elicit relaxation via either activation of adenylate cyclase, release of NO, or activation of potassium channels.

In addition to its potent vasomotor activity, AM plays critical roles in cell growth regulation. Its effects on cell growth and apoptosis depend predominantly on the cell type and the experimental conditions under examination. For example, AM has been well-characterized to promote proliferation in a number of human tumor cell lines, as well as Swiss 3T3 fibroblasts, human oral keratinocytes, rat gastric epithelial cells, and human retinal pigment epithelial cells [51-55]. Conversely, in several cardiorenal cell types AM has been reported to be anti-proliferative. These cell types include rat vascular smooth muscle cells, hypertrophy in cultured myocytes and fibroblasts, rat mesangial cells, and human proximal tubule epithelial cells [56-59]. In parallel to reports for the vascular cell types, AM is expressed by a wide variety of renal cells where it is capable of yielding numerous biological effects. Based on reverse transcriptase- polymerase chain reaction (RT-PCR) studies in the rat nephron segments, Owada *et al.* have detected AM expression in the glomerulus, cortical collecting duct, outer medullary collecting duct, and intermedullary collecting duct, but not in the proximal convoluted tubule or medullary thick ascending limb [30]. In opposition, a different study, reported by Jensen *et al.*, describes mRNA expression for AM and AM-R colocalized in renal vessels, glomeruli, and inner medullary collecting ducts. AM mRNA was also detected in proximal tubules, whereas AM-R mRNA was found in distal convoluted tubules [60]. The previous study also detected high levels of expression of AM in the rat mesangial cells, as well.

Based on the wide expression patterns of AM in the kidney, it is not surprising that AM exerts profound effects on renal function. Studies by Ebara and colleagues demonstrated that intrarenal infusion of AM at concentrations suboptimal for heart rate and blood pressure alterations resulted in an increase in renal blood flow (RBF), total urine output, and urinary sodium excretion. At higher concentrations of AM, MAP shows marked decreases, while GFR is now increased, vasodilation of efferent and afferent arterioles occurs, and distal tubular sodium reabsorption is further decreased, further increasing sodium excretion [30, 32, 45, 61]. Later studies established these AM-stimulated renal vasodilatory and natriuretic effects were NO-dependent [45, 46, 62]. In contrast to these studies and accepted AM actions in the kidney, Leclerc et al. described a cAMP-dependent sodium-sparing capacity of AM, by a mechanism of regulating the Na+/H+ exchangers of the distal tubules [63].

Generalized Biological Actions of AM:

Vascular Effects:

- AM administration results in sustained hypotension via NO, cAMP, and/or PG generation (depends on vascular bed) [13, 38, 44, 64]
- Positive ionotrophic and chronotrophic effects on coronary artery dilation [65-67]

Renal Effects:

- Intrarenal infusion of AM produces increases in RBF, GFR, Na+ excretion, and urine flow [45, 68, 69]
- Inhibits PDGF-induced (MAPK-dependent) mesangial cell proliferation [70, 71]
- Stimulates intrarenal renin release [72]

Effects in Bone:

- Promotes osteoblast growth and protein synthesis (yielding increased area of mineralized bone) [73, 74]

Effects in Lung:

- Inhaled AM reduces histamine-, acetylcholine-, and antigen-induced bronchoconstriction [12, 64]

Effects in Endocrine System:

- Inhibits ACTH release [75, 76]
- Inhibits insulin secretion (increases blood glucose) [23]
- Inhibits aldosterone production/secretion [77]

Effects in Central Nervous System:

- Central stimulation of the sympathetic nervous system (increases blood pressure and heart rate) [78, 79]
- AM administration inhibits thirst drive and salt appetite, attenuates AVP release, and is pro-anorexic [80-82]

Table I. Biological actions of adrenomedullin.

2.1.4. Adrenomedullin expression in pathophysiological states

Because of AM's diverse biological actions, acting in almost any system tested, it is not surprising that AM's role in pathophysiologies is quite extensive and extends beyond the scope of discussion in this literature review. I will focus on the involvement of AM in pathologies of the cardiovascular and renal systems, the systems of particular interest to the laboratory of my thesis research.

Hypertension

Plasma levels of AM have been demonstrated to be elevated in patients with primary arterial hypertension, with even higher levels in patients with complications of hypertension, such as left ventricular hypertrophy and nephrosclerosis [83]. AM gene expression is increased in the Dahl salt-sensitive rats fed a high Na+ diet [84], but the most dramatic elevation of AM gene expression is in the spontaneously hypertensive rats (SHR) treated with deoxycorticosterone acetate (DOCA) and fed a high Na+ diet (DOCA-salt SHR) and the stroke-prone SHR [85-87]. These two animal models also show increased CL-R and RAMP2/3 gene expression. It has been suggested that the up-regulation of the cardiac AM system in hypertension is a protective mechanism, acting to decrease myocardial overload with the vasodilatory and natriuretic properties of AM, as well as limiting further myocardial hypertrophy and remodeling due to the proliferative regulation by AM.

Heart Failure

AM plasma concentration is increased in patients with congestive heart failure, and this elevation is correlative with disease severity [88, 89]. AM concentration in myocardial tissue obtained from heart transplant recipients with severe heart failure is

higher than in donors, suggesting that increased plasma AM levels in heart failure patients is a result of increased myocardial production of AM [90]. In addition, AM, CL-R, RAMP2, and RAMP3 gene expression is markedly up-regulated in different animal models of heart failure (induced by volume or pressure overload) [91-95]. AM up-regulation may be acting in a protective manner, in this situation, to increase myocardial contraction due to its positive ionotrophic properties, or as a compensatory mechanism to decrease cardiac preload and afterload. Additional protective aspects of AM expression are a decrease in myocardial remodeling, due to AM's role in attenuating myocyte hypertrophy, proliferation of myocardial fibroblasts, and production of extracellular matrix. Finally, AM acts in accordance with natriuretic peptides to counteract the vasoconstriction and sodium retention by renin-angiotensin-aldosterone, endothelin, and the sympathetic nervous system. AM infusion in heart failure patients has had beneficial effects, including an increase in cardiac output and natriuresis, reduction of peripheral resistance, pulmonary capillary wedge pressure, left ventricular end diastolic pressure and plasma aldosterone, and an increase in ejection fraction [96]. Some studies have suggested, though, that the vasodilatory and natriuretic properties of AM are impaired in heart failure, with no mechanism for the abated function apparent at the present time [41, 97].

Atherosclerosis

AM levels in the plasma of patients with chronic ischemic stroke are elevated and correlate with the extent of carotid artery atherosclerosis [98]. In addition, AM has been detected in macrophages found within atherosclerotic plaques [99]. Due to AM's inhibitory effect on migration and proliferation of vascular smooth muscle cells,

inhibition of endothelial cell apoptosis, and anti-inflammatory activity, it could be hypothesized that AM could have beneficial effects in atherosclerosis[100]. AM's role in atherosclerosis, in classic models of the disease, has yet to be tested. Data does exist that indirectly suggests AM may have atheroprotective roles, including enhanced intimal thickening observed following arterial injury in $AM^{+/-}$ mice and attenuation of restenosis with AM gene overexpression following balloon-induced or cuff-induced arterial injury in rodents [101-103].

Myocardial Infarction

During the acute phase of myocardial infarction, plasma AM levels have been demonstrated to be increased, reaching their maximum after 2-3 days and returning to baseline after a duration of about 3 weeks [104]. Increased AM levels are associated with hemodynamic impairment. Evidence for this is a positive correlation between plasma AM and central venous pressure, left ventricular and diastolic pressure, and pulmonary capillary wedge pressure, and negative correlations of plasma AM with ventricular ejection fraction [105]. AM, CL-R, and RAMP2 expression increases in ischemic and non-ischemic myocardium following coronary artery ligation in the rat [106, 107]. AM gene expression is also shown to be increased with hypoxia, due in part, to an oxidative stress mechanism [108]. AM production by myocytes may also be stimulated in the infracted region by mechanical stretch, angiotensin II, and proinflammatory cytokines. Due to AM's effects of local coronary vasodilation and reduction of oxidative stress-induced myocardial cell injury, it is believed that AM is playing a protective role in myocardial ischemia [109, 110]. Supporting this concept, AM overexpression before ischemia/reperfusion injury will decrease the superoxide anion generation in the

hypoperfused myocardium, decrease the infart area, inhibit apoptosis of cardiomyocytes, and reduce the number of ventricular fibrillation incidents [111]. Additionally, when AM is infused at suboptimal levels for effect on blood pressure, it retains the ability to inhibit cardiac remodeling following experimental myocardial infarction in the rat [112].

Renal Diseases

In patients with chronic renal failure, plasma AM levels gradually increase. This is thought to be due to decreased peptide clearance, but an elevation due to chronic volume overload cannot be disqualified. In various types of glomerulonephritis, while plasma AM levels are increased, AM excretion is decreased [113, 114]. Hypoxia is known to up-regulate AM gene expression, suggesting that AM may protect the kidney from ischemia-reperfusion injury [115]. In fact, it has been shown that in AM $^{+/-}$ mice, the AM deficiency aggravates histological lesions and functional impairment in experimental ischemic acute renal failure, while AM overexpression has been shown to be protective against these effects [116].

2.1.5. Effects of adrenomedullin gene alteration and adrenomedullin gene delivery

Two labs have produced AM knockout mice, and while some differences exist, AM's role in development is very evident. Both labs demonstrated that homozygosity of the AM knockout is embryonically lethal. The first report disrupted the AM gene so that neither AM or PAMP were produced [117, 118]. The homozygous AM knockout embryos showed poorly developed vitelline vessel vasculogenesis, hemorrhages, myocardial hypertrophy, and hydrops fetalis. These mice typically died between embryonic day 13.5 and 14.5. The heterozygous AM $^{+/-}$ mice are able to survive to

adulthood and are fertile, but they are characterized arterial hypertension due to NO deficiency.

The second group to generate AM knockout mice did so by placing a stop codon at the starting point for the AM coding sequence in the preproAM gene [119]. This resulted in mice that did not express AM, but show normal levels of PAMP. While the homozygous AM mutation in these mice was also lethal (although these AM-/- mice did not show the same placental and vascular defects or hydrops fetalis), some differences were seen in the heterozygous knockout mice. These animals also reached adulthood and were fertile, but in this case, the AM +/- mice showed normal blood pressure regulation. In experimental hypertension induced with angiotensin II administration and high-salt diet, the AM+/- mice showed similar increases in blood pressure as wild-type mice, but did show greater organ damage, due to greater hypertrophy of the left ventricle and coronary arteries, and higher vascular oxidative stress. This data indicates a role for AM in protecting against end-organ damage in Na+-induced hypertension, independent of BP regulation. When comparing the two animal models of AM knockout, it could be hypothesized that PAMP plays a primary role in blood pressure regulation (through NO-dependent mechanisms), but it must be considered that both animal models were generated using different genetic backgrounds.

2.2. Adrenomedullin Receptor Complex

2.2.1. Discovery of adrenomedullin receptor complex

The numerous biological activities of AM (discussed previously, Table 1) had been suggested to be mediated by a cell-surface receptor. Until recently, identification of

this receptor had been quite elusive, due to conflicting reports in both pharmacological inhibition and cloning studies. While AM's vasodilatory response was inhibited in some tissues with the CGRP antagonist, $CGRP_{8-37}$, in other tissues no effect was seen, even at concentrations that potently inhibited the CGRP-mediated vasodilatory response [42, 120-122]. This observation led to the hypothesis that an AM-specific receptor existed. The AM-specific vasodilation was observed, for example, in guinea pig pulmonary artery, hypotensive effects in Long-Evans rats, control of aldosterone production in rat adrenals, and other AM-mediated actions [123-126]. Meanwhile, other groups demonstrated AM-stimulated responses that were sensitive to both CGRP and AM peptidal inhibitors ($CGRP_{8-37}$ and AM_{22-52}, respectively) [127].

Unfortunately, the molecular cloning attempts of the AM receptor initially yielded data equally confusing as the pharmacological inhibition studies. The AM receptor was first reported to have been cloned from the rat lung by Kapas *et al.* This receptor was capable of binding ^{125}I-AM and eliciting an elevation in cAMP in response to AM stimulation when transfected into COS-7 cells [128]. This receptor and a canine receptor, identified a few months later by Kapas and Clark, RDC-1, showed considerable homology to a previously described orphan receptor, termed L1 or G10d [129-131]. Part of the confusion arose when subsequent attempts by other laboratories to replicate and further these studies, resulted in an inability of these labs to reproduce the previously reported observations [132-134].

Later an alternative for the AM receptor was proposed in studies by Aiyar and colleagues. They identified a previous orphan receptor, now termed calcitonin receptor-like receptor (CL-R), which exhibited well-characterized $CGRP_1$ receptor pharmacology

with a weak AM cross-reactivity. When this human CL-R was expressed in human embryonic kidney (HEK 293) cells, a robust increase in CGRP-stimulated cAMP accumulation was observed, as was specific ^{125}I-CGRP binding and dose-dependent inhibition of cAMP production by $CGRP_{8-37}$ [135]. Similar results were seen for rat and porcine CL-R [136, 137]. Propagating the classification of CL-R as an orphan receptor, additional labs were unable to reproduce the results of Aiyar and colleagues in different cell lines [43, 138, 139]. CL-R was characterized as a seven transmembrane-spanning G-protein-coupled receptor, but its native ligand remained unidentified. Based on structural and amino acid homology, CL-R was classified as a family B GPCR (Secretin family of GPCRs). Other members of this GPCR family include receptors for secretin, gastric inhibitory peptide, glucagon, pituitary adenylate cyclase activating hormone, vasoactive intestinal peptide, growth hormone releasing hormone, parathyroid hormone, and calcitonin. CL-R is comprised of 461 and 464 amino acids and shares 50% and 54% amino acid sequence identity with the rat and human calcitonin receptors, respectively [43, 139].

Reforming the study of this receptor system, a critical study was published in 1998 by McLatchie et al. that clarified the elusiveness of the AM and CGRP receptors, while also providing an entirely novel form of GPCR regulation. Utilizing a Xenopus oocyte system that expressed endogenous CGRP receptor and an exogenous cAMP-sensitive cystic fibrosis transmembrane regulator (CFTR), they systematically screened complementary RNAs derived from the SK-N-MC (human neuroblastoma cell line endogenously expressing CGRP receptor characteristics) cell's DNA library [134, 140]. This screen yielded cloning of a 148 amino acid protein, termed receptor activity

modifying protein-1 (RAMP-1), that was capable of significant CGRP-mediated cAMP accumulation in the oocytes. Yet when expressed in HEK 293T, COS-7, or Swiss 3T3 cells, this protein was unable to elicit a CGRP-stimulated cAMP response. Dual expression of CL-R and RAMP-1 was found to restore the CGRP-mediated cAMP production in oocytes and HEK 293T cells. This presumably was the result of these cells not expressing endogenous CL-R, so both the receptor and RAMP expression were required for the cAMP response to be observed. Database search identified two additional isoforms of RAMPs (RAMP-2 and RAMP-3), which together the RAMP proteins only show approximately 31% sequence identity. Repeating similar experiments with RAMP-2/3 in oocytes, as performed for RAMP-1, yielded a quite surprising result. For the first time, a GPCR showed a differing receptor phenotype when expressed with different accessory proteins. Namely, when CL-R was expressed with RAMP-1, a CGRP receptor was produced, whereas when CL-R was expressed with RAMP-2 or RAMP-3 the receptor was responsive to AM (AM1 receptor and AM2 receptor, respectively) [134]. These results were confirmed by a number of additional labs in human endothelial and vascular smooth muscle cells, rat osteoblast-like UMR-106 and COS-7 cells, and Drosophila Schneider 2 cells [141-143]. This was a revolutionizing concept for the GPCR field.

Notably, while RAMP2 and RAMP3 share only approximately 30% sequence identity, they generate virtually pharmacologically and biologically identical AM receptors when co-expressed with CL-R in HEK 293T cells [144]. Until recently published by our laboratory, no other laboratories detected differences in receptor phenotype or signaling between the AM1R and AM2R. Additionally, it was presumed

that differences in receptor regulation must exist between the two receptor isoforms. A report from our lab recently described the differential regulation of RAMP2 and RAMP3 expression by platelet-derived growth factor (PDGF) and lipopolysaccharide (LPS) in rat glomerular mesangial cells [145, 146]. Moreover, RAMP expression studies in animal disease models show variable RAMP2 and RAMP3 mRNA expression (see section 2.2.6).

2.2.2. RAMP gene and protein structure

RAMP-1 and RAMP-2 were initially cloned from human neuroblastoma cell (SK-N-MC) DNA library, whereas RAMP-3 was isolated from the human spleen. Comparison of sequences of the RAMPs with the genomic map predicted their chromosomal location, as well as gene organization. RAMP-1 was shown to reside on chromosome 2, the RAMP-2 gene on chromosome 17, and RAMP-3 gene is on chromosome 7 [134]. A scan of the human genome revealed no more sequences similar to the RAMPs [147]. RAMP-1 and -3 share some similarities in their gene composition, being comprised of three exons divided by large introns. The RAMP-1 and -3 genes are large in comparison to that of RAMP-2 (approximately 24 kilobases vs. 5 Kb, respectively). RAMP-2 has four exons, but shorter introns regions. All three genes have similar localization of the 5'UTR and the signal peptide sequence on the first exon and the C-terminal and transmembrane domains on the last exon of the corresponding RAMP genes [148].

Despite the relatively low amino acid sequence identity between the RAMP isoforms (approximately 30%), the hydrophobicity plot analysis suggested a substantial

similarity of protein structure. The sequence similarity of the RAMP isoforms between species is quite well conserved, at approximately 90% between mouse and rat. Sequence identity between the rodent and human sequences is 70, 65, and 85% for the RAMP1, RAMP2, and RAMP3, respectively [149, 150]. RAMP-1 and RAMP-3 are 148 amino acid proteins, while RAMP2 is composed of 175 amino acids, but all isoforms are made up of large extracellular domains, an approximately 20 amino acid transmembrane domain, and a roughly 10 amino acid intracellular domain [134]. The RAMPs have a molecular weight of only approximately 14-17 molecular weight. Several sequences and/or residues are conserved over the RAMP isoforms and different species, indicating an importance in regulatory functions or preservation of secondary structure. Included are four cysteine residues located in the extracellular domain and two sequences localized to the N-terminal and C-terminal regions, respectively: DPPXX and LVVWXSK [134]. Several consensus sites for N-glycosylation have also been identified on RAMP2 and RAMP3. Four N-glycosylation sites identified on human, mouse and rat RAMP3 are conserved in the mouse RAMP2. Suggesting differential post-translational modifications of the RAMP isoforms, RAMP1 has no consensus sites for N-glycosylation [151]. Protein kinase A and C phosphorylation consensus sites also are present on the C-terminal intracellular domains of RAMP1 and RAMP3, but not RAMP2 [150, 152]. Our laboratory has also recently reported a function for the previously-identified PDZ recognition motif on the extreme C-terminus of RAMP3, but not RAMP1 or RAMP2, that is responsible for protein-protein interactions important in the regulation of trafficking of the receptor complex [153, 154]. Further investigation is clearly required

to determine the functional consequences of the discussed conserved regulatory sequences.

2.2.3. Tissue and cell specific RAMP expression

RAMP mRNA distribution has been analyzed in several species to date, including mouse, rat, and human, with differences existing, but general expression patterns have been established. RAMP-1 has shown predominant expression in the heart, brain, skeletal muscle, thymus, spleen, fat, and kidney; RAMP-2 expression is abundant in the heart, aorta, kidney, spleen, fat, and lung. RAMP-3 shows the widest distribution, but tends to exhibit prevalent expression in the kidney, heart, brain, and lung [134, 148-150, 155, 156].

In addition to tissue distribution, the RAMPs' expression has been studied in a variety of cell types. Differential expression of RAMP isoforms in cell types has been reported, especially in the initial characterization of the CGRP and AM receptors, leading to the identification of CL-R, a previous orphan receptor capable of signaling through both ligands, depending on RAMP expression. Endogenous expression of RAMPs in a particular cellular background has been well-documented to affect the functional character of the observed receptor subtype. Endogenous expression of the RAMPs also affects the functional receptor observed when overexpressing RAMP isoforms in a cell type. For example, rabbit aortic endothelial cells (RAECs) endogenously express RAMP-2, but not RAMP-1, and are selectively responsive to AM and blockade by AM_{22-52}, but not $CGRP_{8-37}$. While transfection of hRAMP-1 into RAECs will yield a CGRP response that is selectively inhibited by $CGRP_{8-37}$, overexpression of hRAMP-1 and

hRAMP-3 into the RAECs results in decrease of CGRP-mediated cAMP response. This work suggests a greater affinity of the rabbit CL-R for RAMP-3 as compared to RAMP-1. However, the overexpression of hRAMP-3 does not alter the responsiveness of the CL-R in RAECs to AM, suggesting an intraspecies preference for RAMP isoforms [157]. Variable RAMP isoform-CL receptor affinity has been demonstrated in additional cell types, namely UMR 106-06 cells and COS-7 cells [139, 142].

RAMP distribution has also been elucidated in several organ systems to date, namely the central nervous system and the kidney. In the central nervous system, using *in situ* hybridization to detect expression, RAMP1 mRNA was predominantly expressed in the cortex, caudate putamen and olfactory tubercles; RAMP2 mRNA was most abundant in hypothalamus; and RAMP3 expression was restricted to the thalamic nuclei [158]. Notably, in specific brain areas only a single RAMP isoform was often detected, suggesting mutual exclusivity in expression. Of particular interest to our laboratory, renal RAMP mRNA expression has been evaluated. RAMP-2 and RAMP-3 mRNA has been detected in abundance in the rat kidney, showing similar expression patterns in both the cortical and medullary parts of the kidney [159]. Using quantitative RT-PCR, Totsune and colleagues measured the mRNA expression levels of RAMP-2, RAMP-3, and CL-R in the kidney of normal Munich-Wistar rats. RAMP-2 expression was 26.5±1.9 mmol per mole of GAPDH and RAMP-3 mRNA levels were 7.7±0.4 mmol per mole of GAPDH. CL-R was detected in the kidney in this study, but at significantly lower concentrations [160]. Our lab has characterized RAMP and CL-R mRNA expression in several renal cell lines. Please see the chart below for RAMP isoform and CL-R distribution (mRNA expression) in the various renal cell lines. The differential

expression of the RAMPs (especially RAMP-2 and RAMP-3) in the different regions of the kidney suggests additional, yet to be identified, regulatory roles for the RAMPs.

CELL LINE	RAMP-1	RAMP-2	RAMP-3	CL-R
Human primary proximal tubule cells	+	+	+	+
Rat mesangial cells	+	+	+	+
Rat kidney fibroblasts	+	+		+
Rabbit cortical collecting duct cells	+			+

Table II. RAMP isoform and CL-R receptor expression in renal cell lines.

2.2.4. Mechanism of RAMP-receptor interaction

Several studies have now established that RAMPs initially interact with their receptor partners in the ER/Golgi and maintain this association throughout the life-cycle of the receptor complex. The RAMP and receptor are trafficked together to through the endocytic pathway to the recycling endosome or lysosome after agonist activation of the receptor [153, 161-164].

The interaction of the RAMP isoform with CL-R results in modification of the terminal glycosylation of the receptor [134, 163]. RAMP-1 and RAMP-2 are both known to modulate CL-R through glycosylation, although only the RAMP-2/CL-R complex requires glycosylation for expression of the receptor complex at the cell surface [163]. While glycosylation may determine cell surface expression for the receptor complex, glycosylation is not required for determination of receptor phenotype by the RAMPs.

Experiments in insect cells, where glycosylation state of the receptor is not altered, show no alteration in ligand recognition or receptor activation, as compared to similar studies in mammalian cell lines [141].

Evidence exists that indicates that the N-terminal, extracellular domain of the RAMP is critical to determination of receptor phenotype, while the transmembrane domain is important in the stabilization of the RAMP-receptor interaction [144]. Chimera studies with the RAMP-1 and RAMP-2 extracellular domains suggest that the N-terminal regions of the RAMPs determined ligand selectivity of CL-R [165]. This work surprisingly suggests that the extracellular domains of RAMPs are sufficient to maintain a fully functional receptor for CGRP. Chimera of the extracellular domain of RAMP-1 with the transmembrane and intracellular domains of PDGF-R only showed a ten-fold decrease in potency for CGRP signaling and binding. However, the extracellular domain alone showed a greatly diminished (approximately 4000-fold) responsiveness to CGRP. These studies aren't entirely consistent with data reported by Steiner *et al.* with respect to the transmembrane domain of RAMP-1 [166]. Steiner and colleagues reported that deletion of only a portion of the RAMP-1 transmembrane domain caused a dramatic decrease in the potency of CGRP, regardless of functional trafficking of the receptor to the cell surface. Further work will be needed, possibly studying the extracellular and transmembrane interactions of RAMP-2 and -3 as well, to determine the interactions necessary for receptor trafficking, activation, and heterodimer stability.

As mentioned above, a role has been indicated for the transmembrane domain of RAMPs in the stabilization of the RAMP interaction with CL-R and the calcitonin receptor (CTR). Chimera experiments with RAMP-1 and RAMP-2 defined the

extracellular domain as having the responsibility for receptor phenotype determination. Because of a selective recognition of amylin by RAMP-1 or RAMP-3 complexed with the CTR, RAMP-1 and -2 chimeras is an excellent tool to delineate the domains responsible for agonist binding, receptor activation, and heterodimer stability. Levels of amylin binding, induced with the wild-type RAMPs, were paralleled by the chimeras according to the transmembrane domain/C-terminus present in the heterodimer [140]. This led to the theory that the transmembrane domain of the RAMPs was the primary interaction site for the RAMP and receptor (at least for the CTR). This model may be rather simplistic or receptor-specific, though, in light of additional recent studies on the N-terminus of RAMP-1 that have identified a stretch of aromatic residues within RAMP-1 that are probably important in the interaction between RAMP-1 and the CL-R. These N-terminal residues (F93, Y100, or F101) act independently of determination of receptor phenotype [167]. While not altering the EC50 value for the CGRP-mediated cAMP response, mutation of any of the above aromatic residues to alanine rendered the receptor complex unable to express at the cell surface and bind agonist (CGRP). In addition, recent work by Sexton and colleagues, with a more diverse set of RAMP-1/2 chimeras, indicates a strong induction of amylin binding, even in the presence of the transmembrane domain of RAMP-2 [168]. Further work is required to establish the regions responsible for interaction between the RAMP and receptor, with differences possibly existing for different receptor partners of the RAMPs.

Segments of all three RAMPs have been identified that are required for generation of a functional receptor phenotype. On RAMP-2 the extracellular region from amino acid 77 to 101, in particular residues 86-92, are critical for adrenomedullin receptor

phenotype when the RAMP is expressed with CL-R [169]. The equivalent region on RAMP-3, residues 59-65, is also required for AM receptor phenotype, although no homology between the crucial sequences of RAMP-2 and RAMP-3 exists. This suggests that this sequence plays a structural role in the RAMPs or this region possesses an allosteric effect on CL-R conformation. Data from RAMP-1 deletion studies suggests a role for these short sequences in the extracellular region on the proper conformation of CL-R to bind ligand [167]. Deletion of the short N-terminal sequence from RAMP-1, residues 101-103, abolishes the induction of CGRP receptor phenotype by the receptor complex, but if the residues are replaced with alanine residues, the potency of CGRP remains unaltered.

Disulfide bond formation also appears critical to ligand binding and receptor activation for the RAMPs and their heterodimeric partners (CL-R and CTR). While four cysteine residues are conserved between all three RAMP isoforms, RAMP-1 and RAMP-3 contain an additional 2 conserved cysteine residues [170]. Data from Flahaut *et al.* suggests that all cysteine residues on RAMP-3 are crucial for ligand binding and receptor activation. It could be speculated that the additional disulfide bond formation on RAMP-1 and RAMP-3 allow for the increased affinity of the RAMP-1/CL-R and RAMP-3/CL-R (to a lesser extent) receptor complexes for CGRP.

2.2.5. Additional RAMP-interacting receptors

One of the first questions raised after the discovery of RAMPs and their role in trafficking and ligand selectivity of CL-R was if additional receptors can interact with the RAMPs. Given the significant sequence identity between the CTR and CL-R, the CTR

was an obvious first choice. Strong evidence exists to establish that RAMP association is not required for CTR binding and activity induced by calcitonin [161, 171]. However, RAMP-1 and RAMP-3 isoforms were found, by several independent laboratories, to interact with the CTR to form a high affinity amylin receptor. These results were confirmed in exogenous systems in COS-7 and rabbit aortic endothelial cells and with an endogenous CTR in Chinese hamster ovary (CHO)-K1 cells [161, 172].

RAMPs have a relatively ubiquitous distribution, with at least one RAMP expressed in most tissues examined to date. Noteworthy, this distribution extends beyond that of the characterized receptor partners (CL-R and CTR), suggesting a more widespread role for RAMPs in receptor regulation [134, 148, 149]. The discovery of the RAMPs led to an interest in the potential of the RAMPs to interact with additional GPCRs to determine cell surface expression or receptor phenotype, especially for receptors that had been thus far difficult to characterize. RAMPs were thought to maybe be the missing link in pairing numerous orphan receptors with their ligands. A recent study by Christopoulos *et al.* identified additional interacting receptors for the RAMPs by screening the capacity of the receptors to traffic the RAMPs to the cell surface [134, 161, 172, 173]. Screened in this study were 10 of the GPCRs of the family B of GPCRs, the family to which CL-R and CTR belong. Of the 10 GPCRs tested, 6 of the receptors were capable of trafficking at least one of the RAMP isoforms to the cell surface [174]. In addition to CL-R and CTR, the newly identified RAMP-interacting receptors include the vasoactive intestinal peptide/pituitary adenylate cyclase activating peptide (VPAC)-1 receptor, parathyroid hormone receptor (-1 and -2), and the glucagon receptor. While the CL-R, CTR, and VPAC-1 receptors interact strongly with all three RAMP isoforms, the

PTH1R and glucagon receptor only interact with RAMP-2, and PTH2R only interacts with RAMP-3. The VPAC2, glucagon-like peptide (-1 and -2), and growth hormone-releasing hormone receptors were unable to interact with any of the RAMP isoforms [174]. This study, while suggesting a more widespread role for the RAMPs in receptor regulation, also reveals additional specificity of RAMP-receptor interactions.

Of note, with the exception of CL-R, RAMP association with interacting receptors is not required for chaperoning of the receptors. The most compelling consequence of the newly-discovered RAMP-receptor interactions is the augmentation of VPAC1 receptor-mediated signaling [174]. RAMP-2 interaction with VPAC1-R does not alter the agonist binding properties or cAMP signaling of the receptor, however RAMP-2 association with the VPAC1-R does enhance the receptor-mediated phosphoinositol hydrolysis pathway. While no change is seen in the EC_{50} for the agonist-mediated PI hydrolysis, the maximal VPAC1-mediated PI signaling (E_{max}) is increased. The physiological relevance of this observation has yet to be defined.

The data reported in this project suggests additional roles RAMPs may be fulfilling with their interacting receptors. Beyond roles in cell surface trafficking and receptor phenotype determination, results from this study propose roles for RAMPs in sorting of receptors after ligand activation to determine the receptor's fate.

2.2.6. Regulation of RAMP gene expression

The changes in RAMP gene expression have been studied under many disease models, physiological changes, and drug treatment. Below is a table summarizing the findings of these studies, focusing on the cardiovascular and renal system changes (Table

III adapted from review by Udawela *et al.*) [168]. Dramatic expression pattern alterations are seen, indicating an important role for dynamic RAMP regulation in these systems. These results suggest the potential for RAMP regulation as a means of modulating some of the pathophysiological conditions associated with the RAMP-interacting GPCRs.

Model	Tissue	R1 Exp.	R2 Exp.	R3 Exp.	CL-R Exp.	AM Exp.	Ref.
Heart Failure (HF) in rats (coronary artery ligation)	Atrium Ventricle Kidney	N/D N/D N/D	↑↑ ↑ --	N/D N/D N/D	↑↑ ↑ --	↑↑ ↑↑↑ --	[94]
Rat ischemic heart failure (left coronary ligation)	Non-ischemic left ventricle Ischemic LV	N/D N/D	↑ ↑↑↑	N/D N/D	N/D N/D	↑ ↑↑↑	[107]
LV hyptertrophy to heart failure in rat (KCl injection)	LV at hypertrophy LV at HF	N/D N/D	↑↑ ↑↑	↑↑↑ ↑↑↑	↑↑↑ ↑↑↑	↑ ↑↑↑	[93]
Chronic HF in rats (aortic banding)	Atria Ventricles	↑ ↑↑	-- --	↑↑↑ ↑↑	-- --	-- --	[91]
Myocardial ischemia in rats (isoproterenol induced)	Myocardium Aorta	N/D N/D	↑ ↑	N/D N/D	N/D N/D	↑↑↑ ↑↑	[175]
Blood pressure changes in rat Increase by phenylephrine Decrease by nitroprusside	PVN Nucleus of solitary tract PVN Nucleus of solitary tract	N/D N/D N/D N/D	↓↓ ↓↓ -- ↑↑↑	N/D N/D N/D N/D	N/D N/D N/D N/D	-- -- ↓↓ --	[176]
Hypertensive rat							[85]
Salt loaded	Renal cortex Renal medulla	N/D N/D	↓↓↓ ↓↓	↑↑ --	↓ --	-- --	
DOCA treated- salt loaded	Renal cortex Renal medulla	N/D N/D	↓ ↓↓	-- ↑↑	-- --	-- ↑↑	
DOCA- salt loaded	LV	N/D	↑	↑	↑	↑	[86]
Chronic salt loading in rat	Adrenal gland Kidney	↑ --	↑↑ --	-- ↑↑	↑ ↑	↑↑↑ ↑↑	[177]
SHR	LV	N/D	↑	--	↑	↑	[87]
Ang II treatment in rat	Cardiomyocytes	↑↑	--	↑↑	--	N/D	[178]
Endothelin treatment	Cardiomyocytes	--	↓	↓	↑	↑	[179]
Hypoxic rat	Lung	↑↑↑	--	↑	--	N/D	[180]
Hypoxia	IMR-32 NB-96	N/D N/D	↓↓↓ --	N/D N/D	N/D N/D	↑↑ ↑↑	[181]
Renal injury in rat (mass ablation)	Kidney (remnant tissue)	N/D	--	↓↓	↓↓	--	[160]
Renal injury in rat (obstructive nephropathy)	Kidney	↑↑↑	↑↑	--	↑↑	--	[150]
Renal fibrosis in rat	Kidney	↑↑↑	↑↑	--	↑	--	[182]

Table III. Regulation of expression of RAMP isoforms and CL-R in various pathophysiologies and animal models. (N/D, not done; --, no change).

2.2.7. Receptor Component Protein (RCP)

During the process of trying to clone the CGRP receptor, Dickerson and colleagues identified a novel 148 amino acid protein that appeared to play a role in the regulation of CGRP signaling [183]. This protein was identified with a system similar to that used to identify the receptor activity modifying proteins, a system utilizing the PKA activation requirement of CFTR to register cAMP responses to particular ligands [134]. In this case, CGRP stimulation allowed the cloning of a protein later named CGRP receptor component protein (RCP). RCP, now cloned in several species, shows highly conserved (82%) protein sequence homology between species thus far sequenced [184, 185]. While RCP is detected primarily in membrane fractions prepared from cells, it shows no amino acid structure for hydrophobic regions or consensus motifs for lipid attachment [186]. This data concluded that RCP is a cytosolic, yet peripheral membrane protein. RCP has also been shown to be immunoprecipitated with RAMP1 and CL-R in complex from NIH3T3 cells or lysates of the cerebellum [186, 187]. There are no conserved glycosylation sites in the RCP sequence, but several conserved protein phosphorylation sites do exist [184, 185]. This raises the possibility of regulation of RCP function in an *in vivo* setting.

RCP expression has been studied in several *in vivo* settings, with expression correlating to tissues known to contain CGRP receptors. CGRP is present in the spinal cord in primary afferent fibers and mediates nociception [188]. In these tissues, RCP expression is found juxtaposed to that of the CGRP receptors [189]. CGRP receptors are also known to be expressed on the walls of blood vessels, in this situation mediating

vasodilation, and again RCP expression is found in this tissue, correlating with a role for RCP in CGRP signaling and biological effects [184].

The role of RCP was tested in NIH3T3 cells, a cell line that endogenously expresses the CGRP receptor system, as well as RCP. RCP expression was inhibited in these cells, to test the affect, with antisense constructs for RCP. The antisense inhibited the expression of RCP to that below detection with Western blot. Loss of RCP in the NIH3T3 cells did not affect either the affinity or the receptor density of CGRP receptor in the antisense cells, as determined by radioligand binding with ^{125}I-CGRP [186, 187]. Interestingly, the RCP antisense cells showed a 70% reduction in cAMP response, as compared to wild-type NIH3T3 cells. Recent results from the Dickerson laboratory have suggested a role for RCP in adrenomedullin signaling, as well [190]. Lack of RCP expression did not appear to have any effect on the signal transduction for other GPCRs, including the β_2-adrenergic receptor or the A_{2b} adenosine receptor [186, 187]. These results suggested that RCP was not working in a chaperoning capacity, but had more influence on coupling of the receptor to its signaling pathway.

The model proposed by Dickerson et al. for a functional CGRP receptor includes the CL-R receptor, RAMP1, and now RCP. More work is needed to establish the role of RCP in the coupling of the CGRP receptor to its respective G protein.

2.3. GPCR Life-cycle Regulation

2.3.1. GPCR life-cycle

G protein-coupled receptors (GPCRs) are known to convey environmental signals to the intracellular environment via heterotrimeric G proteins to affect the cellular

behavior in response to a stimulus. Their form of regulation had in the past been thought of as short-term control, whereas more recently they have been shown to play roles in longer-term regulation of such physiological processes as proliferation, apoptosis, cellular migration, and hypertrophy [191, 192]. Their long-term regulation is partially a result of the desensitization and endocytosis processes and the role of these processes in the intracellular signaling. This attenuation of signaling and removal of the receptor from the plasma membrane can decrease the receptor numbers capable of signaling for minutes to hours, depending on the cell type and receptor. Thus, desensitization is the process of an attenuation of signaling as a result of prolonged exposure of the receptor to its agonist (see Table IV and Figure 1 below). Desensitization of a receptor often signals for the removal of the receptor from the plasma membrane by the pinching off of the plasma membrane and internalization of the receptor, termed endocytosis. Many forms of regulation control these processes, some of which being phosphorylation of the receptor intracellularly, interaction of the receptor with additional proteins (caveolin, arrestin molecules), and interaction of the receptor with the endocytic machinery (clathrin, dynamin, adaptor protein-2 (AP-2), etc.). The receptors, regardless of the type, are often endocytosed into the early endosomes where their fate is determined. From this point receptors are targeted for recycling to the plasma membrane or sorted for degradation in the lysosomes. Protein-protein interactions and post-translational modifications are being elucidated that serve as sorting signals from the early endosome, to target proteins for either recycling to the plasma membrane to promote receptor signaling or shuttling of the receptor to the lysosomes for proteolytic degradation and a down-regulation of the

receptor. The factors that determine the regulation of the GPCR life-cycle will be discussed in the following sections.

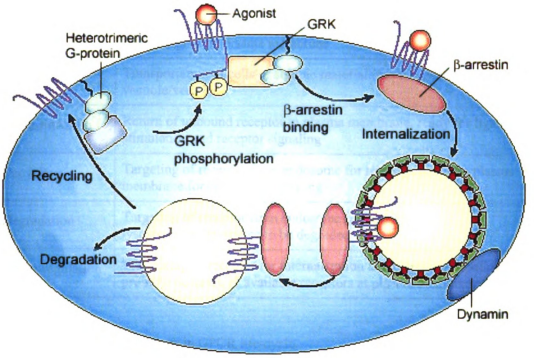


Figure 1. Depiction of GPCR life-cycle (modeled for prototypical GPCR life-cycle of the β_2-adrenergic receptor) [193].

Term:	Definition:
Desensitization	Attenuation in receptor signaling in response to prolonged agonist exposure
Endocytosis	Uptake by a cell of material from the environment by invagination (infolding) of its plasma membrane
Internalization	Transportation of cells or soluble material into the cell via a vacuole/vesicle
Resensitization	Return of unbound receptor to plasma membrane, ready for ligand stimulation and receptor signaling
Recycling	Targeting of receptor from endosome for return pathway to plasma membrane for continued signaling
Degradation	Targeting of receptor from endosome for pathway to lysosomes/proteosomes to be degraded
Down-regulation	Trafficking of receptor for internalization and degradative pathway to promote decrease of available receptors at plasma membrane

Table IV: Terminology for steps in GPCR life-cycle.

2.3.2. Regulation of receptor desensitization

A prolonged exposure of cells to a particular ligand results in an attenuation of responsiveness to subsequent stimulation with that ligand. This phenomenon is called desensitization, which can be homologous or heterologous. Homologous desensitization is classified as a decrease in the response that is specific for the stimulated receptor. Because G-protein regulated kinases (GRKs) are activated and only phosphorylate GPCRs in the agonist-bound state, GRKs are capable of attenuating receptor signaling by homologous desensitization [194, 195]. An attenuation of receptor signaling that is the result of second messenger signaling, and in principal not specific for the activated

receptor, is termed heterologous desensitization. Protein kinase A and C are kinases activated through second messenger signaling of GPCRs, and therefore can activate heterologous receptor desensitization [196].

The most comprehensive study of desensitization, thus far, has been in elucidating the molecular mechanisms of this process for the β-adrenergic receptors [195, 197]. Using concepts from the examination of desensitization of the β-adrenergic receptors, it has been shown in numerous membrane-bound receptor systems, particularly GPCRs, that phosphorylation is an integral step in the attenuation of receptor signaling, termed desensitization. Virtually all GPCRs thus far studied have specific serine, threonine, and/or tyrosine amino acid residues in the third intracellular loop or C-terminus of the receptor that require phosphorylation for the efficient desensitization of the receptor. In most cases, phosphorylation of these key residues allows for interaction with non-visual arrestins. This interaction disrupts the coupling of the receptor to the G-protein, thus halting signaling of the receptor through the G-protein uncoupling. In addition, β-arrestin interaction with the GPCR promotes clathrin-mediated receptor internalization. Without phosphorylation of required residues on the third intracellular loop or C-terminus of the GPCR, signaling will be maintained and desensitization of receptors is attenuated, or prevented.

The mechanism of desensitization of CL-R has been studied in several cell lines to date. Desensitization of CL-R was found to involve protein kinase A activation in rat mesangial cells (RMCs) stimulated with AM, SK-N-MC (neuroblastoma cell line) cells stimulated with CGRP, and vascular smooth muscle cells (VSMCs) stimulated with CGRP [198-200]. Whereas, in HEK 293 cells, desensitization of the CGRP receptor

(CL-R/RAMP1) has been shown to be dependent on the activity of G-protein receptor kinase(GRK)-6 [201]. In HEK 293 cells, CL-R has been shown to be phosphorylated when stimulated with agonist (AM or CGRP) via in vivo phosphorylation assays [202]. Given the published data for the β-adrenergic receptor and additional GPCRs supporting a phosphorylation-dependent mechanism for receptor desensitization, and the above-described studies with CL-R, it is hypothesized that phosphorylation of CL-R is required for receptor desensitization after prolonged agonist exposure. The studies in this project will focus on the requirement of phosphorylation in the desensitization process for the AM receptor subtypes.

With respect to physiological effects of the desensitization process, the inhibition of the process of desensitization for particular GPCRs has been shown to elicit protective therapeutic effects. Lefkowitz *et. al.* have shown that the inhibition of β-adrenergic receptor kinase (kinase that phosphorylates the β-adrenergic receptor to cause desensitization) in the heart can delay the development of heart failure in multiple animal models, in some cases even restoring cardiac function [203, 204]. Others have also shown inhibition of desensitization of the μ-opioid receptor to be beneficial in preventing morphine tolerance [205, 206]. Because of the potential therapeutic effect of prolonged AM signaling in a system where it exerts protective effects, understanding the regulation of the desensitization process for the AM receptor is crucial.

2.3.3. Regulation of receptor internalization/endocytosis

Predominantly, endocytosis of G-protein coupled receptors is enhanced by agonist binding, whereas many nutrient receptors, such as the transferring and LDL receptors are

constitutively endocytosed regardless of ligand occupancy status [207-209]. The best-characterized pathway for receptor internalization is mediated by clathrin-coated vesicles. Often, clathrin-mediated internalization of mammalian GPCRs requires agonist stimulation and interaction with β-arrestin molecules. The β-arrestin proteins not only disrupt G-protein coupling with the receptor, but also serve as adaptors to link the receptor to the endocytic machinery [210, 211]. Clathrin-mediated endocytosis generally also requires the activity of dynamin GTPases for proper vesicle formation and cleavage from the plasma membrane [212].

A second pathway for receptor internalization is now emerging. Data now confirms that receptors are internalized via non-coated vesicles, such as the flask-shaped caveolae and other pinocytic mechanisms [207, 212]. Caveolin-mediated internalization also requires the activity of dynamin, but internalization mechanisms independent of dynamin have even been suggested [212, 213]. In fact, internalization of a GPCR by different endocytic pathways in single cell has even been demonstrated for the cholecystokinin (CCK) receptor [214]. Caveolin-mediated endocytosis involves receptor complex localization in a lipid raft domain enriched in caveolin proteins. These domains are termed caveolae. Internalization of receptors by the different pathways may be determined by the phosphorylation state of the receptor. The β1-adrenergic receptor has been demonstrated undergo agonist-stimulated internalization via caveolae when phosphorylated by protein kinase A and internalization by clathrin-coated pit endocytosis when phosphorylated by GRKs during the desensitization process [215]. The mechanism of internalization may also have an important effect on the endocytic sorting of the receptors. The receptor tyrosine kinase, TGF-β, is degraded when internalized by a

caveolae-mediated mechanism, but is recycled to promote receptor signaling when internalized by clathrin-coated pit endocytosis [216].

NHERF-1, PDZ domain-containing protein discussed previously, has been shown to associate with the epidermal growth factor receptor (EGFR), a receptor tyrosine kinases (RTK) to regulate receptor internalization. In this case, NHERF-1 association acts to stabilize the receptor at the plasma membrane and decrease the endocytosis rate of the receptor [217]. NHERF-1 has also been shown to attenuate the constitutive endocytosis of the PTH1-R by a mechanism of tethering the receptor to the actin cytoskeleton through protein-protein interactions [218]. Using an endogenous system, I have also shown in this project that NHERF-1 is essential to 'hold' the receptor-complex at the membrane, the absence of which leads to internalization of the receptor complex. These studies suggest a critical role for protein-protein interactions and NHERF-1 in regulation of receptor internalization and maintenance of receptor numbers at the plasma membrane.

While many GPCRs utilize similar mechanisms for endocytosis, the functional consequence of endocytosis varies from receptor to receptor. Internalized receptors that are trafficked through a rapid recycling pathway are restored to the plasma membrane in a functional state to achieve resensitization. On the other hand, receptors that are internalized and targeted to late endosomes and lysosomes experience proteolytic degradation, thus promoting attenuation of receptor signaling and down-regulation of the receptor.

2.3.4. Regulation of receptor recycling vs. degradation

After internalization, factors influencing the sorting of receptors in the early endosome are largely unknown, but some of the critical players are beginning to be identified for the GPCRs. Receptor ubiquitination, interaction of the receptor with PDZ domain-containing proteins, and/or interaction of the receptor with additional, newly-identified proteins has been shown to be required in some GPCR systems for efficient targeting of the GPCR for either degradation or recycling pathways [219-221].

Agonist-induced endocytosis is a means of regulating signaling for a multitude of membrane-bound receptors, particularly for G-protein coupled receptors. For some receptors in distinct cell backgrounds, internalization is a means of rapid recycling, characterized by dephosphorlyation and dissociation from agonist in intracellular vesicles for restoration at the plasma membrane in a functional state to achieve resensitization. On the other hand, some receptors that are internalized and targeted to late endosomes and lysosomes experience proteolytic degradation, thus promoting down-regulation of the receptor at the plasma membrane and attenuation of receptor signaling.

Much effort is currently being placed on the signal that sorts the internalized receptors for recycling or degradation. Ubiquitination of GPCRs has recently been shown to contribute to the sorting fate of the receptors for degradation. Ubiquitination is a process that involves the addition of multiple ubiquitin molecules on targeted lysine residues of a protein marked for degradation by an ubiquitinating enzyme complex. This addition of ubiquitin subunits to a particular protein acts as a degradation sorting signal for the degradation machinery [219, 222]. Much of the mechanism in the recognition of the degradation signal is unknown, but the mechanism for the addition of ubiquitin

molecules to targeted lysine residues on proteins has been determined. Ubiquitin addition to a protein is a result of the coordinated activity of three enzymes: the ubiquitin-activating enzyme (E1), ubiquitin-carrying enzyme (E2), and the ubiquitin ligase (E3). Marchese and Benovic have recently shown that ubiquitination of the chemokine receptor, CXCR4, by the ubiquitin E3 ligase, atrophin-interacting protein 4 (AIP4), is mediated by lysine residues in the C-terminus of the receptor. In this study, mutation of lysine residues to arginine on the C-terminus of the receptor inhibits ubiquitination and subsequent receptor degradation, but did not alter the internalization of the receptor upon agonist binding [219, 223]. Immunoprecipitation with an ubiquitin antibody also showed that the receptor was ubiquitinated, as well. In addition to the CXCR4 receptor, the β2-AR has been shown to be targeted for degradation upon ubiquitination of the receptor. Lefkowitz's group also showed that rapid ubiquitination of associated β-arrestin plays a role in the internalization of the ubiquitinated β2-AR. Mutations of lysine residues on the C-terminus of the receptor not only inhibited the degradation of the receptor, but also promoted the recycling of the receptor back to the plasma membrane after agonist-induced internalization [220].

It has been shown in several GPCR systems that interactions with PSD-95/Discs-large/ZO-1 homology (PDZ) domain containing proteins are responsible for the efficient targeting of the receptor after internalization [224-226]. This is a relatively new area of research in the GPCR field, but it is currently thought that the presence of a PDZ recognition sequence on the C-terminus of the receptor is the sorting signal for the interaction of the receptor with the PDZ domain-containing protein and targeting of the receptor for recycling to the plasma membrane from the early endosome. Recycling of

the β2-Adrenergic receptor (β2-AR) is dependent on the interaction of β2-AR, via its PDZ motif, with a protein called N-ethylmaleimide sensitive factor (NSF) [224, 225]. In addition, via the PDZ domain, PDZ domain-containing protein called Na+/H+ Exchange Regulatory Factor (NHERF) interaction with the κ-opioid receptor has recently been shown to increase the recycling efficiency of this particular GPCR [226]. It has also been shown that the δ-opioid receptor (DOR) is targeted for degradation following agonist-induced internalization, yet when a chimera of the DOR and the C-terminal PDZ motif from the β2-AR are treated with DOR agonist, the receptor is rapidly recycled to the plasma membrane after endocytosis [221, 227]. This suggests that the lack of the PDZ recognition motif in the C-terminus of the DOR inhibits binding of the DOR with proteins to target the receptor for recycling, and subsequently the receptor is degraded. Recent data suggests that the C-terminus of the DOR interacts with a novel protein, termed G-protein accessory sorting protein (GASP), to target the receptor for degradation [221], suggesting that sorting for degradation is not the default pathway for GPCRs, but is also a tightly regulated decision. While for most GPCRs the PDZ motif seems to predominantly be required for receptor recycling after agonist-induced endocytosis, the CXCR4 receptor requires an intact PDZ motif on the receptor to be targeted to the lysosomes for degradation. This PDZ recognition motif falls in the region found to be required for proper receptor ubiquitination for degradation. Disruption of this domain decreases the targeting of the receptor to the lysosomes from the early endosomes after internalization [219]. Clearly, more work is required to begin to understand the complex regulation of the sorting of GPCRs for the endosome after agonist-induced internalization.

As described, the fate of the GPCR is quite variable after agonist-induced internalization, with multiple GPCRs shown to both recycle and degrade, depending on the cell type and agonists interacting with the receptor. In the case of CL-R, the receptor fate differs, depending on the cell type where the receptor is expressed. It has been shown that CL-R/RAMP complex will degrade upon prolonged agonist exposure and receptor endocytosis in HEK 293 cells and Rat2 fibroblast cells, while in rat mesangial cells (RMCs) the receptor complex is effectively recycled after internalization [162, 198, 228]. The mechanism that regulates the pathway to which the receptor complex is targeted after agonist-induced internalization was previously unknown. Work from this project has proposed a model that specific RAMP isoform expression characterizes the cell to either recycle or degrade the AM receptor complex after prolonged agonist stimulation.

2.3.5. Protein-protein interaction domains/motifs

Protein-protein interactions are being shown routinely in to regulate numerous biological processes, tying together crucial partners in signaling complexes. This method of regulation has become commonplace the GPCR field. The number of protein-protein interaction domains is ever-growing and for the purpose of this literature review, I will focus on the most common interactions that have been shown to regulate GPCRs (i.e. SH2, SH3, EVH1, WW domains). I will concentrate, in particular, on the post-synaptic density 95, discs large, zonula occludens-1 (PDZ) domain interactions. This dissertation project focuses, in large part, on the role of PDZ interactions in the regulation of the life-cycle and trafficking of the adrenomedullin receptor.

Src-homology 2 (SH2) domains are modules of ~100 amino acids that bind to specific phosphor-tyrosine (pY)-containing peptide motifs. Conventional SH2 domains have a conserved pocket that recognizes pY, and a more variable pocket that binds 3-6 residues C-terminal to the pY, granting specificity. Phosphopeptides of optimal sequence bind to SH2 domains with dissociation constants of ~50-500 nM [229, 230]. The SH2 domain is embedded in a wide variety of metazoan proteins that regulate functionally diverse processes, and for this reason, SH2 domains must display sufficiently high off-rates for rapid and reversible signal transduction. SH2 domain interactions are responsible for the regulation of such varied processes as scaffolding, kinases, cytoskeletal regulation, phosphatases, phosphoinositide signaling, transcription, and ubiquitination, to name just a few [229]. Src plays an active role in the agonist-induced desensitization of beta2-adrenergic receptors via SH2 domain interactions. Src binds, via its SH2 domain, to the beta2-adrenergic receptor on an agonist-mediated phosphor-tyrosine residue, thereby allowing Src to phosphorylate and activate G-protein-coupled receptor kinase 2 (GRK2), a response obligate for agonist-induced desensitization [231].

Src-homology 3 (SH3) domains generally bind to Pro-rich peptides that form a left-handed helix, with the minimal consensus Pro-X-X-Pro. Each Pro is usually preceded by an aliphatic residue. Each of these aliphatic-Pro pairs binds to a hydrophobic pocket on the SH3 domain. From this, two classes of SH3 domains have been defined (Class I and Class 2) which recognize RKXXPXXP and PXXPXR motifs, respectively. The ligand can, in principle, bind in either orientation. Such peptides usually bind to the SH3 domain with a K_d in the mM range [232, 233]. Dopamine (D_4) receptor has recently been reported to bind SH3 domains of proteins, such as Grb2, at its proline-rich putative

third cytoplasmic loop. GPCRs have also been demonstrated to bind via internal SH3 domains, namely the P2Y(2) receptor, binds Src via an agonist-dependent SH3 domain interaction that facilitates Src activation, which recruits the EGFR into a protein complex with the P2Y(2) receptor and allows Src to efficiently phosphorylate the EGFR. The functional significance of these interactions is presently unknown [234].

WW domains are small 38 to 40 amino acid residue modules that have been implicated in binding to Pro-rich sequences. WW domains and SH3 domains can potentially bind overlapping sites, with both binding a left-handed poly-proline type II helix. In addition, the Pin1 WW domain functions as a phosphoserine- or phosphothreonine-binding module. In its function, the WW domain shares elements of SH3 and SH2 domains by recognizing proline-rich ligands and, in some cases, by being regulated by phosphorylation. The domain name is derived from two conserved Trp residues spaced 20 to 22 residues apart within the consensus sequence. The dissociation constants (K_d) for WW–ligand complexes lie in the high nM to low mM range for proline-rich ligands, and in the low mM range for phospho-SP- or phospho-TP-containing ligands [233, 235]. WW domains have attracted attention because the signaling complexes they mediate have been implicated directly or indirectly in several human diseases including muscular dystrophy, Alzheimer's and Huntington's diseases, and, more recently, cancer.

Drosophila enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) domains are 115 residue protein-protein interaction modules which provide essential links for their host proteins to various signal transduction pathways. Like Src homology 3, WW and GYF domains and profilin, EVH1 domains recognize and bind specific

proline-rich sequences [233]. The binding is of low affinity, but tightly regulated by the high specificity encoded into residues in the protein:peptide interface [236]. Many EVH1-containing proteins are associated closely with actin-based structures and are involved in re-organization of the actin cytoskeleton. EVH1 domains are also present in proteins enriched in neuronal tissue, thus implicating them as potential mediators of synaptic plasticity, linking them to memory formation and learning. For example, homer, an EVH1 domain protein forms an adaptor system that regulates coupling of group 1 metabotropic glutamate receptors with intracellular inositol trisphosphate receptors and is modified by neuronal activity [237].

The discovery of the PDZ domain was based on the recognition of sequence repeats in several proteins. The first initial of these three proteins (Postsynaptic density 95, Discs large, Zonula occludens-1) fashioned the name PDZ domain. PDZ domains are quite widespread throughout the metazoans, yet the several yeast species thus far sequenced show surprisingly few PDZ domains.

Classification of PDZ domains, based on their structure and their recognition peptide sequences, has been more difficult than originally thought. Bezprozvanny and Maximov proposed classifying PDZ domains into 25 groups, based on the nature of the residues in two positions, the αB1 position and the position immediately after the βB strand [238]. Instead, classification of PDZ domains has been focused primarily on their specificity for C-terminal peptides. Class I PDZ domains recognize a C-terminal sequence of X-S or T-X-V or L. Examples of proteins containing these interaction sites include the $NMDA_{2A,B}$ receptor, the β2-adrenergic receptor, δ-catenin. The PDZ domain-containing proteins in this class, sharing this interaction include PSD-95, Erbin, and

NHERF [239-241]. The second class of PDZ domains recognizes a C-terminal peptide with the sequence of X-ψ-X-ψ. Interacting proteins with Class II PDZ domains include neurexin, syndecan, and EphB2. Examples of members of the PDZ Class II domain-containing proteins are CASK, PICK1, and syntenin [242-245]. The final class of PDZ domains is Class III and classified by a X-D or E-X-ψ recognition sequence. The melatonin receptor interacts with the Class III PDZ domain-containing protein, nNOS [246].

In many cases, as we have observed, PDZ domain interactions are constitutive, with binding affinities in the 1 to 10 μM range. There are demonstrations for regulation of this interaction, as well. For example, the interaction of the β_2-adrenergic receptor with NHERF has been shown to be dependent on the receptor activation by agonist [240]. In addition, phosphorylation has been shown to regulate some PDZ domain interactions. For example, the interaction between the inwardly rectifying K^+ channel Kir 2.3 and PSD-95 is disrupted by PKA-dependent, serine phosphorylation of the channel at the -2 position [247]. Also, the β_2-adrenergic receptor, when serine-phosphorylated at its -2 position by GRK5, is unable to interact with NHERF [224].

Solving the structural basis of the PDZ domain interaction was critical in determining the specificity with which the PDZ domain-containing proteins bound their ligands. PDZ domains are comprised of 80 to 90 amino acids that make up six β strands (βA-βF) and two α-helices (αA and αB), arranged in a compact globular structure. Several PDZ domains, both complexed with their ligands and ligand-free, have been solved for their three-dimensional structure to clarify the mechanism of the PDZ domain interactions. In short, the binding of the peptide ligand to the PDZ domain takes place in

an elongated surface groove as an antiparallel β strand interacts with the βB strand and the αB helix [248]. PDZ domains have been shown in interact with their peptides in four different types of interactions: recognition of C-terminal motifs in peptides, recognition of internal motifs in peptide binding partners, PDZ-PDZ interactions, and recognition of lipids [249].

PDZ domains were originally thought to simply scaffold signaling complexes and/or receptors at the cell surface. It has become evident that PDZ domain interactions regulate a multitude of functions in cells of many types. A few examples to be discussed briefly in this review are: adaptors for tyrosine kinase receptors, epithelial polarity, and mediators of protein networks. PDZ interactions of Na+/H+ Exchanger Regulatory Factor (NHERF) have been shown to regulate the clustering and autophosphorylation of the platelet-derived growth factor (PDGF) receptor. More potent receptor autophosphorylation is seen when the receptor is complexed with NHERF, leading to a more robust activation of MAPK signaling [250]. In terms of epithelial polarity, NHERF interactions also determine the expression of the Na+/H+ Exchanger-3 (NHE3) in the polarized proximal tubule cells and tissue. NHE3 is an antiporter responsible for the majority of the Na+ reabsorption in the proximal tubule, with the Na+/H+ exchange dependent on proper NHERF interactions to localize the transporter on the correct side of the epithelial cell for Na+ reabsorption [251]. Protein networks maintained by PDZ domain interactions are demonstrated many times over at the neural synapse. For example, through PDZ interactions, the NMDA receptor is tied to the Ca^{2+}-ATPase channel, the kainate receptor, the Shaker K+ channel, and the metabotropic glutamate receptor. To portray the complexity of these interactions, in the literature the

postsynaptic NMDA receptor has been reported to have 17 primary interactions leading to 385 interactions with secondary proteins [249] . A major challenge for the future will be to build models of multiprotein networks and explain the complexity and regulation of these "multiprotein machines".

2.3.6. Na+/H+ Exchanger Regulatory Factor-1 (NHERF-1)

NHERF-1 was initially identified as a regulator of the Na+/H+ exchanger (NHE), mediating the activity of the transporter through cAMP-induced inhibition of NHE3. Through the identification of numerous targets of NHERF-1, some membrane-bound receptors and transporters, the role for this protein has been expanded to that of a scaffolding protein in membrane physiology, as well. NHERF-1 has been characterized as a 55 KDa protein containing two tandem PDZ domains, type I and type II domains, respectively [251]. NHERF-1 has also been shown to associate with members of the ezrin/radixin/moesin family of actin-binding proteins, and thus has the ability to link PDZ domain-interacting proteins to the actin cytoskeleton [252]. The two PDZ domains and MERM domain also allow NHERF to facilitate the formation of multiprotein signaling complexes. For example, NHERF-1 is known to bind NHE3 and the PTH1-receptor, and through interactions with cytoskeletal proteins ties NHE3 to an activated PKA that phosphorylates and inactivates NHE3. Disruption of any of the previous interactions will abate NHE3 inactivation and allow improper Na+/H+ exchange [253].

Yeast two-hybrid experiments identified an additional protein that bound the C-terminal region of NHE3. This protein shared structural homology with NHERF-1, and was thus termed NHE3 kinase A-regulated protein, or NHERF-2. Differential expression

of NHERF-1 and -2 has been observed throughout the kidney. NHERF-1 was detected in the proximal tubules, while NHERF-2 was detected in the glomeruli, peritubular capillaries, collecting duct principal cells, and low levels in the proximal tubules [254]. The differential expression suggests distinct physiological roles for each protein in the kidney. NHERF expression has been detected in tracheal, pancreatic, intestinal, and kidney epithelia.

Determined by immunofluorescence microscopy, MERM cytoskeletal proteins are typically localized at the apical membranes of polarized cells. Because of their interactions with MERM proteins, both NHERF-1 and -2 are localized primarily in the apical membranes of kidney cells [255]. This apical localization is critical for the scaffolding of signaling complexes to function properly in a physiological setting. For example, changes in the cystic fibrosis transmembrane regulator (CFTR) trafficking are seen in the majority (~90%) of humans with cystic fibrosis. Mutants of CFTR lacking the C-terminal PDZ interaction motif are unable to interact with NHERF and therefore show altered trafficking of the mutant CFTR to basolateral membranes, in contrast to the wild-type CFTR expression at apical membranes [256]. Studies have implicated the interaction of CFTR with NHERF-1 with the correct apical localization and proper intracellular trafficking of CFTR, and lack of NHERF-1 interaction with contributing to the disease process.

Several studies have shown NHERF-1 to play differing roles in various cellular processes, including receptor trafficking [257, 258]. NHERF-1 has been demonstrated to *bind* the extreme carboxy-terminus of several G-protein coupled receptors (GPCRs), namely the β_2-adrenergic receptor, the κ-opioid receptor, PTH-R, and the P2Y purinergic

receptor [154, 259]. Agonist exposure promotes NHERF-1 association with the β_2-adrenergic and the κ-opioid receptors. NHERF-1 association with these receptors enhances the recycling of the receptors after agonist stimulation [240, 259]. In addition to GPCRs, NHERF-1 has also been shown to associate with the epidermal growth factor receptor (EGFR), a receptor tyrosine kinases (RTK). In this case, NHERF-1 association acts to stabilize the receptor at the plasma membrane and decrease the endocytosis rate of the receptor [217]. While utilizing different mechanisms, collectively these data suggest that NHERF-1 interaction with both RTKs and G-protein-coupled receptors is mandatory to enhance the portion of receptors present at the cell surface.

2.3.7. N-ethylmaleimide Sensitive Factor (NSF)

NSF is a 76 KDa protein with functions in vesicle exocytosis and membrane fusion events. NSF is composed of three domains: an N-terminal NSF-N domain followed by two ATP-binding domains, NSF-D1 and NSF-D2. The N-terminal NSF-N domain is critical for binding to the SNAP-SNARE complex during vesicle fusion and exocytosis. Contained in the two ATP-binding domains is a 230-250 amino acid motif that is characteristic of the ATPases Associated with various cellular Activities (AAA) family of ATPases, and the orientation of this domain classifies NSF as a class II AAA protein [260]. NSF forms a homo-hexamer and requires binding of ATP for its active conformation.

NSF is commonly known to interact with αSNAP (soluble NSF attachment protein) and SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins to form the 20S particle, a complex that plays a critical role in

intracellular membrane fusion and exocytosis [261-263]. Monomeric α-SNAP binds to NSF only upon binding SNAREs. The C-terminal 45 amino acids of α-SNAP are required for binding and for stimulation of NSF's ATPase activity [264-266]. The ATPase activity of NSF is responsible for the dissociation of α-SNAP and the SNAREs to complete the fusion of a vesicle with the cell membrane.

Factors influencing the sorting of receptors in the early endosomes are largely unknown, but interactions with PSD-95/Discs-large/ZO-1 homology (PDZ) domain proteins are being suggested to regulate the receptor-targeting after internalization [259, 267, 268]. The life cycle of the β2-adrenergic receptor (β2-AR) was reported to be altered in the presence of NSF [268]. It has been shown that the β2AR interacts with NSF via a PDZ type I recognition motif (-DSLL) at its extreme C-terminus. Binding of the β2AR with NSF enhances the recycling of the β2AR after agonist-stimulated receptor internalization. In addition, binding of NSF to the Glu2 subunits of the AMPA receptor was demonstrated to be crucial for the recycling of the α-amino-3-hydroxy-5-methyl-isoxazolepropionate (AMPA) receptor [269, 270]. NSF binds the $GluR_{2C}$ subunit, but binding is enhanced by binding of another PDZ domain protein, PICK1. PICK1, $GluR_{2C}$, and NSF form a tripartite complex that also binds α-SNAP to increase the disassembly of the complex upon vesicle fusion with the membrane [271]. NSF plays a chaperoning role for SNAREs in the majority of membrane fusion events in a cell, but when targeting membrane receptors for recycling, NSF acts independently of the SNARE complex to promote rapid resensitization of the receptors at the plasma membrane [261-263]. A report, published from our laboratory recently, demonstrated a role for NSF in the sorting of the AM2 receptor at the endosome for a recycling pathway. In this case, NSF's

interaction with an accessory protein (RAMP3) directs the trafficking of a receptor from the sorting endosome [153].

NSF has been shown to interact with another protein critical to receptor trafficking, the β-arrestin molecule. β-arrestin is responsible for two steps in the desensitization process for many GPCRs. First, the β-arrestin binds the receptor, upon receptor phosphorylation, and disrupts further signaling by the G-protein. Second, the β-arrestin molecule interacts with proteins to target many receptors for clathrin-mediated endocytosis [272, 273] . β-arrestin binding to NSF appears to play a role in the turnover of the receptor at the plasma membrane. Overexpression of NSF causes an increase in the receptor clearance from the plasma membrane after agonist-stimulation of the β2-adrenergic receptor [274].

At the present time, there is no obvious sequence that could be described as the NSF binding motif. It will be important to determine, through mutational analysis, the domains on NSF important for these protein-protein interactions.

3. Characterization of AM-stimulated desensitization response in transfected HEK 293 cells

3.1. Introduction

The recent discovery of receptor activity modifying proteins (RAMPs) new alternatives in the regulation of G-protein coupled receptors (GPCRs). RAMPs are characterized as single transmembrane-spanning accessory proteins required for the function of an orphan GPCR, now termed the calcitonin receptor-like receptor (CL-R)[275]. Through structural homology, three RAMP isoforms (1-3) have been identified and are synthesized as distinct gene products. RAMPs are required for the cell surface expression, as well as for ligand selectivity of CL-R [275, 276]. A heterodimer of RAMP1 and CL-R yields a calcitonin gene-related peptide-1 (CGRP-1) receptor, whereas RAMP2 or -3 coexpressed with CL-R produces adrenomedullin receptors, AM1 and AM2 receptors, respectively [277, 278]. Both AM and CGRP are multi-functional peptides with many overlapping functions, ranging from potent vasodilation to proliferation regulation to regulation of salt and water balance [279]. It has been suggested in the literature that differential expression of RAMP isoforms regulates both physiological and pathological states.

Upon agonist binding, the CL-R/RAMP receptor complex causes cyclic AMP activation in most systems, regardless of whether the ligand is AM or CGRP. In addition, the receptor complex is phosphorylated by protein kinases and subsequently undergoes desensitization and internalization in response to a prolonged agonist stimulation [280]. Internalization of all three RAMP isoforms with CL-R has been reported to be clathrin-mediated and dynamin-dependent. In addition, the CL-R/RAMP1 receptor complex is

known to internalize with agonist pretreatment in a complex with the β-arrestin molecule and all three components colocalize to the endosomes. From this stage, the receptor complex is targeted for a recycling or degradation pathway, depending on the cell type and RAMP expression profile.

The findings in this study characterized the AM-stimulated cAMP accumulation and desensitization of the two AM receptor subtypes in response to AM treatment. These experiments represent pilot studies performed to determine agonist doses and time points for remaining studies in this thesis.

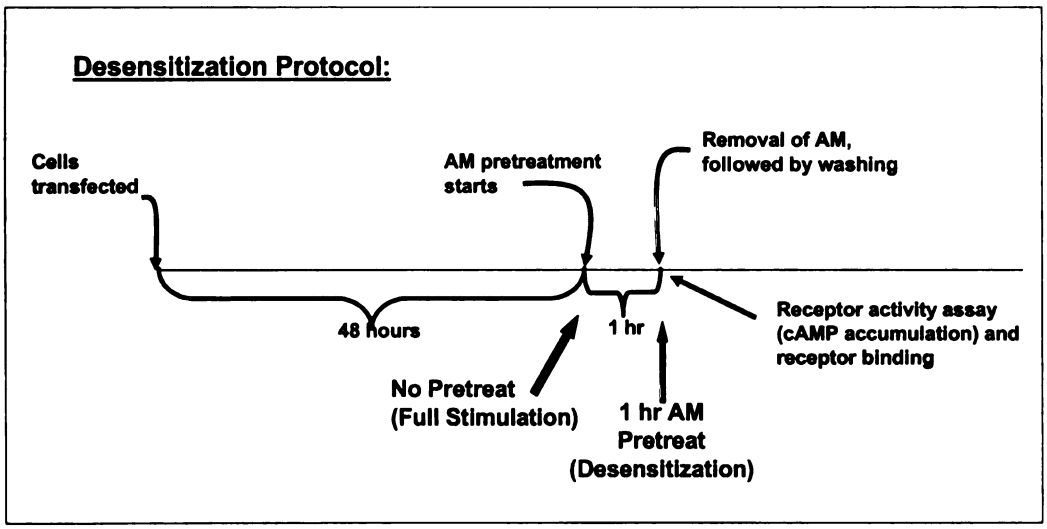
3.2. Materials and Methods

3.2.1. Materials: Adrenomedullin was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). ^{125}I-labeled adrenomedullin was purchased from Amersham Biosciences Corp. (Piscataway, NY). Cell culture media, fetal bovine serum, penicillin/streptomycin, trypsin-EDTA were purchased from GibcoBRL® (Grand Island, NY). All other reagents were of highest quality available.

3.2.2. Cell Culture and Transfection protocols: HEK-293T cells (obtained from ATCC) are maintained in DMEM low glucose media containing 10% FBS, 1% penicillin-streptomycin. Transfection of HEK293T was performed using Lipofectamine Plus protocol (Invitrogen Life Technologies, Carlsbad, CA). Cells were transfected with the DNA and Lipofectamine Plus as per manufacturer's protocol. Cells were collected for assays after 48 hours of transfection.

3.2.3. RAMP cloning and expression: Full length cDNA of human RAMPs1, 2 and 3 and bovine CL-R were described before [281, 282]. CL-R, cloned into N1-EGFP and also in pcDNA3.1 expression vectors, was used for transfection in HEK 293T cells.

3.2.4. Desensitization assays: 48 hours post-tranfection cells were pretreated with or without 10 nM AM in DMEM containing 0.2% BSA for indicated time periods (up to 4 hr). After agonist exposure, cells were washed three times with Dulbecco's phosphate buffered saline (dPBS, Gibco BRL) containing 0.2% BSA and either frozen for membrane preparation for adenylate cyclase assays or used immediately for intact-cell radioligand binding.



3.2.5. Receptor binding: Homologous competition radioligand binding assays were performed as described by Aiyar et al and as established in our laboratory [283]. HEK 293T cells were transfected and approximately 200,000 cells/well seeded in poly-D-

lysine precoated 24-well plates (BD Biosciences, Palo Alto, CA). 48 hours post-tranfection cells were treated for desensitization or resensitization assays as described above. After agonist exposure, cells were washed three times with dPBS buffer containing 0.2% BSA then incubated with increasing concentrations (1 pM to 100 nM) of competing ligand (rAM) and 175-250 pM ^{125}I-rAM for 30 min at 37°C. After incubation, plates were washed three times with ice-cold assay buffer and the reactions were terminated by the addition of 2M NaOH. Cells were then harvested and associated radioligand activity is counted on a γ-counter. All binding assays were performed in duplicate, with each experiment repeated at least three times. Nonspecific binding was determined in the presence of 100 nM of unlabeled rAM. Data was analyzed by LIGAND (assuming radioligand and competitor both bind reversibly to a single binding site; MacLigand, Version 4.97, NIH, Bethesda, MD) using the following equation:

$$B = \frac{B_{max} \bullet [\text{hot ligand}]}{K_D + [\text{hot ligand}] + [\text{cold ligand}]}$$

Where B represents specific binding, [hot ligand] the single concentration of [^{125}I]rAM studied, [cold ligand] the concentration of unlabeled rAM competing with the radiolabel for AM receptor binding, B_{max} the maximum number of binding sites and KD the equilibrium dissociation constant (the equation was solved where the "cold ligand" IC_{50} = [hot ligand] + K_D). Analysis of all binding data was performed by computer-assisted nonlinear least square fitting using GraphPad PRIZM version 4 (GraphPad Software, San Diego, CA). Binding sites/cell was calculated and data was expressed as percent of the control.

3.2.6. Adenylate cyclase assays: Cyclase activity was done as described before with slight modifications [146, 284]. Cells were harvested from P100 or P60 plates and homogenized in Tris HCl (10mM)/EDTA (10mM) buffer. Membranes were prepared by homogenization and centrifugation in Tris HCl (50mM)/MgCl (10mM) buffer. Final concentration of 20 μg of protein/assay tube was obtained. Membranes were incubated for 15 min at 30°C with appropriate concentrations of drugs (100nM AM, 10μM Forskolin) and assay mix containing ATP regeneration system and α^{32}P-ATP. After the reaction was stopped (with stop solution containing ^{3}H-cAMP) contents of the assay tubes were passed through Dowex and subsequently through alumina columns to separate the degradation products of ATP, by washing the dowex with water and alumina with imidazole. Elution profile was done to determine the amount of water and imidazole needed to wash and elute the products. Product eluted from alumina column was counted for the presence of ^{3}H-cAMP and α^{32}P-cAMP in a β-counter. Each experiment was done in triplicates and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. Basal cyclase activity and forskolin stimulation did not show statistically significant differences between treatments.

3.2.7. cAMP accumulation assays: HEK 293 cells were seeded on a 24-well plate until reaching 80-90% confluency, then incubated in serum-free media overnight before experiment. Desensitization experiments were carried out as described in Materials and Methods section, with cells pretreated with 10nM rAM and subsequently challenged for 10 min at 37°C with appropriate concentrations of drugs (100nM AM, 10μM Forskolin) in the presence of 200μM 3-isobutyl-1-methylxanthine. Determination of cAMP level

was measured using the biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. Basal cyclase activity and forskolin stimulation did not show statistically significant differences between treatments.

3.2.8. Statistics: Data are presented as mean ± S.E.M. Single group comparisons exercised a paired Student's t-test method. Statistical significance was set at $P<0.05$.

3.3. Results/Discussion

We and other groups have previously shown that the AM1 receptor (CL-R + RAMP2) and AM2 receptor (CL-R + RAMP3 complex) undergo agonist-stimulated desensitization, internalization, and degradation in HEK 293 cells [153, 276, 285]. An initial 100 nM AM challenge to HEK 293 cells expressing CL-R and RAMP2 or RAMP3 results in an approximate 9 and 8-fold increase, respectively, in cAMP accumulation over basal. Pretreatment of HEK 293 cells expressing CL-R and RAMP2 or RAMP3 with 10 nM AM for one hour and subsequent challenge with 100 nM AM resulted in desensitization of the cAMP accumulation response. AM pretreatment did not significantly alter the basal or forskolin-stimulated (10 μM) cAMP accumulation (Figure 2A, B). Because AM pretreatment was not shown to alter the basal or FK-stimulated cAMP accumulation, the remaining cAMP accumulation and adenylate cyclase activity data throughout the thesis will be expressed as percent maximal response, or percent

forskolin stimulation. Dose response experiments experiments for receptor desensitization indicate that maximal desensitization is observed with a 10 nM AM pretreat and 100 nM AM challenge (Figure 3A, B). These will be the AM doses used for pretreatment and challenge in the desensitization and resensitization assays employed throughout the remaining chapters of the thesis. The time course for desensitization shows a maximal attenuation of cAMP accumulation between one and two hours of agonist pretreatment (Figure 4A, B), and for this reason a one hour agonist pretreatment is used throughout the remaining thesis chapters to measure receptor desensitization.

Homologous competitive binding experiments were optimized by altering ligand and competing ligand concentrations, temperature, and duration of binding experiments. Figure 5 depicts a representative competition curve for the whole-cell, homologous competitive binding experiments employed throughout this thesis. Receptor binding sites per cell were determined from scatchard plots, as described in Materials and Methods section. A representative scatchard plot is shown in Figure 6. Table V shows receptor affinity and receptor density calculations for HEK 293 cells expressing CL-R and RAMP3 with NSF or NHERF-1 expression. This table depicts a representative experiment and indicates no change in receptor affinity with agonist pretreatment or with expression of additional proteins (NSF or NHERF-1). In addition, co-expression of NSF or NHERF-1 with CL-R/RAMP3 does not significantly alter receptor density in the HEK 293 cells. For the remaining homologous competitive binding experiments, these calculations will be followed and data expressed as percent of control.

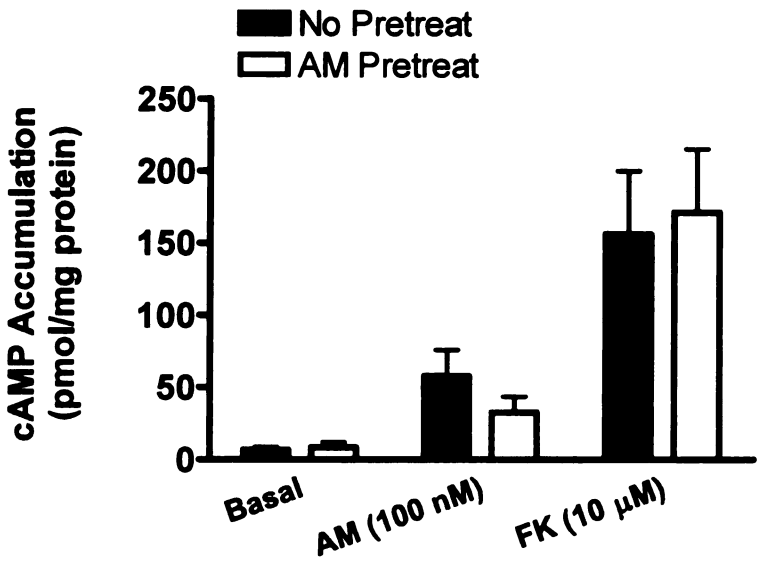


Figure 2A: Representative desensitization experiment of AM1 receptor in HEK 293 cells, measured by cAMP accumulation. HEK 293 cells were transiently transfected with CL-R and RAMP2 and pretreated with 10 nM AM for one hour. After agonist pretreatments, cells were washed repeatedly, and cAMP accumulation in response to 100 nM AM challenge was measured. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. cAMP accumulation is shown on the left y-axis, raw data shown. Representative experiment is shown in figure.

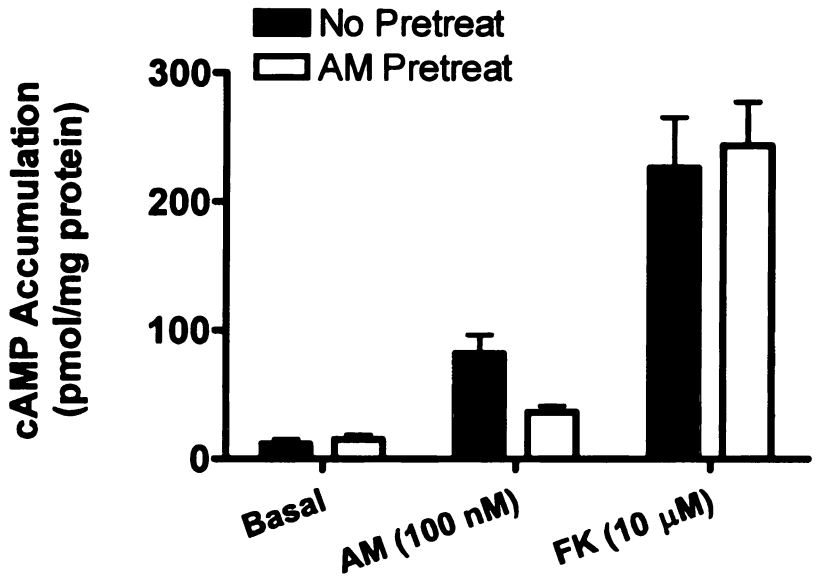


Figure 2B: Representative desensitization experiment of AM2 receptor in HEK 293 cells, measured by cAMP accumulation. HEK 293 cells were transiently transfected with CL-R and RAMP3 and pretreated with 10 nM AM for one hour. After agonist pretreatments, cells were washed repeatedly, and cAMP accumulation in response to 100 nM AM challenge was measured. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. cAMP accumulation is shown on the left y-axis, raw data shown. Representative experiment is shown in figure.

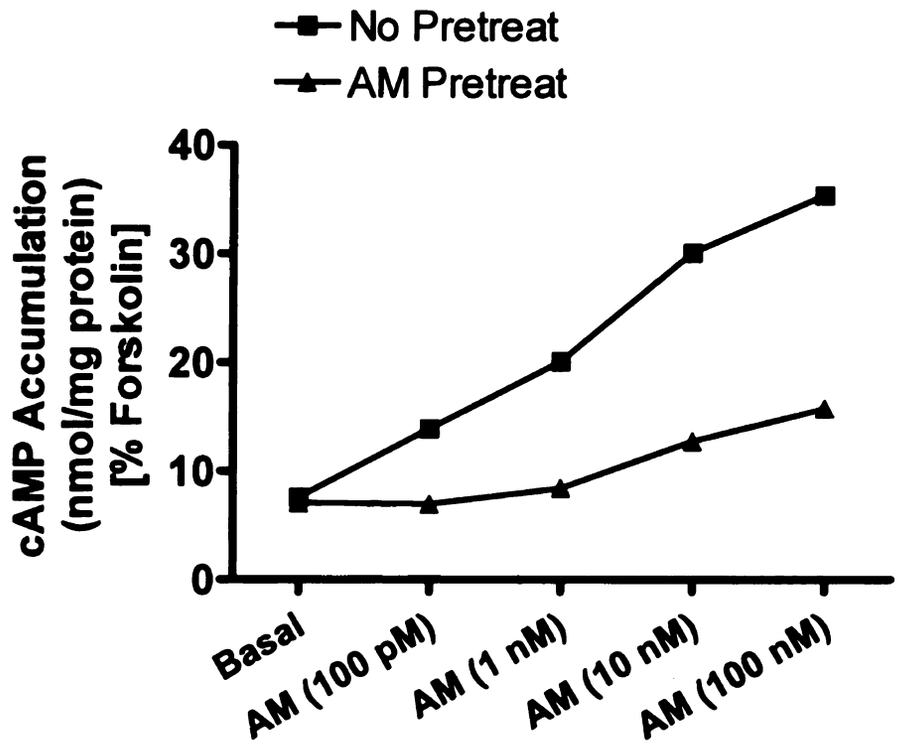


Figure 3A: Desensitization dose response of AM1 receptor in HEK 293 cells. CL-R/RAMP2 complex (AM1 receptor) desensitizes in HEK 293 cells after prolonged agonist stimulation. HEK 293 cells transiently transfected with CL-R and RAMP2 were treated for one hour with AM (10 nM), then washed repeatedly, and after repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. cAMP accumulation is shown on the left y-axis, expressed as percent maximal response (% forskolin stimulation). Representative experiment shown in figure.

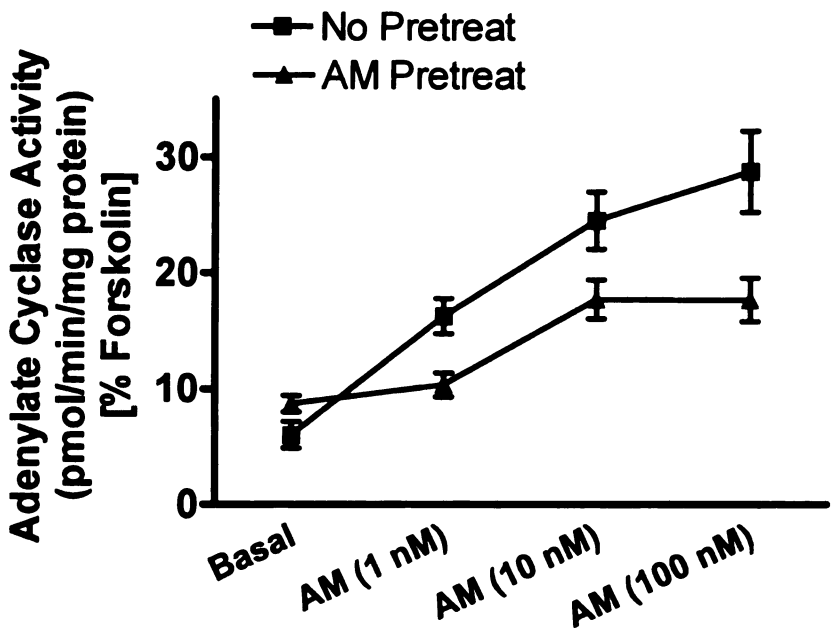


Figure 3B: Desensitization dose response of AM2 receptor in HEK 293 cells. CL-R/RAMP3 complex (AM2 receptor) desensitizes in HEK 293 cells after prolonged agonist stimulation. HEK 293 cells transiently transfected with CL-R and RAMP3 were treated for one hour with AM (10 nM) and then washed and receptor desensitization (adenylate cyclase activity) was measured. Adenylate cyclase activity is shown on the left y-axis, expressed as percent maximal response (% forskolin stimulation). $n \geq 3$ experiments.

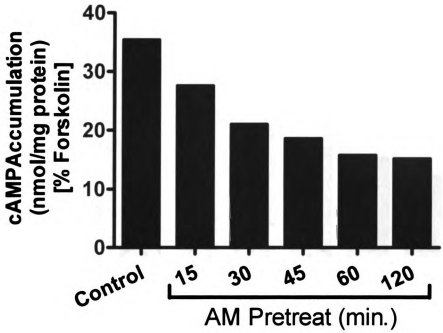


Figure 4A: Desensitization time course for AM1 receptor in HEK 293 cells. CL-R/RAMP2 complex (AM1 receptor) desensitizes in a time-dependent manner in HEK 293 cells after agonist stimulation. HEK 293 cells transiently transfected with CL-R and RAMP2 were treated for one hour with AM (10 nM), then washed repeatedly, and after repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. cAMP accumulation is shown on the left y-axis, expressed as percent control (no pretreatment) sample. Representative experiment shown in figure.

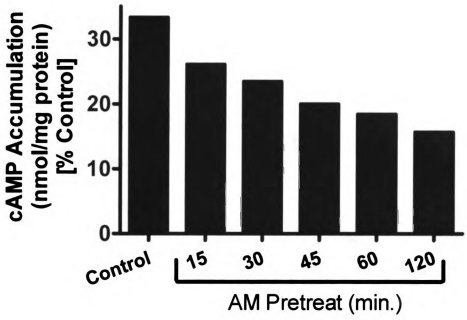


Figure 4B: Desensitization time course for AM2 receptor in HEK 293 cells. CL-R/RAMP3 complex (AM2 receptor) desensitizes in a time-dependent manner in HEK 293 cells after agonist stimulation. HEK 293 cells transiently transfected with CL-R and RAMP3 were treated for one hour with AM (10 nM), then washed repeatedly, and after repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. cAMP accumulation is shown on the left y-axis, expressed as percent control (no pretreatment) sample. Representative experiment shown in figure.

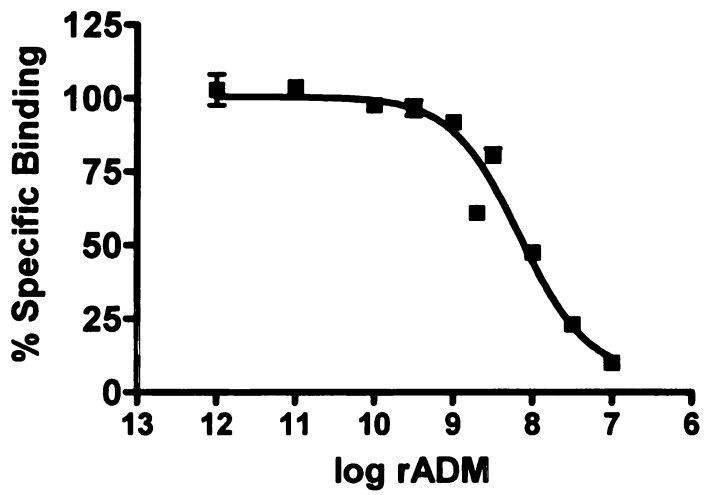


Figure 5: Competition curves for rAM on intact HEK-293T cells expressing CL-R and RAMP3 with [^{125}I] rAM as radioligand were generated as described in Materials and Methods section. Representative experiment for homologous competition binding employed throughout remaining thesis chapters.

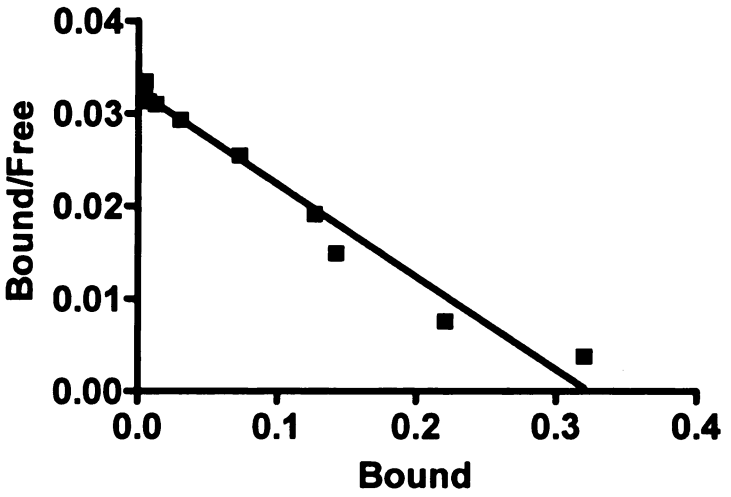


Figure 6: Scatchard plot for homologous competition binding of rAM on intact HEK-293T cells expressing CL-R and RAMP3, with [^{125}I] rAM as radioligand, generated as described in Materials and Methods section. Representative experiment for homologous competition binding employed throughout remaining thesis chapters.

Experiment:	Kd (nM)	Ki (nM)	Bmax (pmol/assay)	cells/well	Binding sites/cell	% control
CL-R+RAMP3						
Control	4.00	2.66	0.122	200000	368123	100.00
1 hr Pretreat	3.44	2.29	0.078	231500	203848	55.37
4 hr Recovery	3.73	2.49	0.071	246800	173477	47.12
CL-R+RAMP3+NSF						
Control	3.44	2.29	0.093	209300	267894	100.00
1 hr Pretreat	5.35	3.57	0.031	189400	97324	36.33
4 hr Recovery	4.45	2.97	0.076	177200	259451	96.85
CL-R+RAMP3+NHERF1						
Control	3.80	2.62	0.129	230000	336335	100.00
1 hr Pretreat	2.44	1.68	0.138	220000	363389	108.04
4 hr Recovery	2.76	1.90	0.108	200000	323876	96.30

Table V: Binding properties for homologous competition binding of rAM on intact HEK-293T cells expressing CL-R and RAMP3 +/- NSF or NHERF1, with [^{125}I] rAM as radioligand, generated as described in Materials and Methods section. No statistically significant changes in receptor affinity or receptor density observed in homologous competitive binding experiments. Representative experiment for homologous competition binding to determine binding sites per cell, employed throughout remaining thesis chapters.

4. Differential regulation of adrenomedullin receptor desensitization and internalization by receptor activity-modifying proteins.

4.1. Introduction

The recent discovery of receptor activity modifying proteins (RAMPs) has raised new possibilities for modes of regulation of G-protein coupled receptors (GPCRs). RAMPs are characterized as single transmembrane-spanning accessory proteins requisite to the function of an orphan GPCR, now termed the calcitonin receptor-like receptor (CL-R)[275]. Through structural homology, three RAMP isoforms (1-3) have been identified as distinct gene products. RAMPs are required for the cell surface expression, as well as for ligand selectivity of CL-R [275, 276]. A heterodimer of RAMP1 and CL-R yields a calcitonin gene-related peptide-1 (CGRP-1) receptor, whereas coexpression of RAMP2 or -3 with CL-R produces adrenomedullin receptors, AM1 and AM2 receptors, respectively [277, 278]. Both AM and CGRP are multi-functional peptides with many overlapping functions, ranging from potent vasodilation to proliferation regulation to regulation of natriuresis and diuresis [279]. It has been suggested in the literature that differential expression of RAMP isoforms plays a regulatory role in both physiological and pathophysiological disease states. Moreover, the recent identification of RAMP interactions with additional members of the Class II GPCR family and RAMP expression in cell lines lacking CL-R have raised the possibility of additional functions for RAMPs in GPCR regulation [286]. In addition, we have recently reported novel roles for RAMPs, through protein-protein interactions with NSF and NHERF, in the trafficking of the AM receptor subtypes.

Upon agonist binding, the CL-R/RAMP receptor complex causes cyclic AMP activation in most systems, regardless of whether the ligand is AM or CGRP. In addition, the receptor complex is phosphorylated by protein kinases and subsequently undergoes desensitization and internalization in response to a prolonged agonist stimulation [280]. Internalization of all three RAMP isoforms with CL-R has been reported to be clathrin-mediated and dynamin-dependent. In addition, the CL-R/RAMP1 receptor complex is known to internalize with agonist pretreatment in a complex with the β-arrestin molecule and all three components colocalize to the endosomes. From this stage, the receptor complex is targeted for a recycling or degradation pathway, depending on the cell type and RAMP expression profile.

Phosphorylation has been demonstrated to be a requisite step in the desensitization process of the majority of GPCRs. The phosphorylation of the receptor, upon agonist exposure, allows interaction of the receptor with the β-arrestin molecules to promote an attenuation of receptor signaling, or receptor desensitization. Receptor desensitization can occur by two mechanisms, termed homologous and heterologous desensitization. Homologous desensitization is classified as desensitization specific for the agonist-occupied receptor. This occurs as a result of G-protein coupled receptor kinase (GRK) specificity for only agonist-occupied receptors. On the other hand, heterologous desensitization occurs by activation of second messenger kinases, PKA and PKC for example, and has no specificity for agonist-occupied receptors. Heterologous desensitization raises the possibility of "cross-desensitization" for unoccupied receptors.

Upon ligand binding, it has been reported that CL-R undergoes phosphorylation, while the RAMP partner remains unphosphorylated. Moreover, PKA has been shown to

regulate the phosphorylation and desensitization of the CL-R/RAMP1 receptor complex in SK-N-MC and vascular smooth muscle cells, while GRK-6 was reported to phosphorylate and promote desensitization of the CL-R/RAMP1 complex in HEK 293 cells [200-202]. Kinases responsible for the phosphorylation of the CL-R/RAMP2 or -3 receptor complexes have yet to be specifically characterized.

The findings in this study reveal that different RAMP isoform association with CL-R may lead to differential phosphorylation of the receptor complex. The Ser 421 residue (a putative PKA phosphorylation site) on CL-R was found to be critical for the desensitization and internalization of the CL-R/RAMP2 receptor complex, whereas the Thr 423 residue (a putative PKA phosphorylation site) on the C-terminus of CL-R was found to be crucial for the CL-R/RAMP3 receptor desensitization and internalization. CL-R-R2 complex desensitization and internalization was blocked by H89, a PKA inhibitor, while CL-R-R3 complex desensitization and internalization was blocked by RO 32-0432, a PKC inhibitor. Taken together, these results suggest that in addition to the various roles of RAMPs in the regulation of CL-R/RAMP complex as reported by us and others, the isoform of RAMPs could also be involved in the differential phosphorylation of CL-R.

4.2. Materials and Methods

4.2.1. Materials: Adrenomedullin was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). 125I-labeled adrenomedullin was purchased from Amersham Biosciences Corp. (Piscataway, NY). Cell culture media, fetal bovine serum,

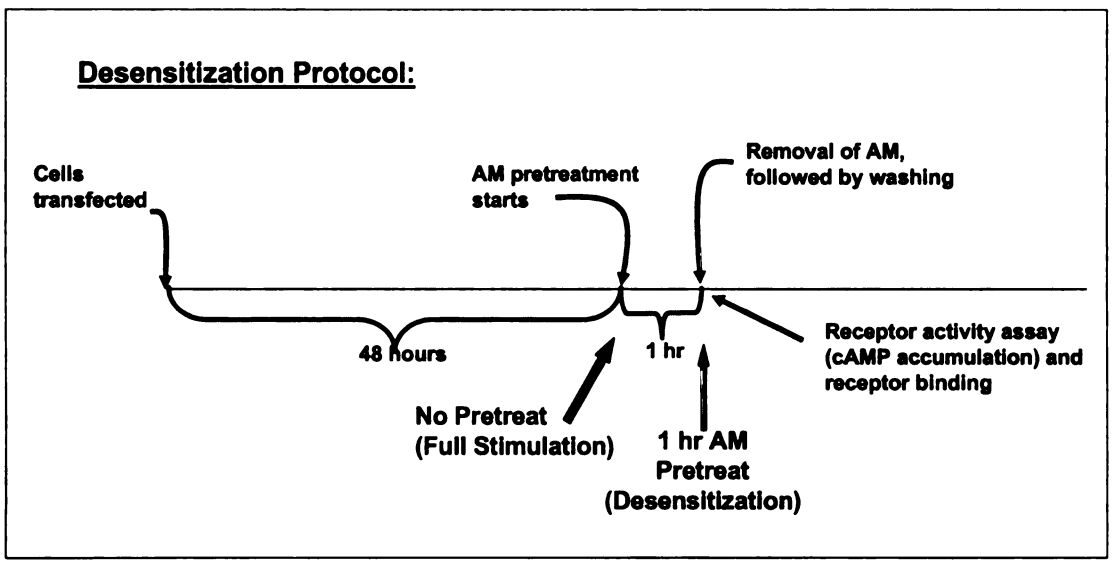
penicillin/streptomycin, trypsin-EDTA were purchased from GibcoBRL® (Grand Island, NY). RAMP3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit Cy3 secondary antibody was from Jackson Immunoresearch Laboratories (West Grove, PA). PKA (H-89) and PKC (Ro 32-0432) inhibitors were purchased from Sigma (St. Louis, MO). All other reagents were of highest quality available.

4.2.2. Cell Culture and Transfection protocols: HEK-293T cells (obtained from ATCC) are maintained in DMEM low glucose media containing 10% FBS, 1% penicillin-streptomycin. Rat-2 fibroblast cells (obtained from ATCC) are maintained in DMEM high glucose media containing 10% FBS, 1% penicillin-streptomycin. Transfection of HEK293T and Rat-2 fibroblast cells was performed using Lipofectamine Plus protocol (Invitrogen Life Technologies, Carlsbad, CA). Cells were transfected with the DNA and Lipofectamine Plus as per manufacturer's protocol. Cells were collected for assays after 48 hours of transfection.

4.2.3. RAMP cloning and expression: Full length cDNA of human RAMPs1, 2 and 3 and bovine CL-R were described before [281, 282]. CL-R, cloned into N1-EGFP and also in pcDNA3.1 expression vectors, was used for transfection in HEK 293T cells.

4.2.4. Desensitization assays: 48 hours post-tranfection cells were pretreated with or without 10 nM AM in DMEM containing 0.2% BSA for indicated time periods (up to 4

hr). After agonist exposure, cells were washed three times with Dulbecco's phosphate buffered saline (dPBS, Gibco BRL) containing 0.2% BSA and either frozen for membrane preparation for adenylate cyclase assays or used immediately for intact-cell radioligand binding.



4.2.5. Receptor binding: Homologous competition radioligand binding assays were performed as described by Aiyar et al and as established in our laboratory [283]. HEK 293T cells were transfected and approximately 200,000 cells/well seeded in poly-D-lysine precoated 24-well plates (BD Biosciences, Palo Alto, CA). 48 hours post-tranfection cells were treated for desensitization or resensitization assays as described above. After agonist exposure, cells were washed three times with dPBS buffer containing 0.2% BSA then incubated with increasing concentrations (1 pM to 100 nM) of competing ligand (rAM) and 175-250 pM ^{125}I-rAM for 30 min at 37°C. After incubation, plates were washed three times with ice-cold assay buffer and the reactions were

terminated by the addition of 2M NaOH. Cells were then harvested and associated radioligand activity is counted on a γ-counter. All binding assays were performed in duplicate, with each experiment repeated at least three times. Nonspecific binding was determined in the presence of 100 nM of unlabeled rAM. Data was analyzed by LIGAND (assuming radioligand and competitor both bind reversibly to a single binding site; MacLigand, Version 4.97, NIH, Bethesda, MD) using the following equation:

$$B = \frac{B_{max} \bullet [\text{hot ligand}]}{K_D + [\text{hot ligand}] + [\text{cold ligand}]}$$

Where B represents specific binding, [hot ligand] the single concentration of [^{125}I]rAM studied, [cold ligand] the concentration of unlabeled rAM competing with the radiolabel for AM receptor binding, B_{max} the maximum number of binding sites and KD the equilibrium dissociation constant (the equation was solved where the "cold ligand" IC_{50} = [hot ligand] + K_D). Analysis of all binding data was performed by computer-assisted nonlinear least square fitting using GraphPad PRIZM version 4 (GraphPad Software, San Diego, CA). Binding sites/cell was calculated and data was expressed as percent of the control.

4.2.6. Adenylate cyclase assays: Cyclase activity was done as described before with slight modifications [146, 284]. Cells were harvested from P100 or P60 plates and homogenized in Tris HCl (10mM)/EDTA (10mM) buffer. Membranes were prepared by homogenization and centrifugation in Tris HCl (50mM)/MgCl (10mM) buffer. Final concentration of 20 μg of protein/assay tube was obtained. Membranes were incubated for 15 min at 30°C with appropriate concentrations of drugs (100nM AM, 10μM

Forskolin) and assay mix containing ATP regeneration system and α^{32}P-ATP. After the reaction was stopped (with stop solution containing ^{3}H-cAMP) contents of the assay tubes were passed through Dowex and subsequently through alumina columns to separate the degradation products of ATP, by washing the dowex with water and alumina with imidazole. Elution profile was done to determine the amount of water and imidazole needed to wash and elute the products. Product eluted from alumina column was counted for the presence of ^{3}H-cAMP and α^{32}P-cAMP in a β-counter. Each experiment was done in triplicates and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. Basal cyclase activity and forskolin stimulation did not show statistically significant differences between treatments.

4.2.7. cAMP accumulation assays: HEK 293 cells were seeded on a 24-well plate until reaching 80-90% confluency, then incubated in serum-free media overnight before experiment. Desensitization experiments were carried out as described in Materials and Methods section, with cells pretreated with 10nM rAM and subsequently challenged for 10 min at 37°C with appropriate concentrations of drugs (100nM AM, 10μM Forskolin) in the presence of 200μM 3-isobutyl-1-methylxanthine. Determination of cAMP level was measured using the biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in HEK 293 cells were calculated using a standard curve ranging from 10 to 10^{4} fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. Basal cyclase activity and forskolin stimulation did not show statistically significant differences between treatments.

4.2.8. Mutagenesis procedure: Site-directed mutagenesis was performed using a PCR-based strategy that employs the *pfu* Turbo polymerase (Stratagene, La Jolla, CA). A pair of complementary oligonucleotides containing the appropriate point mutations in the sequence of RAMP or a premature stop codon at position 145 or 147 codon of RAMP-3 for deletion mutants were synthesized (Michigan State University Macromolecular structure facility). The PCR for the mutation was as follows: 94°C for 2 minutes; 30 cycles of 94°C for 30 sec., 50°C for 30 sec., 68°C for 8 min.; final cycle of 68°C for 8 minutes. PCR product was digested for 4 hours with DpnI enzyme (Invitrogen) and transformed in to DH5α cells. Mutations were confirmed by automated sequencing (Michigan State University Genomic Technology Support Facility). Putative phosphorylation sites and kinase consensus sites were identified with NetPhosK Server 1.0 database (http://www.cbs.dtu.dk/services/NetPhosK).

4.2.9. Immunofluorescence microscopy: HEK 293 cells were transfected as described above and seeded at 24hr post-transfection onto collagen type I-coated coverslips. Resensitization assays were performed as described and reactions were stopped by fixing cells in 4% paraformaldehyde for 30min. at room temperature. Samples were permeablized with 0.1% v/v Triton X-100 in PBS and blocked overnight in 0.1% v/v Triton X-100 in PBS + 10% goat serum. Samples were incubated in primary antibody in blocking buffer for 2h at room temperature (NSF at 1:250 and RAMP3 at 1:200). Appropriate secondary antibodies were applied for 1h at room temperature (Goat anti-mouse Cy3 at 1:500 and Goat anti-rabbit Cy5 at 1:400). Coverslips were mounted in

Shandon Permafluor mounting medium and slides stored at 2-8°C until analysis. Cells were visualized on a Zeiss 210 laser confocal microscope at a zoom of 2. Images presented are representative single optical sections of a z-series taken from at least twenty fields per experiment and at least three individual experiments. Images in this thesis/dissertation are presented in color.

4.2.10. Statistics: Data are presented as mean ± S.E.M. Single group comparisons exercised a paired Student's t-test method. Statistical significance was set at $P<0.05$.

4.3. Results

We and other groups have previously shown that the AM2 receptor (CL-R + RAMP3 complex) undergoes agonist-stimulated desensitization, internalization, and degradation in HEK 293 cells [153, 276, 285]. Furthermore, we have recently shown that in the presence of NSF, the AM2 receptor complex undergoes recycling, instead of following a degradation pathway, in HEK 293 cells [153]. We have also demonstrated that the presence of the adaptor protein, NHERF, in HEK 293 cells can inhibit the internalization, but not the desensitization, of the AM2 receptor complex through protein-protein interactions. Given the recognized role of RAMPs in the trafficking of the AM receptor subtypes, in this study we have examined the role of RAMPs in regulating the phosphorylation of the AM receptor subtypes in the desensitization and internalization processes.

Pretreatment of HEK 293 cells expressing CL-R and RAMP2 or RAMP3 with 10 nM AM for one hour resulted in desensitization of the cAMP accumulation response

from approximately 40% (of forskolin stimulation) in untreated cells to 15% in AM-treated cells (Figure 7A, B). In addition, as shown in Figure10C and 11C, respectively, CL-R/RAMP2 or CL-R/RAMP3 complex underwent agonist-induced internalization as determined by receptor binding (Figure 7A, B) and immunofluorescence microscopy. These findings are in agreement with those of Kuwasako *et al.* and as reported by us recently [153, 276]. Using site-directed mutagenesis, we mutated clusters of putative phosphorylation residues in the C-terminus of CL-R to alanine residues and tested the effect of these mutations on desensitization and internalization of the receptor complexes (Figure 8). Mutant CL-R/RAMP receptor complexes showed similar levels of receptor stimulation and receptor complex expression levels as compared to wild-type CL-R/RAMP receptor complex, as determined by cAMP accumulation and whole-cell ligand binding experiments, respectively (Figure 9A, C; data not shown). Additionally, the CL-R cluster mutants showed similar receptor stimulation and receptor complex expression when co-expressed with RAMP2 or RAMP3 (determined by cAMP accumulation and whole-cell binding assays, respectively). Interestingly, when desensitization assays were performed on the cluster mutants of CL-R in complex with RAMP2 or RAMP3, the same cluster mutant of CL-R showed reduced levels of receptor desensitization when complexed with RAMP2 or RAMP3 (Figure 9A, C, respectively). Cluster mutant #2 of CL-R, when in complex with RAMP2 or RAMP3, showed no significant receptor desensitization with AM pretreatment, as compared to wild-type CL-R and cluster mutants #1 and #3. In addition, when receptor complex internalization was measured with whole-cell binding experiments, AM pretreatment also failed to promote significant internalization of the cluster mutant #2 of CL-R when it was complexed with RAMP2 or

RAMP3 (Figure 9B, D, respectively). Wild-type CL-R and cluster mutants #1 and #3 showed similar levels of receptor internalization with one hour AM pretreatment. Individual residues within the second cluster of CL-R were then examined for their role in regulation of receptor desensitization and internalization of the CL-R/RAMP receptor complex.

Site-directed mutagenesis was again employed to create the single mutations of putative phosphorylation residues within the second cluster of CL-R, determined to be critical for agonist-stimulated receptor complex desensitization and internalization, regardless of RAMP in association with CL-R. Point mutants of putative phosphorylation residues to alanine residues were co-expressed with RAMP2 or RAMP3 in HEK 293 cells and desensitization and internalization assays were performed. Point mutants of CL-R when complexed with RAMP2 or RAMP3 showed similar levels of receptor stimulation and receptor complex expression as compared to wild-type CL-R/RAMP complexes (determined by cAMP accumulation and whole-cell binding assays, respectively). Additionally, no differences in receptor stimulation or receptor complex expression were seen when CL-R point mutants were expressed with RAMP2 or RAMP3. Using cAMP accumulation assays to determine receptor desensitization, only Ser 421 mutant of CL-R, when complexed with RAMP2, showed a loss of receptor desensitization as compared to wild-type CL-R/RAMP2 receptor complex (Figure 10A). Whole-cell binding and immunofluorescence microscopy determined Ser 421 of CL-R to also be critical to the agonist-stimulated internalization of the CL-R/RAMP2 receptor complex (Figure 10B, C). The additional point mutants of CL-R, when co-expressed with RAMP2, showed no significant changes in agonist-induced receptor desensitization

or internalization as compared to wild-type CL-R/RAMP2 receptor complex (determined by cAMP accumulation and whole-cell binding assays, respectively).

Meanwhile, co-expression of the point mutants of CL-R with RAMP3 showed a different putative phosphorylation residue critical to receptor desensitization and internalization. While all point mutants of CL-R, when complexed with RAMP3, showed similar levels of receptor stimulation and receptor complex expression as compared to wild-type CL-R/RAMP3 complexes (determined by cAMP accumulation and whole-cell binding assays, respectively), the Thr 423 mutant of CL-R showed a lack of significant receptor desensitization when pretreated with AM, compared to an approximate 50% attenuation of receptor signaling seen for the additional point mutants and wild-type CL-R co-expressed with RAMP3 (as measured by cAMP accumulation assays) (Figure 11A). In addition, mutation of Thr 423 of CL-R to alanine inhibited the agonist-stimulated receptor internalization when co-expressed with RAMP3 in HEK 293 cells (as measured by whole-cell binding and immunofluorescence microscopy) (Figure 11B, C, respectively). The additional point mutants of CL-R, when co-expressed with RAMP3, showed no significant changes in agonist-induced receptor desensitization or internalization as compared to wild-type CL-R/RAMP3 receptor complex (determined by cAMP accumulation and whole-cell binding assays, respectively). These findings indicate that the two RAMP isoforms differentially regulate the CL-R receptor in the process of receptor desensitization and internalization.

To determine if mutation of putative phosphorylation residues on the third intracellular loop of CL-R altered receptor desensitization or internalization when complexed with RAMP2 or RAMP3, site-directed mutagenesis of these residues to

alanine was performed. Desensitization assays, measured by cAMP accumulation, showed no effect of third intracellular loop mutations on desensitization of CL-R/RAMP2 or CL-R/RAMP3 receptor complex. The receptor activity was attenuated to levels similar to wild-type CL-R, when mutant receptors were co-expressed with RAMP2 or RAMP3 (Figure 12A, B). Third intracellular loop mutants of CL-R, in complex with RAMP2 or RAMP3, showed no difference in receptor expression levels at the plasma membrane (as measured with whole-cell binding) in untreated cells, as compared to wild-type CL-R/RAMP2 or CL-R/RAMP3 complex (data not shown). These results indicate that amino acid residues on the third intracellular loop of CL-R are not required for desensitization or internalization of the CL-R/RAMP2 or CL-R/RAMP3 receptor complex.

It was then important to determine if putative phosphorylation residues on the RAMP isoforms contributes to the regulation of receptor complex desensitization and/or internalization. Putative phosphorylation residues on the C-terminus of the RAMP2 and RAMP3 isoforms were mutated to alanine residues and desensitization/internalization assays were performed. The phosphorylation mutant RAMPs co-expressed with wild-type CL-R showed no difference in receptor stimulation or receptor complex expression levels at the plasma membrane (as measured with cAMP accumulation and whole-cell binding, respectively) in untreated cells, as compared to wild-type CL-R/RAMP2 or CL-R/RAMP3 complex (Figure 13A-D). Desensitization of the receptor complex was unaltered with the RAMP phosphorylation mutants, in comparison to wild-type RAMP2 or RAMP3 in complex with CL-R (as measured by cAMP accumulation) (Figure 13A, C). In addition, the RAMP phosphorylation mutations were unable to modify the

internalization of the CL-R/RAMP2 or CL-R/RAMP3 receptor complexes (Figure 13B, D). No significant differences were seen in the desensitization or internalization patterns of the phosphorylation mutant RAMPs, when compared to wild-type RAMP2 or RAMP3 complexed with CL-R. These findings indicate that serine/threonine amino acid residues of the RAMPs do not appear to play a role in regulation of the desensitization and internalization processes of the CL-R/RAMP receptor complex.

Data presented so far supports the hypothesis that different CL-R/RAMP isoform associations could result in differential phosphorylation of the AM receptor subtypes as a means of regulating desensitization and internalization of the receptor complexes. It was then important to identify the kinases regulating the desensitization and internalization of the different CL-R/RAMP2 and CL-R/RAMP3 receptor complexes. Sequence analysis of CL-R in the NetPhosK database (http://www.cbs.dtu.dk/services/NetPhosK) predicted consensus sites for PKA and PKC phosphorylation on amino acid residues Ser 421 and Thr 423 of CL-R, respectively. These amino acid residues were identified in earlier experiments within this study to regulate desensitization and internalization of the CL-R/RAMP2 and CL-R/RAMP3 receptor complexes, respectively. Therefore, it was hypothesized that inhibition of these kinases would block receptor desensitization and internalization of the appropriate AM receptor complex.

The Ser 421 amino acid residue of CL-R that was determined to be critical to the agonist-stimulated desensitization and internalization of the CL-R/RAMP2 receptor complex was predicted to be regulated by protein kinase A. In order to test if this kinase was regulating the CL-R/RAMP2 receptor complex desensitization and internalization, a specific protein kinase A (PKA) inhibitor, H-89, was used to perform desensitization and

internalization assays. As a control, a protein kinase C (PKC) inhibitor, RO 32-0432, was also tested. Cells pretreated with the PKC inhibitor and AM showed similar levels of receptor desensitization and internalization as cells pretreated with only AM (Figure 14A, B). On the other hand, cells pretreated with the PKA inhibitor in the presence of AM showed an inhibition of both receptor desensitization and internalization of the CL-R/RAMP2 receptor complex (Figure 14A, B). No significant differences were observed in receptor cAMP stimulation and receptor complex expression levels in cells co-expressing CL-R and RAMP2 that were pretreated with or without the PKA and PKC inhibitors. These findings indicate that PKA, but not PKC, is involved in CL-R/RAMP2 desensitization and internalization, presumably by phosphorylating Ser 421 of CL-R.

PKC and PKA inhibitors were then used to test their effects on the desensitization and internalization patterns of the CL-R/RAMP3 receptor complex. Because the Thr 423 on CL-R was a PKC consensus site, it was predicted that the PKC inhibitor would alter the desensitization and internalization of the receptor complex. cAMP accumulation and whole-cell binding assays showed that while the PKA inhibitor had no effect on the desensitization and internalization of the CL-R/RAMP3 receptor complex, the PKC inhibitor completely blocked the desensitization and internalization of the CL-R/RAMP3 complex (Figure 15A, B). These studies suggest that PKC, but not PKA, is involved in the desensitization and internalization of CL-R/RAMP3 receptor complex, seemingly by phosphorylation of Thr 423 of CL-R.

To further characterize the role of PKA and PKC in the desensitization and internalization of the AM1 and AM2 receptors, respectively, the kinases were individually activated and tested for their ability to induce receptor desensitization and/or

internalization. No significant alterations were seen in the receptor expression levels or basal or forskolin-stimulated cAMP accumulation when transfected HEK 293 cells were pretreated with forskolin (10 μM) or phorbol-12-myristate-13-acetate (PMA, 1 μM) for the given time periods (data not shown). As would be predicted, activation of PKA with forskolin pretreatment resulted in efficient desensitization and internalization of the AM1, but not AM2 receptors (Figure 16A, B). PKA activation was capable of achieving levels of desensitization and internalization comparable to that induced by AM in cells transfected with CL-R and RAMP2. When transfected HEK 293 cells were pretreated with PMA, to activate PKC, desensitization and internalization was observed for the AM2, but not AM1 receptors (Figure 16A, B). The PKC-induced desensitization and internalization observed for cells expressing CL-R and RAMP3 was comparable to that stimulated by AM. These findings are in agreement with the observations from the kinase inhibitor studies (Figures 14, 15). This data confirms a role for PKA in the desensitization and internalization processes for the AM1 receptor (CL-R+RAMP2), and a role for PKC in the desensitization and internalization of the AM2 receptor.

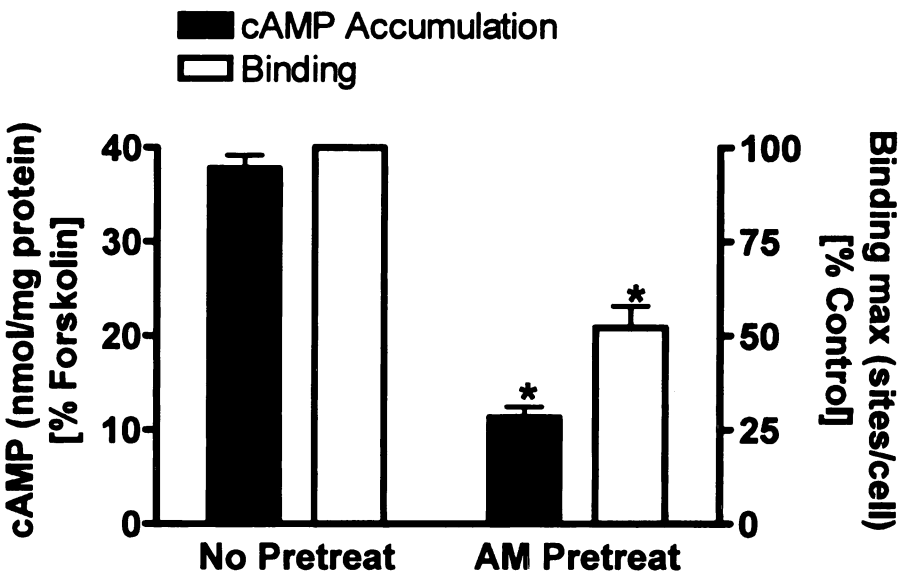


Figure 7A: Desensitization and internalization of the AM1 and AM2 receptor in HEK 293 cells. CL-R/RAMP2 complex (AM1 receptor) desensitizes and internalizes in HEK 293 cells after prolonged agonist stimulation. HEK 293 cells transiently transfected with CL-R and RAMP2 were treated for one hour with AM (10 nM) and then washed and receptor desensitization (cAMP accumulation) and internalization (radioligand binding) were measured. cAMP accumulation is shown on the left y-axis, expressed as percent maximal response (% forskolin stimulation), and radioligand binding is shown on the right y-axis, expressed as percent control. * $p \leq 0.05$; $n \geq 4$ experiments.

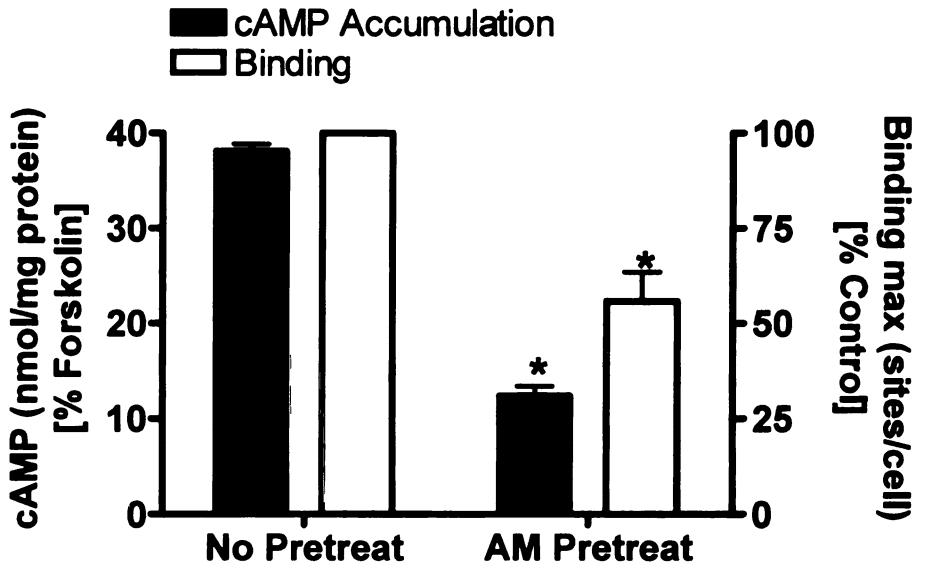


Figure 7B: Desensitization and internalization of the AM1 and AM2 receptor in HEK 293 cells. CL-R/RAMP3 complex (AM2 receptor) desensitizes and internalizes in HEK 293 cells after prolonged agonist stimulation. HEK 293 cells transiently transfected with CL-R and RAMP3 were treated for one hour with AM (10 nM) and then washed and receptor desensitization (cAMP accumulation) and internalization (radioligand binding) were measured. cAMP accumulation is shown on the left y-axis, expressed as percent maximal response (% forskolin stimulation), and radioligand binding is shown on the right y-axis, expressed as percent control. * $p \leq 0.05$; $n \geq 4$ experiments.

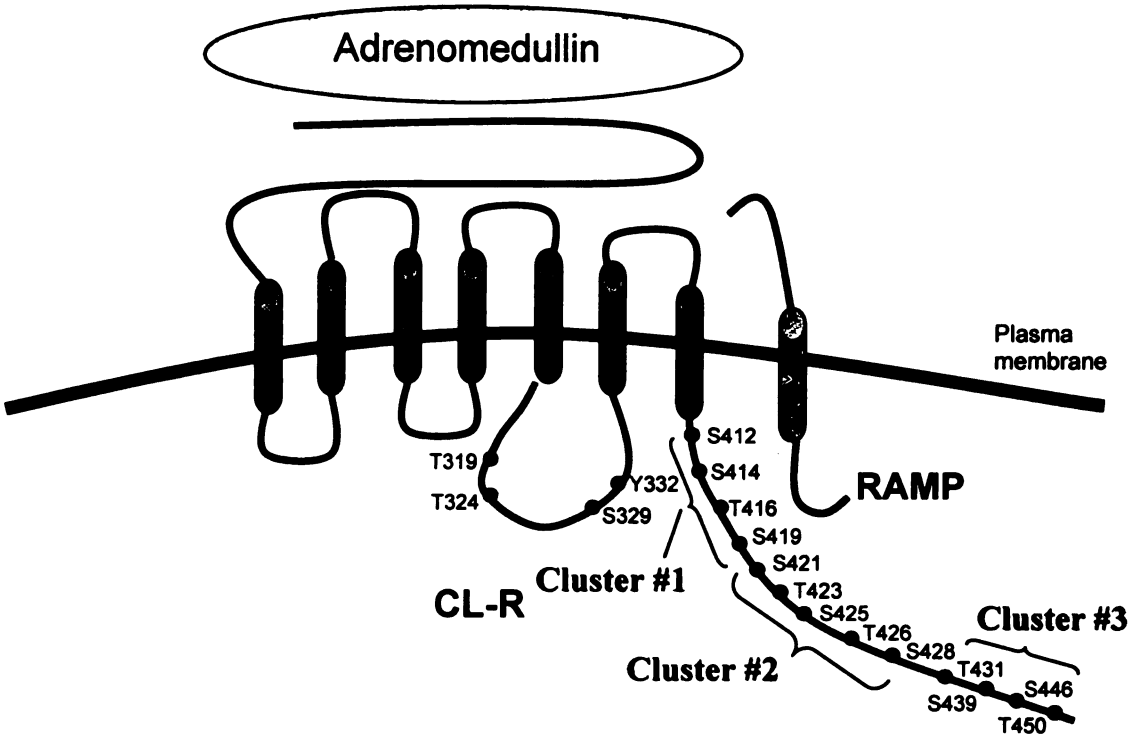


Figure 8: Illustration of cluster mutations of CL-R to investigate role of phosphorylation on AM receptor desensitization and internalization. Site-directed mutagenesis techniques, described in Materials and Methods section, were employed to mutate putative phosphorylation residues on C-terminus of CL-R to alanine residues. Cluster mutants (1-3), as depicted in figure, are used in experiments reported in Figure 9A-D.

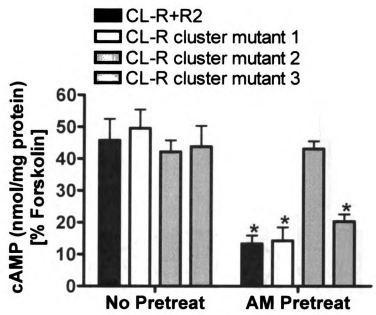


Figure 9A: Role of phosphorylation on AM1 and AM2 receptor desensitization and internalization (cluster mutations). Amino acid residues 421-428 are critical to desensitization of AM1 receptor. HEK 293 cells transiently transfected with CL-R/CL-R cluster mutants and RAMP2 were seeded in 48-well plates and treated for one hour with AM (10 nM) and then washed and receptor desensitization (cAMP accumulation) was measured. After repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

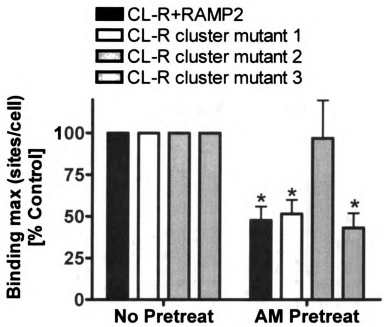


Figure 9B: Role of phosphorylation on AM1 and AM2 receptor desensitization and internalization (cluster mutations). Amino acid residues 421-428 are critical to AM1 receptor internalization. HEK 293 cells were co-transfected with CL-R or CL-R cluster mutants and RAMP2. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

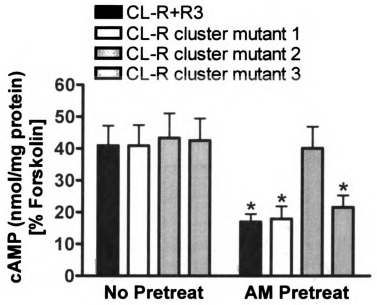


Figure 9C: Role of phosphorylation on AM1 and AM2 receptor desensitization and internalization (cluster mutations). Amino acid residues 421-428 are critical to desensitization of AM2 receptor. HEK 293 cells transiently transfected with CL-R/CL-R cluster mutants and RAMP3 were seeded in 48-well plates and treated for one hour with AM (10 nM) and then washed and receptor desensitization (cAMP accumulation) was measured. After repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

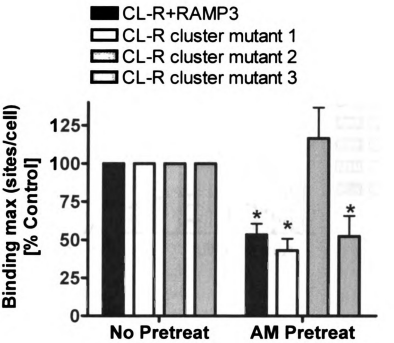


Figure 9D: Role of phosphorylation on AM1 and AM2 receptor desensitization and internalization (cluster mutations). Amino acid residues 421-428 are critical to AM2 receptor internalization. HEK 293 cells were co-transfected with CL-R or CL-R cluster mutants and RAMP3. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

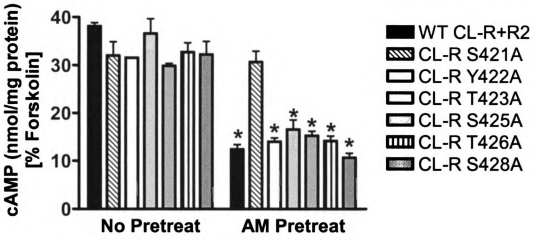


Figure 10A: Role of phosphorylation on AM1 receptor desensitization and internalization (point mutations). Residue Ser 421 is crucial to desensitization of AM1 receptor. HEK 293 cells transiently transfected with CL-R/CL-R point cluster mutants and RAMP2 were seeded in 48-well plates and treated for one hour with AM (10 nM) and then washed and receptor desensitization (cAMP accumulation) was measured. After repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^{4} fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

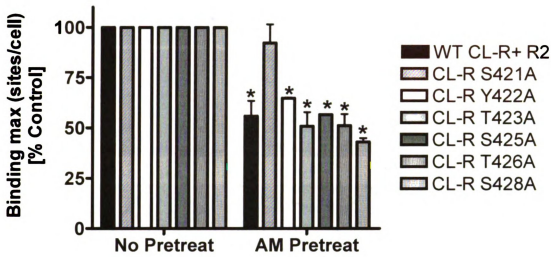


Figure 10B: Role of phosphorylation on AM1 receptor desensitization and internalization (point mutations). Residue Ser 421 is critical to AM1 receptor internalization. HEK 293 cells were co-transfected with CL-R or CL-R point mutants and RAMP2. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

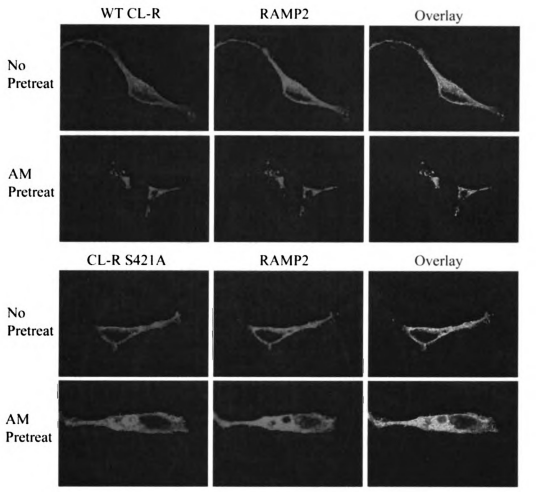


Figure 10C: Role of phosphorylation on AM1 receptor desensitization and internalization (point mutations). Immunofluorescence microscopy shows failure of CL-R S421A/RAMP2 receptor complex to internalize after agonist stimulation. HEK 293 cells transfected with CL-R S421A-GFP and RAMP2 were pretreated with 10nM AM for 1h. After pretreatment with AM, cells were washed, fixed, and components were visualized using anti-RAMP2 antibody (1:150) and detected with Cy3 secondary antibody (1:250), and CL-R is detected with an EGFP tag; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least twenty fields imaged per experiment from at least three experiments. Bar scales on all images represent 100μm.

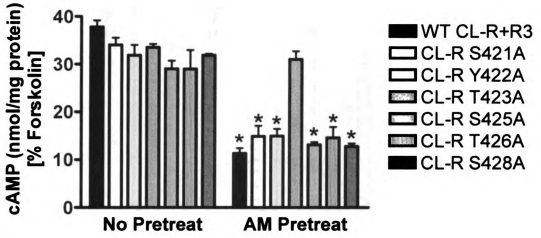


Figure 11A: Role of phosphorylation on AM2 receptor desensitization and internalization (point mutations). Residue Thr 423 is crucial to desensitization of AM2 receptor. HEK 293 cells transiently transfected with CL-R/CL-R point mutants and RAMP3 were seeded in 48-well plates and treated for one hour with AM (10 nM) and then washed and receptor desensitization (cAMP accumulation) was measured. After repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

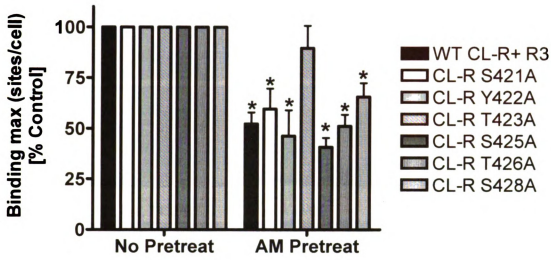


Figure 11B: Role of phosphorylation on AM2 receptor desensitization and internalization (point mutations). Residue Thr 423 is critical to AM2 receptor internalization. HEK 293 cells were co-transfected with CL-R or CL-R point mutants and RAMP3. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

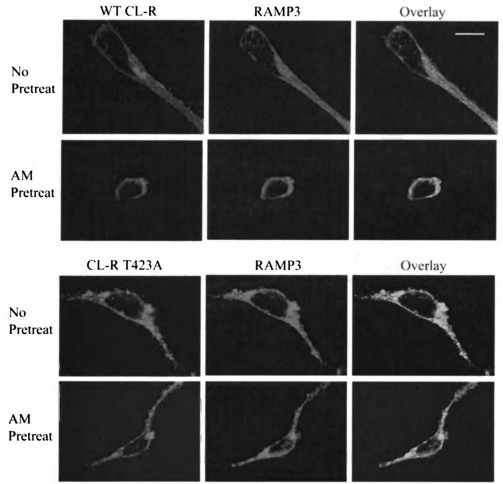


Figure 11C: Role of phosphorylation on AM2 receptor desensitization and internalization (point mutations). Immunofluorescence microscopy shows failure of CL-R T423A/RAMP3 receptor complex to internalize after agonist stimulation. HEK 293 cells transfected with CL-R T423A-GFP and RAMP3 were pretreated with 10nM AM for 1h. After pretreatment with AM, cells were washed, fixed, and components were visualized using anti-RAMP3 antibody (1:150) and detected with Cy3 secondary antibody (1:250), and CL-R is detected with an EGFP tag; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least twenty fields imaged per experiment from at least three experiments. Bar scales on all images represent 100μm.

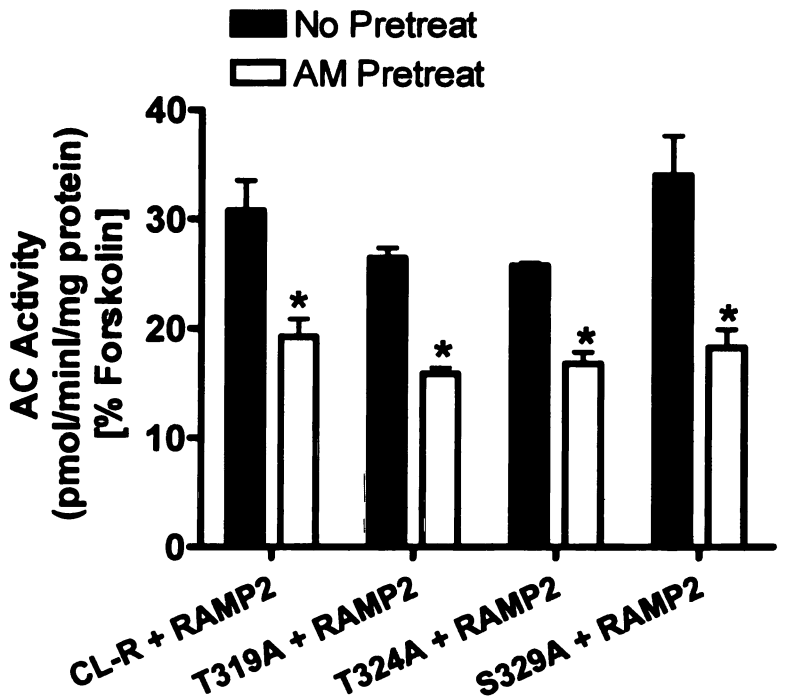


Figure 12A: Role of phosphorylation of residues in third intracellular loop of CL-R on AM1 and AM2 receptor desensitization. Phosphorylation of third intracellular loop residues of CL-R not vital in AM1 receptor desensitization. HEK 293 cells transiently transfected with CL-R/CL-R point mutants and RAMP2 were seeded in 48-well plates and treated for one hour with AM (10 nM) and then washed and receptor desensitization (cAMP accumulation) was measured. After repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

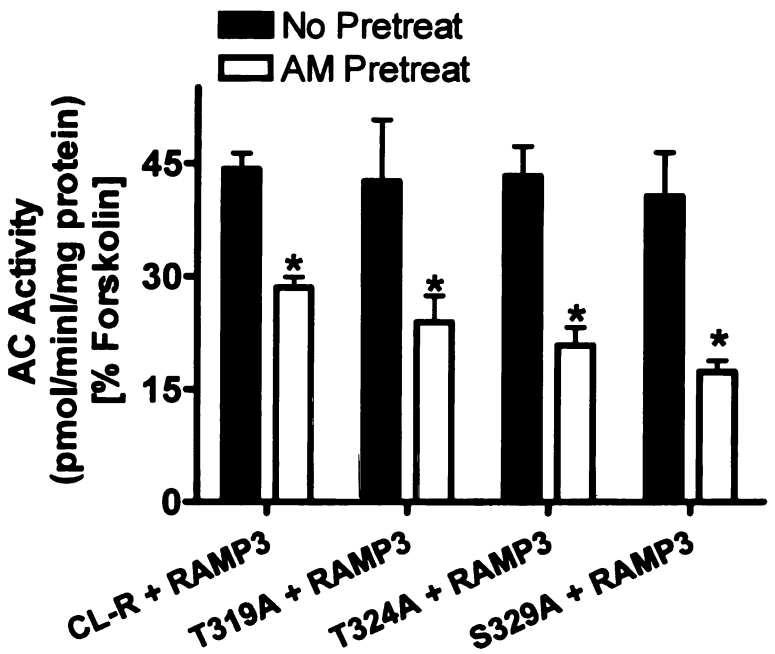


Figure 12B: Role of phosphorylation of residues in third intracellular loop of CL-R on AM1 and AM2 receptor desensitization. Third intracellular loop residues of CL-R not phosphorylated in desensitization of AM2 receptor. HEK 293 cells transiently transfected with CL-R/CL-R point mutants and RAMP3 were seeded in 48-well plates and treated for one hour with AM (10 nM) and then washed and receptor desensitization (cAMP accumulation) was measured. After repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

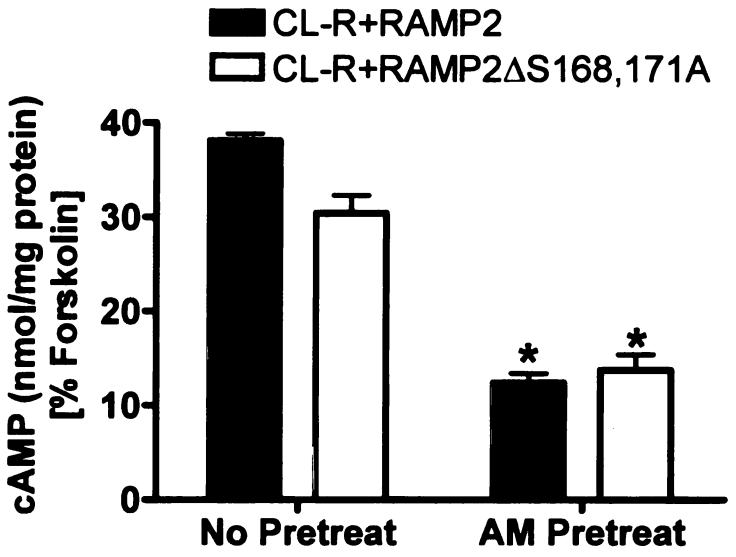


Figure 13A: Role of phosphorylation of RAMPs on AM1 and AM2 receptor desensitization and internalization. Phosphorylation of residues on RAMP2 not essential to desensitization of AM1 receptor. HEK 293 cells transiently transfected with CL-R and RAMP2/RAMP2 phosphorylation mutants were seeded in 48-well plates and treated for one hour with AM (10 nM) and then washed and receptor desensitization (cAMP accumulation) was measured. After repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

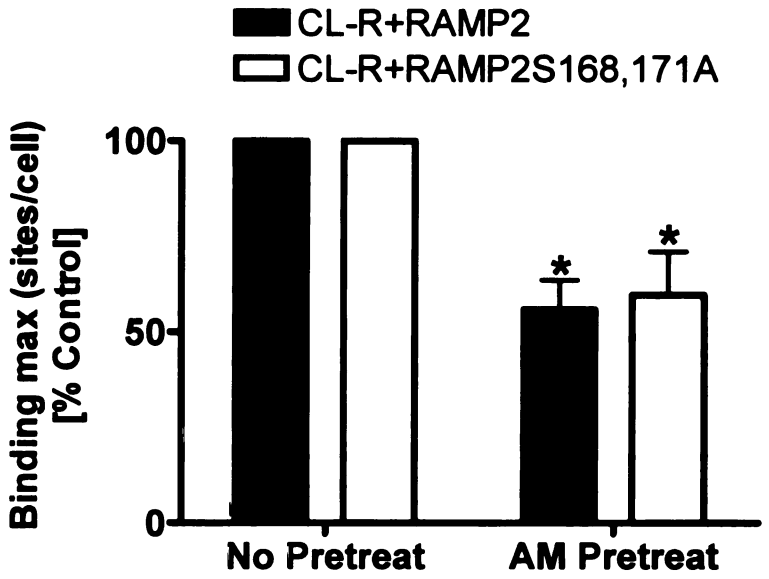


Figure 13B: Role of phosphorylation of RAMPs on AM1 and AM2 receptor desensitization and internalization. Phosphorylation of RAMP2 is not critical to AM1 receptor internalization. HEK 293 cells were co-transfected with CL-R and RAMP2/RAMP2 phosphorylation mutants. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

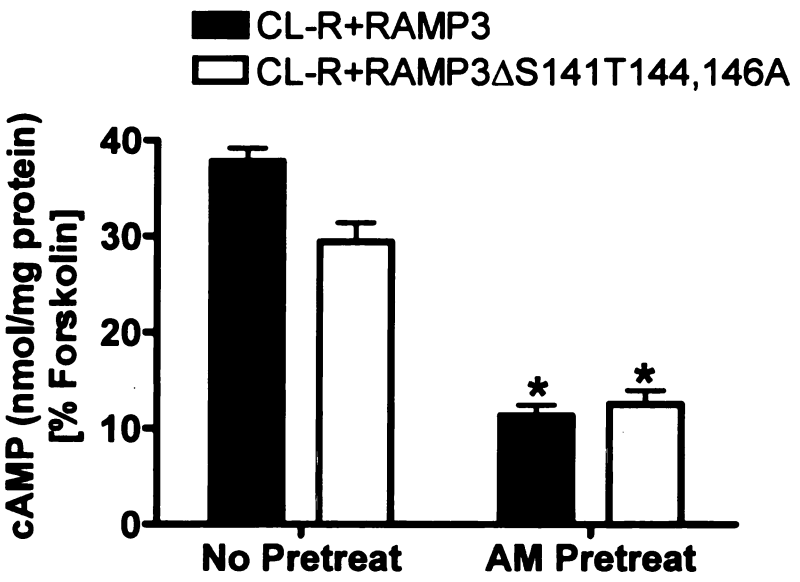


Figure 13C: Role of phosphorylation of RAMPs on AM1 and AM2 receptor desensitization and internalization. Phosphorylation of residues on RAMP3 not required for desensitization of AM2 receptor. HEK 293 cells transiently transfected with CL-R and RAMP3/RAMP3 phosphorylation mutants were seeded in 48-well plates and treated for one hour with AM (10 nM) and then washed and receptor desensitization (cAMP accumulation) was measured. After repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

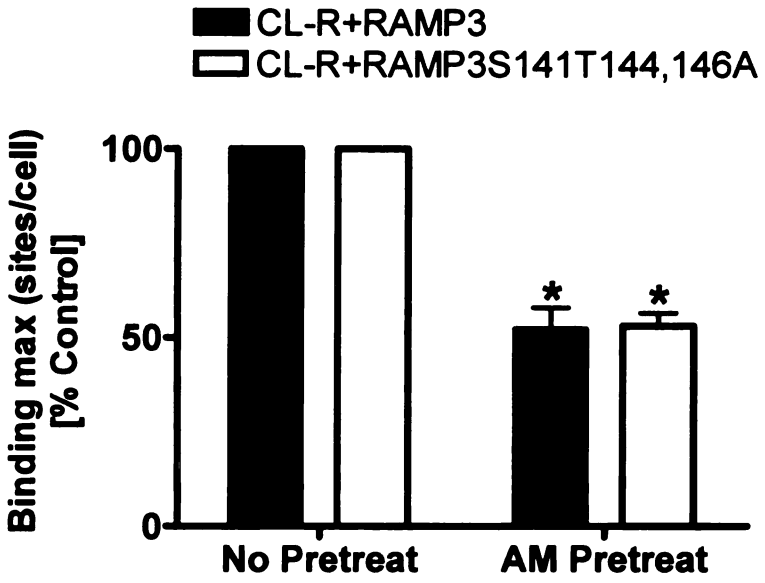


Figure 13D: Role of phosphorylation of RAMPs on AM1 and AM2 receptor desensitization and internalization. Phosphorylation of RAMP3 is not vital for AM2 receptor internalization. HEK 293 cells were co-transfected with CL-R and RAMP3/RAMP3 phosphorylation mutants. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

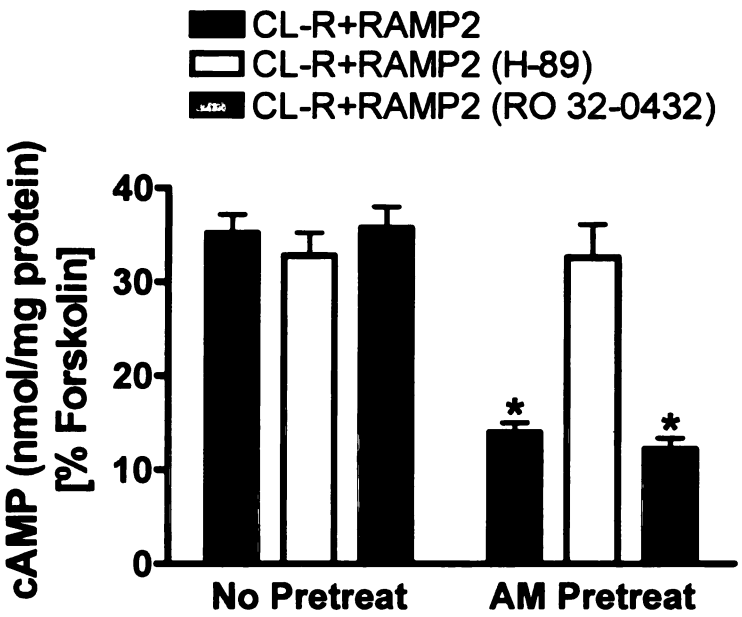


Figure 14A: Role of kinases in AM1 receptor desensitization and internalization. Phosphorylation of residues of CL-R for desensitization and internalization of AM1 receptor is sensitive to H-89 in HEK 293 cells. Cells transiently transfected with CL-R and RAMP2 were seeded in 48-well plates and treated for one hour with AM (10 nM) with and without H-89 (1μM) and Ro 32-0432 (3μM) treatment, then washed, and receptor desensitization (cAMP accumulation) was measured. After repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

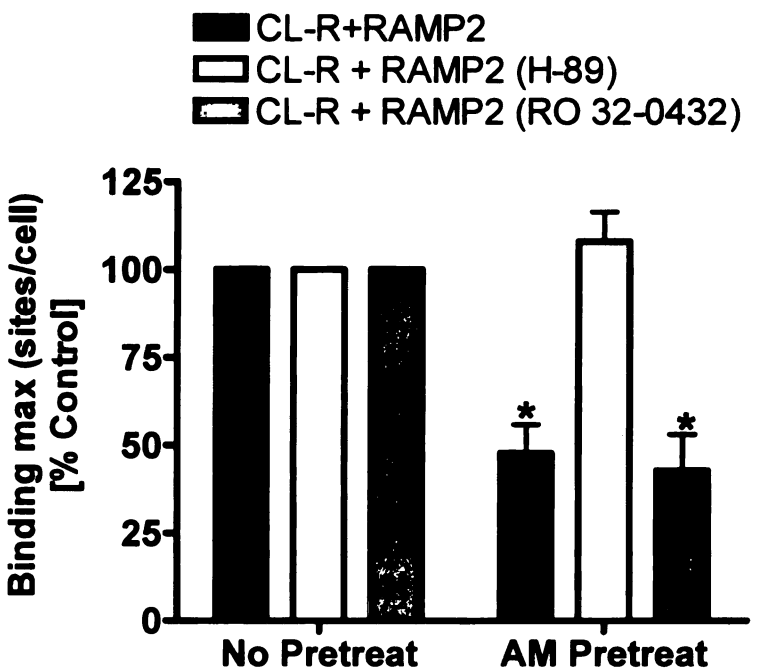


Figure 14B: Role of kinases in AM1 receptor desensitization and internalization. Phosphorylation of residues of CL-R for internalization of AM1 receptor is sensitive to H-89 in HEK 293 cells. HEK 293 cells were co-transfected with CL-R and RAMP2. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour with and without H-89 (1μM) and Ro 32-0432 (3μM) treatment, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

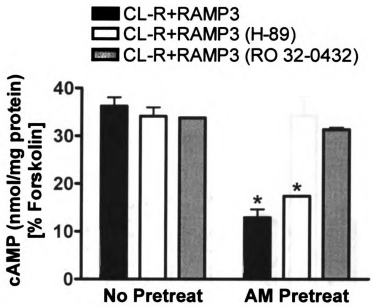


Figure 15A: Role of kinases in AM2 receptor desensitization and internalization. Phosphorylation of residues of CL-R for desensitization and internalization of AM2 receptor is sensitive to RO 32-0432 in HEK 293 cells. Cells transiently transfected with CL-R and RAMP3 were seeded in 48-well plates and treated for one hour with AM (10 nM) with and without H-89 (1μM) and Ro 32-0432 (3μM) treatment, then washed, and receptor desensitization (cAMP accumulation) was measured. After repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

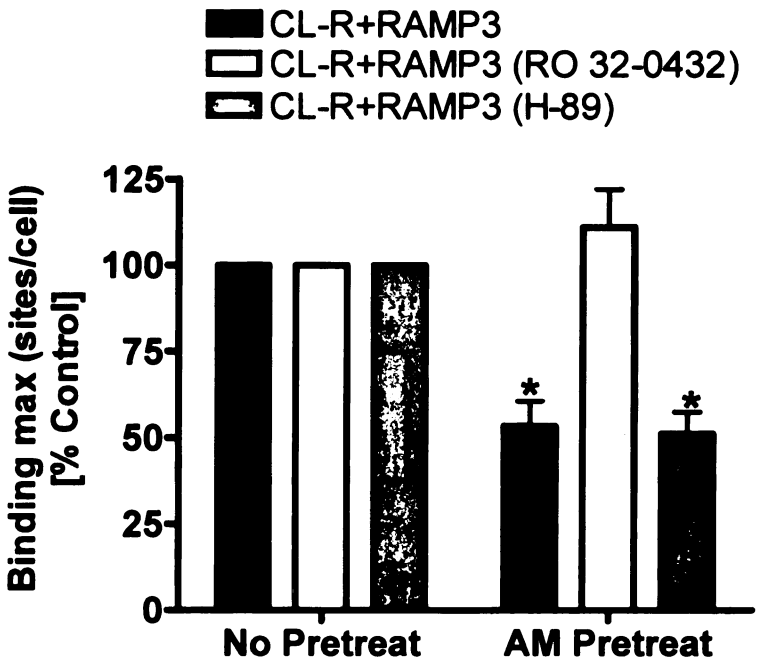


Figure 15B: Role of kinases in AM2 receptor desensitization and internalization. Phosphorylation of residues of CL-R for internalization of AM2 receptor is sensitive to Ro 32-0432 in HEK 293 cells. HEK 293 cells were co-transfected with CL-R and RAMP3. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour with and without H-89 (1μM) and Ro 32-0432 (3μM) treatment, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

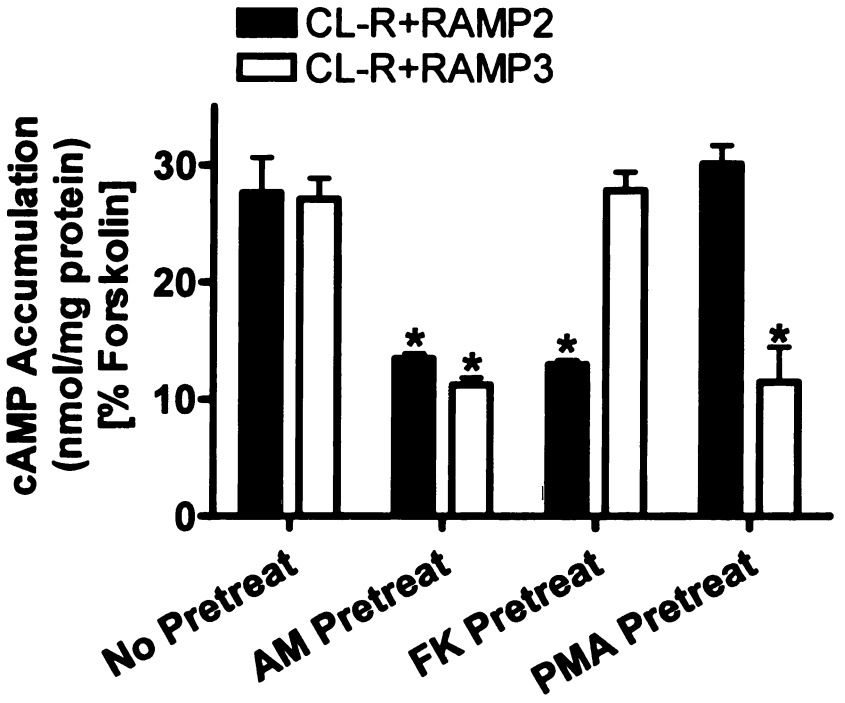


Figure 16A: Role of kinases in AM2 receptor desensitization and internalization. Desensitization and internalization of AM1 receptor is dependent on PKA activation, while the AM2 receptor requires PKC activation. Cells transiently transfected with CL-R and RAMP2 or RAMP3 were seeded in 48-well plates and treated either for one hour with AM (10 nM) or for 30 min. with forskolin (10 μM) or for 10 min. with PMA (1 μM), then washed, and receptor desensitization (cAMP accumulation) was measured. After repeated wash steps, cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in transfected HEK 293 cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

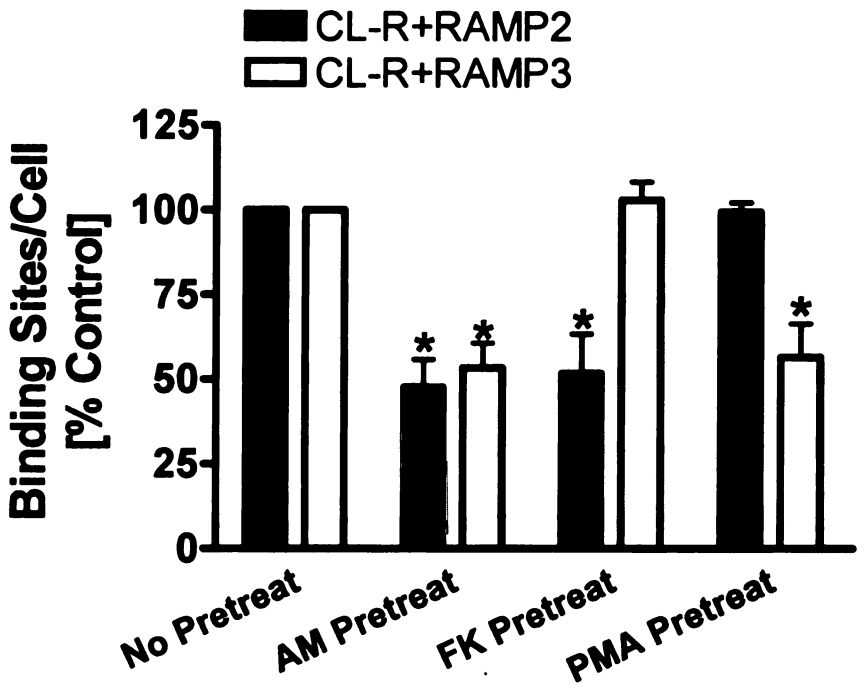


Figure 16B: Role of kinases in AM2 receptor desensitization and internalization. Desensitization and internalization of AM1 receptor is dependent on PKA activation, while the AM2 receptor requires PKC activation. Cells transiently transfected with CL-R and RAMP2 or RAMP3 were seeded in 48-well plates and treated either for one hour with AM (10 nM) or for 30 min. with forskolin (10 μM) or for 10 min. with PMA (1 μM), washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

4.4. Discussion

The process of agonist-promoted receptor desensitization and internalization is an indispensable self-regulation mechanism of the GPCRs. These processes allow receptor signaling to be closely-monitored, providing a mechanism to promote receptor signaling or receptor down-regulation. Receptors preserve this maintenance in many different ways, with phosphorylation of the receptor ranking as one of the most common [196]. Receptor phosphorylation during the process of desensitization is thought to enable β-arrestin molecule interaction with the receptor, thus uncoupling the receptor from its obligate G-protein and signaling for the assembly of the endocytic machinery for receptor internalization. In this study we examined the role of phosphorylation in the desensitization and internalization processes of the AM receptor subtypes. Putative phosphorylation residues were found to play a vital role in the process of desensitization and internalization of both AM receptor complexes. Notably, the two AM receptor subtypes were regulated by different amino acid residues on CL-R. Namely, the serine residue 421 of CL-R and threonine residue 423 of CL-R were responsible for the efficient receptor desensitization and internalization of the CL-R/RAMP2 and CL-R/RAMP3 receptor complexes, respectively. Protein kinase A inhibitor blocked only CL-R/RAMP2 desensitization and internalization, while a PKC inhibitor blocked only CL-R/RAMP3 desensitization and internalization, suggesting different kinases are involved in the phosphorylation of CL-R when different RAMP isoforms are associated. In addition, it was demonstrated the phosphorylation of the RAMP proteins or the third intracellular loop of CL-R was not required for AM receptor complex desensitization or internalization.

A prolonged exposure of cells to a particular ligand results in an attenuation of responsiveness to subsequent stimulation with that ligand. This phenomenon is called desensitization, which can be homologous or heterologous. Homologous desensitization is classified as a decrease in the response to a particular ligand that is specific for the stimulated receptor. Because G-protein regulated kinases (GRKs) are activated and only phosphorylate GPCRs in the agonist-bound state, GRKs are capable of attenuating receptor signaling by homologous desensitization [194, 195]. An attenuation of receptor signaling that is the result of activation of second messenger signaling, and in principal not specific for the agonist-bound receptor, is termed heterologous desensitization. Protein kinase A and C are common kinases activated through second messenger signaling of GPCRs, and therefore can activate heterologous receptor desensitization [196]. While past studies in HEK 293 cells have identified homologous desensitization as a means of regulating CL-R, this was only characterized for the CL-R/RAMP1 receptor complex (CGRP-1 receptor) [201]. Meanwhile, this study has identified heterologous desensitization to play a crucial role in the regulation of AM signaling in the HEK 293 cells, through both AM receptor subtypes.

The current literature characterizes the CL-R/RAMP receptor complex to undergo heterologous desensitization in the majority of cell lines studied. Desensitization of CL-R was found to involve cAMP-dependent protein kinase (PKA) activation in rat mesangial cells (RMCs) stimulated with AM, SK-N-MC (neuroblastoma cell line) cells stimulated with CGRP, Rat2 fibroblast cells stimulated with AM, and vascular smooth muscle cells (VSMCs) stimulated with CGRP [198-200, 287]. No studies to date have reported regulation of desensitization of the CL-R/RAMP receptor complex by PKC.

Although, this kinase is found to promote heterologous desensitization of numerous other GPCRs, for example the D2 dopamine [288], H1 histamine [289], serotonin 5-HT(2A) [290], thromboxane receptor alpha [291], and alpha(2)-adrenergic receptors [292]. Data from this manuscript demonstrates a requirement in HEK 293 cells of PKA phosphorylation for AM1 receptor desensitization/internalization and PKC phosphorylation for AM2 receptor desensitization/internalization.

Further studies are needed to define the implications of differential receptor phosphorylation to the AM signaling pathway. It is tempting to hypothesize that the different phosphorylation sites on CL-R when complexed with RAMP2 or RAMP3 may allow different protein-protein interactions that dictate down-stream receptor signaling or trafficking. It could be predicted that the differential phosphorylation of the AM1 and AM2 receptors allow protein-protein interactions that allow the documented targeting of the two receptor complexes for different pathways after endocytosis, degradative vs. recycling pathways, respectively [153].

Understanding the mechanism of AM receptor desensitization is of great importance given the documented protective role of AM in various cardiovascular and renal disorders. In the cardiorenal disease states where AM is protective, circulating plasma levels of AM have been shown to be increased. For example, in chronic glomerulonephritis, type I diabetes, and type II diabetes plasma AM levels are elevated [293-295]. In addition, AM delivery through adenoviral injection has been shown to decrease cardiac hypertrophy and renal damage in rat models of hypertension and improves cardiac function and prevents renal damage in streptozotocin-induced diabetic rats [296-300]. Because AM levels are chronically elevated in many of the above

cardiovascular and renal disorders, targeting receptor desensitization is a possible therapy for these pathophysiologies. The inhibition of the process of desensitization for additional GPCRs has been shown to elicit protective therapeutic effects for the disease states associated with the receptors. Lefkowitz *et. al.* have shown that the inhibition of β-adrenergic receptor kinase (kinase that phosphorylates the β-adrenergic receptor to cause desensitization) in the heart can delay the development of heart failure in multiple animal models, in some cases even restoring cardiac function [203, 204]. Others have also shown inhibition of desensitization of the μ-opioid receptor to be beneficial in preventing morphine tolerance [205, 206].

This is the first study to show that the RAMP isoforms are capable of dictating differential phosphorylation of CL-R to regulate the AM receptor complex agonist-stimulated desensitization and internalization. In addition, it has not previously been shown that different kinases are responsible for the phosphorylation of the two AM receptor subtypes (PKA for AM1 receptor and PKC for AM2 receptor). This report indicates yet another novel form of regulation of the AM receptor life-cycle by the RAMP proteins. Given the recent report of RAMPs ability to interact with other receptors in the family II of GPCRs, RAMPs may be playing a more prevalent role in the regulation of GPCR life-cycle [286].

5. RAMP isoform-specific regulation of adrenomedullin receptor trafficking by NHERF-1.

5.1 Introduction

The recent discovery of receptor activity modifying proteins (RAMPs) has broadened the field of G-protein coupled receptor (GPCR) regulation. RAMPs were discovered as required accessory proteins to an orphan GPCR, now termed the calcitonin receptor-like receptor (CL-R) [275]. The three RAMP isoforms (1-3) are products of three distinct genes and yield unique single transmembrane accessory proteins. RAMPs are required for the plasma membrane expression and determination of receptor phenotype for CL-R [275, 276]. RAMPs have recently been found to associate with additional members of the Class II family of GPCRs [286]. Co-expression of RAMP1 with CL-R yields a functional calcitonin gene-related peptide (CGRP) receptor, while co-expression of RAMP2 or -3 with CL-R produces a receptor responsive to adrenomedullin (AM) (AM1-R and AM2-R, respectively) [277, 278]. AM and CGRP are multi-functional peptides with many overlapping functions, ranging from potent vasodilation to proliferation to regulation of salt and water balance [279]. The RAMP isoforms have shown a differential expression patterns in different organ systems and in different pathophysiological states, suggesting a regulatory role for RAMPs in both physiological and pathophysiological situations. Furthermore, the identification of RAMP interactions with additional members of the Class II GPCR family and RAMP expression in cell lines lacking CL-R have raised the possibility of novel functions for RAMPs in GPCR regulation.

It has been shown in other GPCR systems that interactions with PSD-95/Discs-large/ZO-1 homology (PDZ) domain proteins are responsible for altering the receptor-trafficking after agonist stimulation [259, 267, 268]. In particular, a protein called Na^+/H^+ Exchange Regulatory Factor-1 (NHERF-1) has been shown to regulate the trafficking of the β2-AR, κ-OR, and PTH-R after agonist activation [240, 276, 301]. NHERF-1 is an adapter protein that is thought to tether membrane receptors to cytoskeletal proteins through PDZ interactions and interactions with MERM family of cytoskeletal proteins [257].

Comparable to the C-terminus of β2-AR, PTH-R, CFTR, and PDGF-R, human RAMP3 C-terminus contains a type-I PDZ recognition motif, whereas CL-R, RAMP1 or RAMP2 do not contain any PDZ recognition sequences [153, 154, 240, 250, 259, 302]. We hypothesized that RAMP3, via its interaction with NHERF-1, can regulate the trafficking of the CL-R/RAMP3 complex. We show here that while CL-R/RAMP1 and CL-R/RAMP2 complexes do not interact with NHERF-1, CL-R/RAMP3 complex interacts with NHERF-1 via the PDZ domain of NHERF-1 [303]. Moreover, we show here that over-expression of NHERF-1 in HEK-293 cells alters the trafficking pattern of the receptor complex to block the receptor's internalization by tethering the receptor complex to the actin cytoskeleton via interactions between RAMP3 and NHERF-1 through a type I PDZ domain. Furthermore, we also demonstrate that in primary human proximal tubule cells (which express endogenous NHERF-1 and CL-R/RAMP3), the CL-R/RAMP3 complex does not internalize upon agonist stimulation. Knocking down NHERF-1 or RAMP3 expression with RNAi causes the receptor to undergo

internalization upon agonist treatment, suggesting critical roles for both NHERF and RAMP3 in receptor internalization.

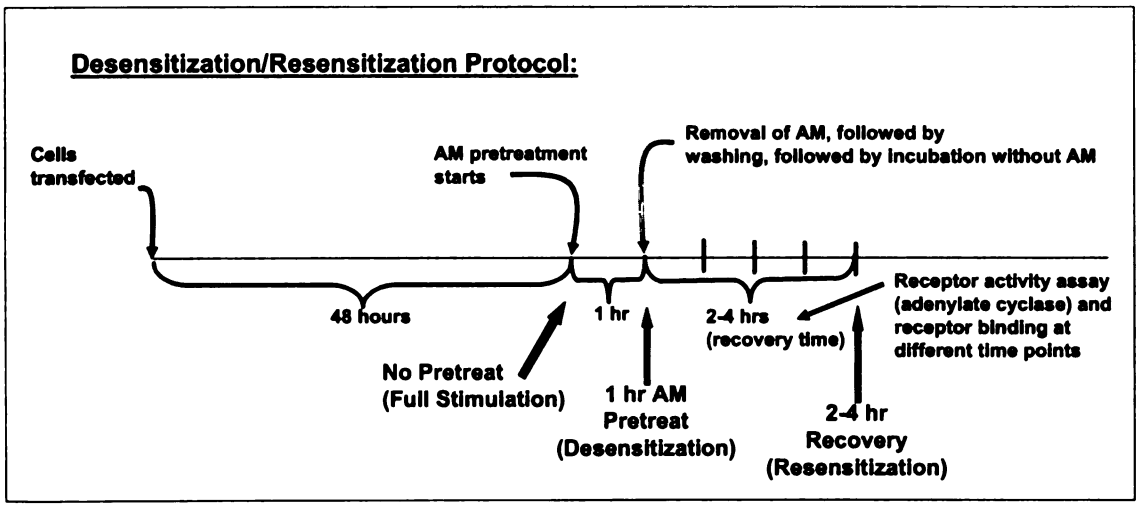
5.2. Materials and Methods

5.2.1. Materials: Adrenomedullin was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). 125I-labeled adrenomedullin was purchased from Amersham Biosciences Corp. (Piscataway, NY). DMEM media, fetal bovine serum, penicillin/streptomycin, trypsin-EDTA were purchased from GibcoBRL® (Grand Island, NY). RAMP3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and NHERF-1 (EBP50) antibody was from Affinity Bioreagents (Golden, CO). Alexa 488-phalloidin was purchased from Molecular Probes (Eugene, OR). Anti-mouse Cy3 and anti-rabbit Cy5 secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). All other reagents were of highest quality available.

5.2.2. Cell Culture and Transfection protocols: HEK-293T cells were obtained from ATCC and are maintained in DMEM low glucose media containing 10% FBS, 1%penicillin-streptomycin. Transfection of HEK293T cells was performed using Lipofectamine Plus protocol (Invitrogen). Cells were transfected with the DNA and Lipofectamine Plus as per manufacturer's protocol. Cells were collected for assays after 48 hours of transfection. Human proximal tubule epithelial (hPTE) cells were acquired from Mediatech and maintained in appropriate media. Transfection of d-siRNA into hPTE cells was performed with Lipofectamine 2000 (Invitrogen) and cells incubated for 48 hours before assaying.

5.2.3. RAMP cloning and expression: Full length cDNA of human RAMPs1, 2 and 3 and bovine CL-R were described before [281, 282]. CL-R, cloned into N1-EGFP and also in pcDNA3.1 expression vectors, was used for transfection in HEK 293T cells.

5.2.4. Desensitization and Resensitization assays: 48 hours post-tranfection cells were pretreated with or without 10 nM AM in DMEM containing 0.2% BSA for indicated time periods (up to 4 hr). After agonist exposure, cells were washed three times with Dulbecco's phosphate buffered saline (dPBS, Gibco BRL) containing 0.2% BSA and either frozen for membrane preparation for adenylate cyclase assays or used immediately for intact-cell radioligand binding. For receptor resensitization assays, after agonist exposure, cells were washed and incubated for indicated time periods in DMEM containing 0.2% BSA and 5 μg/ml cycloheximide to allow receptor recovery.



5.2.5. Receptor binding: Homologous competition radioligand binding assays were performed as described by Aiyar et al and as established in our laboratory [283]. HEK 293T cells were transfected and approximately 200,000 cells/well seeded in poly-D-

lysine pre-coated 24-well plates (BD Biosciences, Palo Alto, CA). 48 hours post-transfection cells were treated for desensitization or resensitization assays as described above. After agonist exposure, cells were washed three times with dPBS buffer containing 0.2% BSA then incubated with increasing concentrations (1 pM to 100 nM) of competing ligand (rAM) and 175-250 pM ^{125}I-rAM for 30 min at 37°C. After incubation, plates were washed three times with ice-cold assay buffer and the reactions were terminated by the addition of 2M NaOH. Cells were then harvested and associated radioligand activity is counted on a γ-counter. All binding assays were performed in duplicate, with each experiment repeated at least three times. Nonspecific binding was determined in the presence of 100 nM of unlabeled rAM. Data was analyzed by LIGAND (assuming radioligand and competitor both bind reversibly to a single binding site; MacLigand, Version 4.97, NIH, Bethesda, MD) using the following equation:

$$B = \frac{B_{max} \bullet [\text{hot ligand}]}{K_D + [\text{hot ligand}] + [\text{cold ligand}]}$$

Where B represents specific binding, [hot ligand] the single concentration of [^{125}I]rAM studied, [cold ligand] the concentration of unlabeled rAM competing with the radiolabel for AM receptor binding, B_{max} the maximum number of binding sites and KD the equilibrium dissociation constant (the equation was solved where the "cold ligand" IC_{50} = [hot ligand] + K_D). Analysis of all binding data was performed by computer-assisted nonlinear least square fitting using GraphPad PRIZM version 4 (GraphPad Software, San Diego, CA). Binding sites/cell was calculated and data was expressed as percent of the control.

5.2.6. Adenylate cyclase assays: Cyclase activity was done as described before with slight modifications [282, 284]. Cells were harvested from P100 or P60 plates and homogenized in Tris HCl (10mM)/EDTA (10mM) buffer. Membranes were prepared by homogenization and centrifugation in Tris HCl (50mM)/MgCl (10mM) buffer. Final concentration of 20 μg of protein/assay tube was obtained. Membranes were incubated for 15 min at 30°C with appropriate concentrations of drugs and assay mix containing ATP regeneration system and α^{32}P-ATP. After the reaction was stopped (with stop solution containing ^{3}H-cAMP) contents of the assay tubes were passed through Dowex and subsequently through alumina columns to separate the degradation products of ATP, by washing the dowex with water and alumina with imidazole. Elution profile was done to determine the amount of water and imidazole needed to wash and elute the products. Product eluted from alumina column was counted for the presence of ^{3}H-cAMP and α^{32}P-cAMP in a β-counter. Each experiment was done in triplicates and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. Basal cyclase activity and forskolin stimulation did not show statistically significant differences between treatments.

5.2.7. cAMP accumulation assays: Human proximal tubule cells were seeded on a 24-well plate until reaching 80-90% confluency, then incubated in serum-free media overnight before experiment. Desensitization experiments were carried out as described in Materials and Methods section, with cells pretreated with 10nM rAM and subsequently challenged for 10 min at 37°C with appropriate concentrations of drugs (100nM AM,

10μM Forskolin) in the presence of 200μM 3-isobutyl-1-methylxanthine. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in human proximal tubule cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. Basal cyclase activity and forskolin stimulation did not show statistically significant differences between treatments.

5.2.8. RNA Interference analysis: Gene-specific d-siRNA for *lacZ* (control), *NHERF-1* and *RAMP3* were generated and purified using BLOCK-iT Dicer RNAi kit from Invitrogen (Carlsbad, CA). hPTE cells were transfected with d-siRNAs using Lipofectamine 2000 as per manufacturer's instructions (Invitrogen). 48 hours after transfection cells were frozen for mRNA analysis, or used for cAMP accumulation assays or immunofluorescence microscopy.

5.2.9. Reverse transcriptase PCR (RT-PCR) analysis: RT-PCR analysis performed as described before [282].Total RNA was isolated from hPTEs using Trizol reagent (GIBCO BRL). After sodium acetate-ethanol precipitation and several ethanol washes, RNA was used as a template in a reverse transcriptase PCR amplification procedure. The RT-PCR reaction was carried out using Superscript One-Step RT-PCR with Platinum *Taq* (GIBCO BRL), in accordance with the manufacturer's specifications. Reactions were carried out, with a Perkin-Elmer model 9600 thermal cycler, in 50 μl of total reaction volumes subjected to the following conditions: *1*) 94°C for 2 min (1 cycle); *3*) 94°C for

30 s, 50°C for 30 s, 68°C for 30 s (30 cycles); and *4*) 68°C for 7min (1 cycle). Products were separated by gel electrophoresis and subsequently visualized by ethidium bromide staining and ultraviolet illumination. Photographs of the gels were taken and digitalized with a UMAX Astra 2000P flat-bed scanner.

5.2.10. Mutagenesis procedure: Site-directed mutagenesis was performed using a PCR-based strategy that employs the *pfu* Turbo polymerase (Stratagene, La Jolla, CA). A pair of complementary oligonucleotides containing the appropriate point mutations in the sequence of RAMP/CL-R or a premature stop codon at position 145 or 147 codon of RAMP3 for deletion mutants were synthesized (Michigan State University Macromolecular structure facility). The PCR for the mutation was as follows: 94°C for 5 minutes; 30 cycles of 94°C for 30 sec., 50°C for 30 sec., 68°C for 8 min.; final cycle of 68°C for 8 minutes. PCR product was digested for 4 hours with DpnI enzyme (Invitrogen) and transformed in to DH5α cells. Mutations were confirmed by automated sequencing (Michigan State University Genomic Technology Support Facility).

5.2.11. Immunofluorescence microscopy: HEK293 and hPTE cells were transfected as described above and seeded at 24hr post-transfection onto collagen type I-coated coverslips. Desensitization assays were performed as described and reactions were stopped by fixing cells in 4% paraformaldehyde for 30min. at room temperature. Samples were permeablized with 0.1% v/v Triton X-100 in PBS and blocked overnight in 0.1% v/v Triton X-100 in PBS + 10% goat serum. Samples were incubated in primary antibody in blocking buffer for 2h at room temperature (NHERF-1 at 1:250 and RAMP3

at 1:200). Appropriate secondary antibodies were applied for 1h at room temperature (Goat anti-mouse Cy3 at 1:500 and Goat anti-rabbit Cy5 at 1:400). Cytoskeletal staining was carried out using Alexa 488-phalloidin antibody at 1:75 (Molecular Probes). Coverslips were mounted in Shandon mounting medium and slides store at 2-8°C until analysis. Cells were visualized on a Zeiss 210 laser confocal microscope at a zoom of 2. Images presented are representative single optical sections of a z-series taken from at least twenty fields per experiment and at least three individual experiments. Images in this thesis/dissertation are presented in color.

5.2.12. Fusion protein overlays and western blotting: Overlay assays and western blotting performed as described before [153]. 10 μg of GST-fusion proteins were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose filters. Filters were blocked with 5% w/v fat-free milk powder in Tris-buffered saline with Tween 20 (TTBS: 20 mM Tris, pH 7.4, 500 mM NaCl, 0.1% v/v Tween 20) and incubated overnight at 4 °C in lysates of HEK293 cells with or without overexpression NHERF-1. Blots were then washed three times with TTBS buffer and incubated with anti-EBP50 (NHERF-1) monoclonal antibody for 2 h at room temperature. After three washes with TTBS, filters were incubated for 1h with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Gibco BRL®, Grand Island, NY), washed again with TTBS, soaked in Supersignal West Pico chemiluminescent substrate (Pierce) and exposed to x-ray film. Same protocol, with the exception of the overnight incubation with cell lysate, was followed for immunoblot analysis of RAMP3.

5.2.13. Statistics: Data are presented as mean ± S.E.M. Single group comparisons exercised a paired Student's t-test method. Statistical significance was set at $P<0.05$.

5.3. Results

5.3.1. Role of NHERF-1 in Internalization of the CL-R/RAMP complex

We and others have previously shown that the AM2 receptor (CL-R + RAMP3 complex) undergoes agonist-stimulated desensitization, internalization, and degradation [153, 276, 285]. Furthermore, we have recently shown that in the presence of NSF, the AM2 receptor complex undergoes recycling, instead of following a degradation pathway, in HEK 293 cells [153]. In this study, we have examined the role of another protein, namely NHERF-1, on agonist-induced trafficking of the CL-R/RAMP3 complex.

Pretreatment of HEK 293 cells expressing CL-R and RAMP3 with 10 nM AM for one hour resulted in desensitization of the adenylate cyclase response from 50% (of forskolin stimulation) in untreated cells to 28% in AM-treated cells (Figure 17A). In addition, as shown in Figure 17B and 18, CL-R/RAMP3 complex underwent agonist-induced internalization as determined by receptor binding and immunofluorescence microscopy. These findings are in agreement with those of Kuwasako *et al.* and as reported by us recently [153, 276]. However, over-expression of NHERF-1 with CL-R/RAMP3 resulted in a remarkable change in the agonist-induced receptor complex trafficking in HEK293 cells.

HEK293 cells transfected with CL-R/RAMP3 and NHERF-1 showed similar levels of adenylate cyclase activity and desensitization patterns as compared to CL-R/RAMP3 alone (Figure 17A). But in the presence of NHERF-1, the receptor complex

failed to internalize with agonist pretreatment (Figure 17B, 18). To determine if NHERF-1 co-expression had changed the kinetics of internalization, agonist pretreatment was carried out for a time course extending to 4 hours. Four hours of agonist pretreatment still yielded a complete inhibition of receptor complex internalization in HEK293 cells co-expressing NHERF-1 and the CL-R/RAMP3 complex, as compared to a continued internalization of the receptor complex in HEK293 cells lacking NHERF-1 over-expression (Figure 17C). These results indicate that over-expression of NHERF-1 alters the trafficking of the CL-R/RAMP3 receptor complex after AM-stimulated desensitization.

5.3.2. RAMP isoform-specific regulation of CL-R/RAMP receptor complex trafficking

To determine if this effect of NHERF-1 was specific for RAMP3, the additional RAMPs (RAMP1 or -2) were tested for their ability to act with NHERF-1 to alter the receptor complex trafficking. Interestingly, in contrast to RAMP3, presence of NHERF-1 did not alter the internalization pattern of the CL-R/RAMP1 or -2 receptor complexes. No significant differences were seen in the receptor numbers from whole-cell binding when CL-R was co-expressed with RAMP1, 2, or 3 (with and without NHERF-1). Desensitization patterns in cells transfected with CL-R+RAMP1 or CL-R+RAMP2 also remained unchanged in the absence or presence of NHERF-1 (Figure 19A-D). These results indicate that RAMP3 must contain a molecular feature distinct from the other RAMPs that allowed its interaction and action with NHERF-1.

5.3.3. Role of PDZ interactions in trafficking of the CL-R/RAMP3 complex

We have observed before that NSF regulated the CL-R/RAMP3 recycling by interacting specifically with the PDZ motif present at the extreme C-terminus of RAMP3. Neither CL-R, nor the other two RAMP isoforms (RAMP1 or RAMP2), contain PDZ recognition motifs. To test the hypothesis that this domain is critical for interaction of the CL-R/RAMP3 complex with NHERF-1, the PDZ motif (-DTLL) on RAMP3 was deleted (RAMP3Δ145-8) and an internalization assay was performed. Deletion of this domain did not affect basal adenylate cyclase activity or the desensitization response of the CL-R/RAMP3 complex in response to AM, even in the presence of NHERF-1. In addition, the RAMP3 PDZ motif mutant (RAMP3Δ145-8) showed no difference in receptor expression levels at the plasma membrane (as measured with whole-cell binding), as compared to wild-type CL-R/RAMP3 complex (in the presence/absence of NHERF-1). Unlike CL-R/RAMP3 complex, the CL-R/RAMP3Δ145-8 receptor complex was now capable of AM-induced internalization, as measured by whole-cell receptor binding and immunofluoresence microscopy (Figure 20A and 21).

To further test the hypothesis that the absence of the PDZ motif on the RAMP2 accounts for the lack of ability of NHERF-1 to interact and therefore inhibit internalization, the PDZ motif of RAMP3, the amino acids -DTLL, were substituted on the C-terminus of RAMP2, in exchange for its original four C-terminal amino acids (-EAQA). The CL-R/RAMP2ΔDTLL mutant complex expression levels were comparable to that of CL-R/RAMP2 complex, as determined by whole-cell binding and adenylate cyclase assays. Additionally, in the absence of NHERF-1, both CL-R/RAMP2 and CL-R/RAMP2ΔDTLL showed similar levels of AM-stimulated internalization. However, co-

expression of NHERF-1 with CL-R/RAMP2ΔDTLL was now capable of inhibiting the internalization of the receptor complex with agonist pretreatment, as compared to wild-type CL-R/RAMP2 complex (Figure 20B). These findings provide additional evidence that the PDZ motif on the RAMP3 interacts with NHERF-1, causing an inhibition of receptor internalization, despite normal desensitization.

To identify the critical amino acids in the PDZ binding sequence that regulate the RAMP3/NHERF-1 interaction, site-directed mutagenesis was performed to mutate the individual amino acids of the PDZ motif to alanine. Mutations of the individual amino acids in the PDZ motif of RAMP3 did not affect the basal levels of receptor expression and function, as measured by whole-cell binding experiments and adenylate cyclase assays, respectively. In addition, the desensitization and internalization in the absence of NHERF-1 were also similar between the wild-type and mutant CL-R/RAMP3 complexes. However, in the presence of NHERF-1, the CL-R/RAMP3T146A complex now underwent agonist-stimulated internalization, similar to when expressed without NHERF-1 (Figure 20C). The other point mutant RAMP3/CL-R complexes behaved like wild-type in the presence of NHERF-1, indicating Thr^{146} in the PDZ domain is critical for the PDZ interaction between RAMP3 and NHERF-1.

As described before, NHERF-1 contains two PDZ domains through which it interacts with numerous proteins [258]. To determine which PDZ domain of NHERF-1 is responsible for the interaction with RAMP3, the two PDZ domains of NHERF-1 were deleted individually and agonist-induced internalization assays, employing whole-cell receptor binding, were performed. Cells expressing wild-type or mutant NHERF-1 with CL-R/RAMP3 receptor complex showed comparable levels of receptor expression and

function, as assessed by whole-cell receptor binding and adenylate cyclase assays, respectively. In addition, they showed similar desensitization patterns. However, internalization assays (with whole-cell binding) showed that the first PDZ domain of NHERF-1 is responsible for the interaction with RAMP3. HEK 293 cells expressing the mutant NHERF-1ΔPDZ1 (lacking only the first PDZ domain) and CL-R/RAMP3 complex underwent AM-stimulated internalization compared to cells expressing the wild-type NHERF-1 (Figure 20D). Deletion of the second PDZ domain of NHERF-1 (NHERF-1ΔPDZ2) had no effect on the internalization pattern of CL-R/RAMP3, as compared to wild-type NHERF-1 (Figure 20D). These findings further confirm that RAMP3 and NHERF-1 are interacting via their PDZ domains to inhibit internalization of the CL-R/RAMP3 receptor complex.

To examine if the PDZ domain on RAMP3 is physically interacting with NHERF-1, overlay assays were performed. This was accomplished using GST-RAMP3 fusion proteins in an overlay assay with cell lysates of HEK293 cells overexpressing NHERF-1. Control experiments run with GST protein showed no detectable bands when incubated with NHERF-1 lysates and probed with an NHERF-1 antibody (Figure 22A). Importantly, GST- RAMP3 fusion proteins showed significant interaction with NHERF-1 in the cell lysates of HEK293 cells overexpressing NHERF-1 in the overlay assay (Figure 22A). As a control, lysates of HEK 293 cells not over-expressing NHERF-1 showed no detectable bands when run with GST-RAMP3 in the overlay assay (Figure 22B). Additionally, when GST-RAMP3Δ145-8 fusion proteins were tested for interaction with NHERF-1 using the above described overlay assay, no bands were detected, in contrast to wild-type GST-RAMP3 (Figure 22A). When blots used in the overlay assay were

stripped and probed for RAMP3, a band for RAMP3 was detected at the exact location as that of the NHERF-1 band detected in the overlay assay (Figure 22C). This data demonstrates a physical interaction between RAMP3 and NHERF-1, an interaction that is dependent on PDZ domain interactions of the two proteins and is capable of regulating CL-R/RAMP3 (AM2R) complex trafficking.

5.3.4. Mechanism of inhibition of CL-R/RAMP3 internalization by NHERF-1

The ERM domain of NHERF-1 is known to interact with MERM cytoskeletal proteins, allowing NHERF-1 to tether proteins to the actin cytoskeleton [304]. Hence, we hypothesized that the CL-R/RAMP3 receptor complex internalization is regulated by NHERF-1 through interactions of NHERF-1 with cytoskeletal proteins. To test this hypothesis, we employed a mutant of NHERF-1 with a deletion of its ERM domain [252]. Control experiments with the ERM domain mutants of NHERF-1 co-transfected with CL-R/RAMP3 showed similar levels of receptor expression and function, as measured by whole-cell binding and adenylate cyclase. As hypothesized, the ERM domain mutant of NHERF-1, when co-expressed in HEK 293 cells with CL-R/RAMP3 complex, was now capable of internalization after agonist pretreatment, differing from the wild-type NHERF-1 that inhibits receptor internalization (Figure 23A). These experiments were repeated and confirmed with confocal immunofluorescence microscopy, using phalloidin to label the actin cytoskeleton and test for NHERF-1 co-localization. While wild-type NHERF-1 colocalized with the actin cytoskeleton (to presumably tether RAMP3 and therefore CL-R to the plasma membrane after agonist pretreatment), the ERM domain mutant of NHERF-1 showed disrupted actin

cytoskeleton co-localization and therefore, did not block the internalization of RAMP3 (and CL-R) with agonist pretreatment (Figure 23B). These findings indicate that NHERF-1 inhibits the internalization of the CL-R/RAMP3 receptor complex by acting as an adaptor to tether the receptor complex to the actin cytoskeleton.

To further test this mechanism for inhibiting internalization, an actin depolymerization agent (Cytochalasin D) was employed to disrupt the actin cytoskeleton and the ability of NHERF-1 to tether the CL-R/RAMP3 complex to the plasma membrane. Treatment of HEK293 cells expressing CL-R/RAMP3 in the presence and absence of NHERF-1 had no effect on the adenylate cyclase activity or desensitization pattern of the receptor complex (Figure 23C). After treatment with Cytochalasin D, HEK293 cells expressing CL-R/RAMP3 and NHERF-1 were now capable of internalization. The Cytochalasin D experimental data further supports that the mechanism of inhibition of internalization of the CL-R/RAMP3 complex by NHERF-1 by tethering of the receptor complex to the plasma membrane by NHERF-1's interaction with the actin cytoskeleton.

5.3.5. NHERF's role in receptor trafficking in primary human proximal tubule epithelial cells

To examine if our observations using the over-expressed system in HEK293 cells could be translated to a more physiological cell type, we chose human proximal tubule cells to test our hypothesis. Because of the many roles of adrenomedullin and NHERF-1 in the kidney, we chose a human primary proximal tubule cell line to perform these studies. The human proximal tubule epithelial cells (hPTEs) were determined to express

CL-R, RAMP2, RAMP3, and NHERF-1 and show high levels of specific adrenomedullin binding and receptor stimulation by AM (data not shown). When pretreated with AM for a period of one hour, desensitization was observed with adenylate cyclase assays (Figure 24A). However, as we would predict, internalization was not observed (by whole-cell receptor binding assays) (Figure 24B). Internalization was not observed when hPTE cells were pretreated with AM for up to four hours, indicating desensitization and internalization were not simply showing different kinetics. In order to test our hypothesis that RAMP3 and NHERF-1 were critical to the inhibition of internalization of the CL-R/RAMP3 receptor complex, RNA interference technology was employed to individually knockdown RAMP3 and NHERF-1. In both mRNA and protein expression studies, RAMP3 and NHERF-1 dramatically decreased in d-siRNA treated samples, while control experiments using *lacZ* knockdown showed no significant alteration in RAMP3 or NHERF-1 expression when compared to wild-type hPTE cells (Figure 25A, B; data not shown). Internalization assays and whole-cell receptor binding assays were performed to determine the effect of RAMP3 and NHERF-1 RNA interference on receptor internalization in hPTE cells. Strikingly, when pretreated with agonist, hPTE cells with RAMP3 or NHERF-1 RNA interference showed a regained ability to internalize the receptor complex, unlike the wild-type hPTE cells were internalization was inhibited (Figure 25C). This finding demonstrates that RAMP3 and NHERF-1 are both critical for the receptor trafficking of the CL-R/RAMP3 complex in hPTE cells, an unaltered cell line absent the issues of overexpression.

To determine if the mechanism of inhibition of internalization in hPTE cells was similar to that in the HEK 293 cells, actin cytoskeletal tethering was examined in the

hPTE cells. hPTE cells were treated with Cytochalasin D, as was used in HEK 293 cells to disrupt the actin cytoskeleton, and internalization assays were performed. Cytochalasin D treatment did not alter the receptor expression levels in the hPTE cells, as measured by whole-cell receptor binding, from the levels in untreated cells. Interestingly, when cells were treated with Cytochalasin D and pretreated with AM for one hour, the receptor complex was now capable of internalization (Figure 26). This data supports the proposed model that NHERF-1 inhibits the internalization of the CL-R/RAMP3 receptor complex after agonist stimulation by tethering the receptor complex to the actin cytoskeleton via NHERF-1's ERM domain interactions with the cytoskeleton and PDZ domain interactions with RAMP3.

Finally, it was important to determine the effect of the inhibition of internalization on AM signaling in the hPTE cells. To this end, a resensitization assay was performed and cAMP accumulation was measured. AM pretreatment of the hPTE cells results in an attenuation of cAMP accumulation from 35% to 15% of maximal response, whereas allowed recovery time in the absence of agonist after the AM pretreatment was shown to recover AM signaling to similar levels as untreated cells. While it was shown in a previous Figure that AM pretreatment causes desensitization of the AM receptor signaling in the hPTE cells, the resensitization assay now showed that resensitization occurs in the hPTE cells, even in the absence of receptor internalization (Figure 24A, 27). This finding suggests the inhibition of internalization of the AM receptor in the hPTE cells is a mechanism for receptor resensitization, not requiring receptor internalization as a prerequisite.

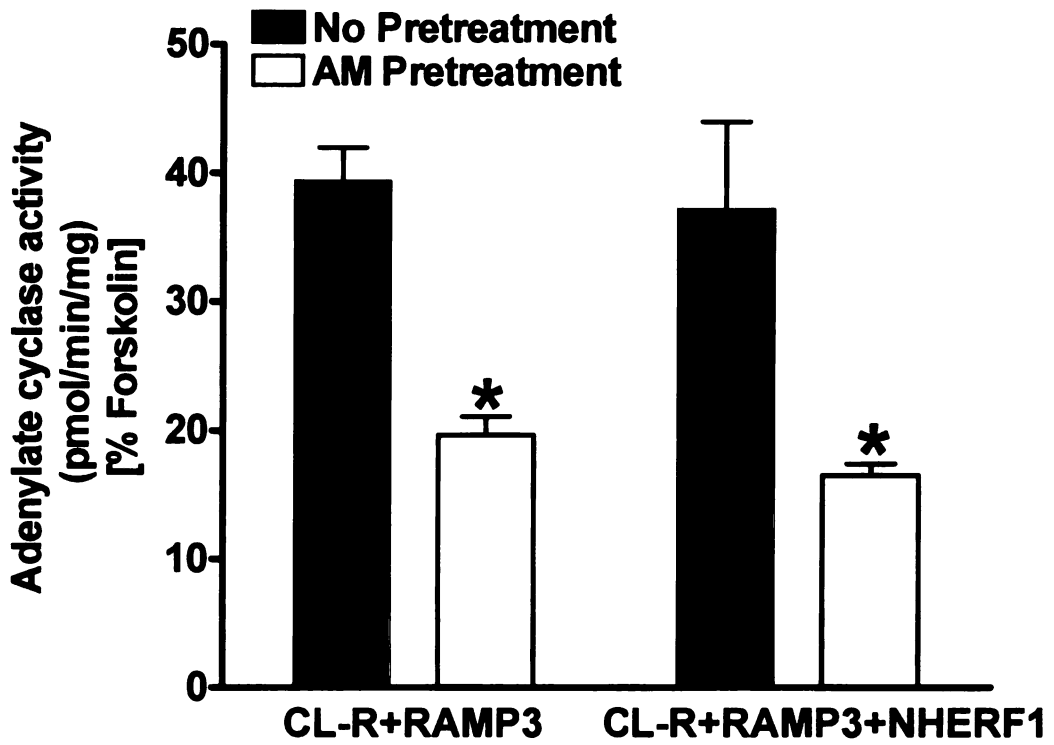


Figure 17A: The role of NHERF-1 in the trafficking of the AM2R (CL-R+RAMP3) in HEK 293 cells. NHERF-1 does not alter desensitization of AM2R in transfected HEK 293 cells. HEK 293 cells transiently transfected with CL-R and RAMP3 with or without NHERF-1. At 48h post-transfection, cells were treated for one hour with AM (10 nM) and then washed and adenylate cyclase activity was measured. After agonist pretreatments, membranes were extracted and AC activity in response to 100 nM AM was measured. NHERF-1 overexpression with AM2R had no significant effect on receptor desensitization. Experiments performed in triplicates and data expressed as percent maximal stimulation (% Forskolin). * $p \leq 0.05$; $n \geq 4$.

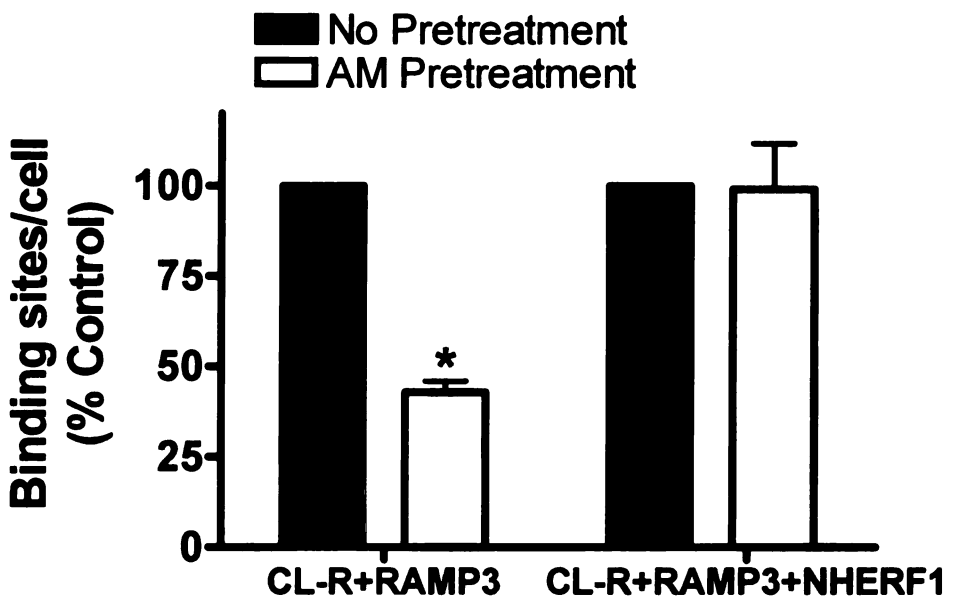


Figure 17B: The role of NHERF-1 in the trafficking of the AM2R (CL-R+RAMP3) in HEK 293 cells. NHERF-1 inhibits internalization of AM2R in HEK 293 cells. HEK 293 cells transfected and pretreated with agonist as described in 17*A*. Receptor internalization measured by whole-cell competition binding assays using ^{125}I-rAM as ligand (cold rAM served as the competitor) and number of binding sites/cell was estimated using the GRAPHPAD PRISM software. "No pretreat" represents samples at maximal radioligand binding that were not pre-incubated with agonist. "1h pretreat" represents samples pretreated with AM (10 nM) for one hour, washed as indicated in Methods section, and tested immediately after wash steps for radioligand binding. NHERF-1 overexpression in cells expressing AM2R caused altered receptor trafficking to inhibit internalization of the receptor complex. * $p \leq 0.05$; $n \geq 4$ experiments.

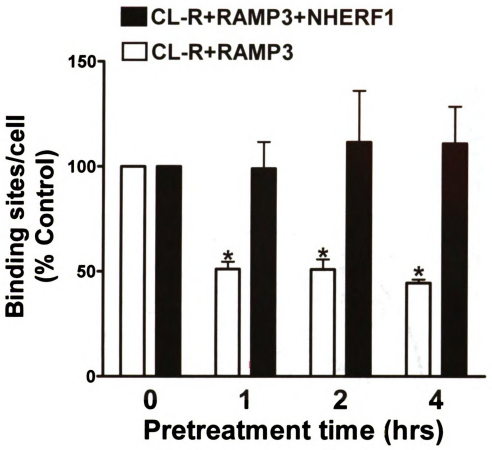


Figure 17C: The role of NHERF-1 in the trafficking of the AM2R (CL-R+RAMP3) in HEK 293 cells. Time course internalization of AM2-R in presence/absence of NHERF-1. Lengthening of agonist pretreatment does not alter inhibition of internalization of CL-R/RAMP3 complex with NHERF-1 co-expression. HEK 293 cells transfected and pretreated with agonist for varying time points, as described in 17*A*. Receptor internalization measured by whole-cell competition binding assays using ^{125}I-rAM as ligand (cold rAM served as the competitor) and number of binding sites/cell was estimated using the GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$ experiments.

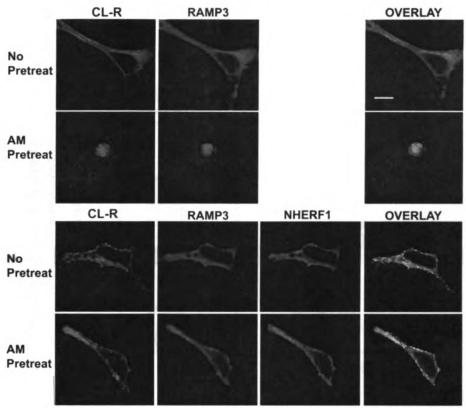


Figure 18: Localization of CL-R, RAMP3, and NHERF-1 in HEK 293 cells during an internalization experiment. After 1h AM pretreatment, CL-R and RAMP3 are internalized in absence of NHERF-1 co-expression. In cells co-expressing NHERF-1 with CL-R/RAMP3 complex, internalization of receptor complex is blocked after AM pretreatment. HEK 293 cells transfected with CL-R-GFP, RAMP3, and NHERF-1 were pretreated with 10nM ADM for 1h. Note: "1 hr pretreatment" indicates time just after AM pretreatment and wash steps. Cells were fixed and components were visualized using anti-RAMP3 antibody (1:200) and anti-NHERF-1 antibody (1:250) with Cy5 anti-rabbit secondary antibody (1:400, in blue) and Cy3 anti-mouse secondary antibody (1:500, in red), respectively; CL-R-GFP is detected with an EGFP tag and shown in green; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least twenty fields imaged per experiment from at least three experiments. Bar scales on all images represent 100μm.

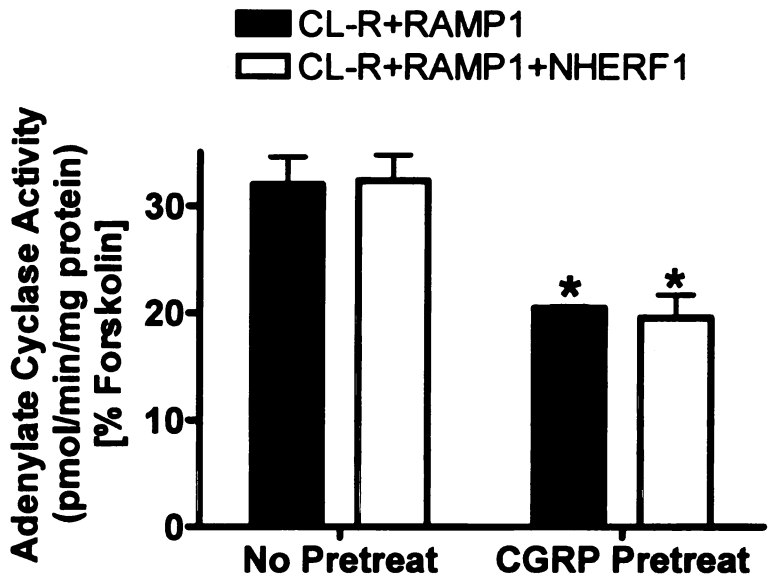


Figure 19A: The role of NHERF-1 in post-endocytic sorting of CGRP1 (CL-R+R1) and AM1 receptor. NHERF-1 overexpression does not alter CGRP receptor desensitization after agonist stimulation. HEK 293 cells were transfected and pretreated as in figure 17*A* and then membranes were extracted. Adenylate cyclase activity was measured in membranes stimulated with 100 nM CGRP for 15 min. Experiments performed in triplicates and expressed as percent maximal stimulation (% Forskolin). * $p \leq 0.05$; $n \geq 3$.

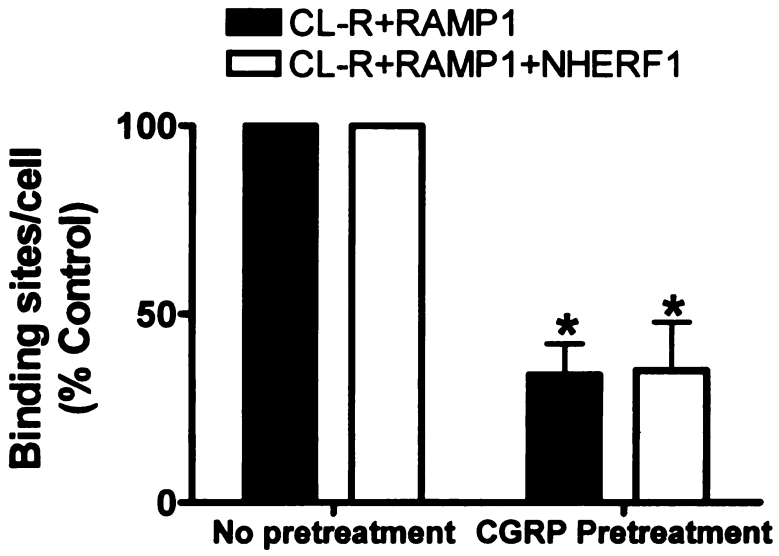


Figure 19B: The role of NHERF-1 in post-endocytic sorting of CGRP1 (CL-R+R1) and AM1 receptor. NHERF-1 overexpression does not alter the trafficking of the CGRP receptor after agonist stimulation. HEK 293 cells were transfected with CL-R and RAMP1, with or without NHERF-1. 48h post-transfection, cells were pretreated with CGRP (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell competition binding using ^{125}I-rCGRP as the ligand and cold rCGRP as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

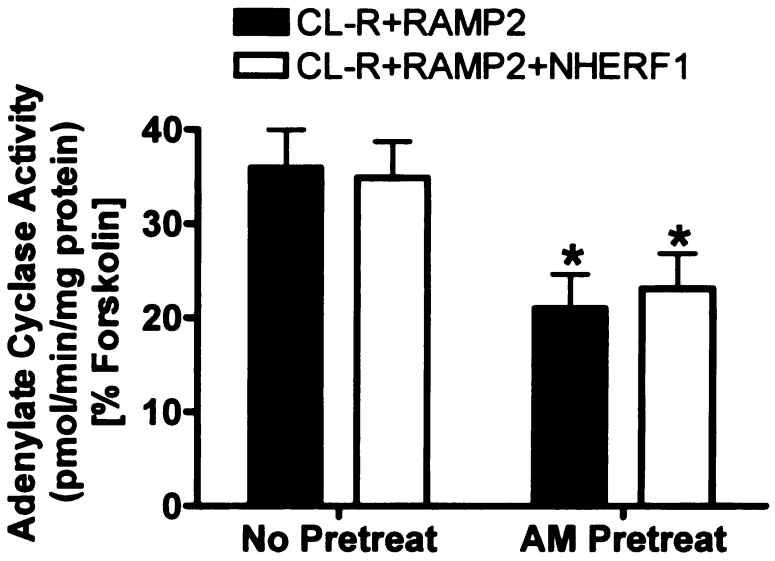


Figure 19C: The role of NHERF-1 in post-endocytic sorting of CGRP1 (CL-R+R1) and AM1 receptor. NHERF-1 overexpression does not alter AM1 receptor desensitization after agonist induction. HEK 293 cells were transfected and pretreated as in figure 17*A* and then membranes were extracted. Adenylate cyclase activity was measured in membranes stimulated with 100 nM AM for 15 min. Experiments performed in triplicates and expressed as percent maximal stimulation (% Forskolin). * $p \leq 0.05$; $n \geq 3$.

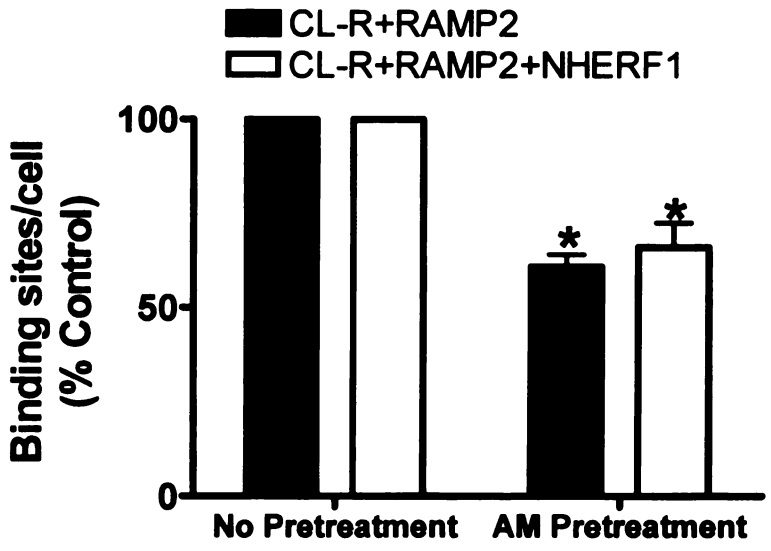


Figure 19D: The role of NHERF-1 in post-endocytic sorting of CGRP1 (CL-R+R1) and AM1 receptor. NHERF-1 overexpression does not alter internalization of AM1 receptor after receptor activation. HEK 293 cells were transfected with CL-R and RAMP2, with or without NHERF-1. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell competition binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

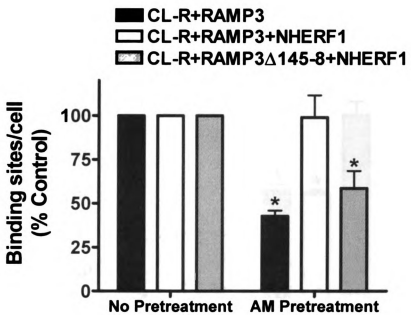


Figure 20A: The effect of RAMP3 PDZ motif deletion on the trafficking of the AM2R (CL-R+RAMP3). Deletion of RAMP3 PDZ motif allowed internalization of the CL-R/RAMP3 complex when co-expressed with NHERF-1. HEK 293 cells were co-transfected with CL-R, wild-type RAMP3 or RAMP3Δ145-8, and NHERF-1. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

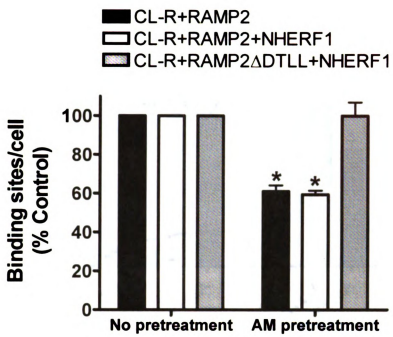


Figure 20B: Effect of PDZ motif substitution on the C-terminus of RAMP2 on internalization of CL-R/RAMP complex. Substitution of PDZ motif (-DTLL) on the C-terminus of RAMP2 caused a change in receptor trafficking to inhibit internalization of the receptor complex when co-expressed with NHERF-1. HEK 293 cells were co-transfected with CL-R, wild-type RAMP2 or RAMP2ΔDTLL, and NHERF-1. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

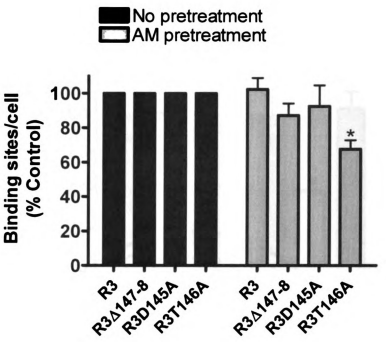


Figure 20C: Effect of RAMP3 PDZ motif point mutations on the internalization of the CL-R/RAMP3 complex in the presence of NHERF-1. Only point mutant RAMP3T146A was critical in the inhibition of the internalization of the CL-R/RAMP3 complex seen when co-expressed with NHERF-1. HEK 293 cells were co-transfected with CL-R, wild-type RAMP3 or RAMP3 point mutants, and NHERF-1. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$..

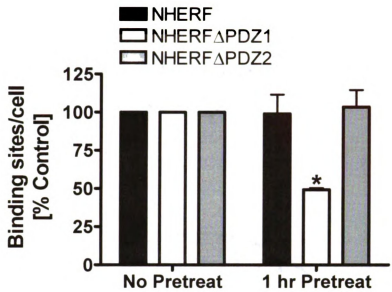


Figure 20D: The effect of NHERF-1 PDZ domain deletion on the trafficking of the AM2R (CL-R+RAMP3). Deletion of NHERF-1 PDZ1 domain allowed internalization of the CL-R/RAMP3 complex. HEK 293 cells were co-transfected with CL-R, wild-type RAMP3, and wild-type or PDZ domain mutants of NHERF-1. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

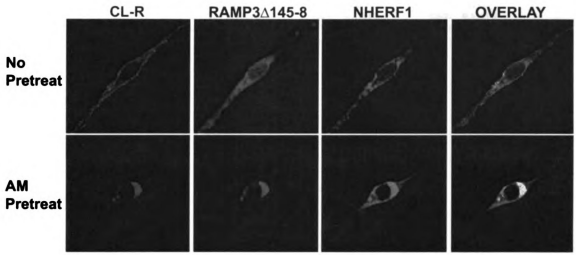


Figure 21: Localization of CL-R, RAMP3Δ145-8, and NHERF-1 in HEK 293 cells during an internalization experiment. Untreated and after one hour ADM (10nM) pretreatment, CL-R and RAMP3Δ145-8 internalize similarly to the wild-type CL-R/RAMP3 complex in the absence of NHERF-1. Experiments performed as described in Figure 18. Fixed cells were stained with anti-RAMP3 antibody (1:200) and anti-NHERF-1 antibody (1:250) with Cy5 anti-rabbit secondary antibody (1:400, in blue) and Cy3 anti-mouse secondary antibody (1:500, in red), respectively; CL-R-GFP is shown in green; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least twenty fields imaged from at least three experiments. Bar scales on all images represent 100μm.

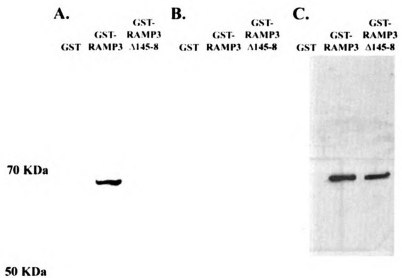


Figure 22: Interaction studies of RAMP3 with NHERF-1. Fusion proteins of GST-RAMP3 demonstrate a physical interaction with NHERF-1 in an overlay assay and western blot analysis. 10μg of GST, GST-RAMP3, and GST-RAMP3Δ145-8 protein was run and separated on an SDS-PAGE gel and transferred to a nitrocellulose filter. The nitrocellulose filter was incubated overnight in lysate of HEK293 cells transfected with or without NHERF-1 at 4°C. Filter was then washed and probed by immunoblot for NHERF-1 (1:250). Identical filters were probed by immunoblot for RAMP3 (1:400). *A*, GST, GST-RAMP3, and GST-RAMP3Δ145-8 overlay assay incubated in NHERF-1-transfected HEK 293 lysates and probed for NHERF-1. *B*, GST, GST-RAMP3, and GST-RAMP3Δ145-8 overlay assay incubated in non-transfected HEK 293 lysates and probed for NHERF-1. *C*, Immunoblot of GST, GST-RAMP3, and GST-RAMP3Δ145-8 probed for RAMP3. Shown are representative blots of at least four experiments.

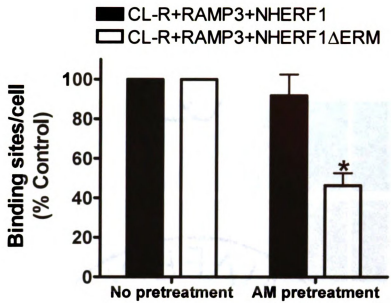


Figure 23A: Mechanism of NHERF-1 inhibition of internalization of CL-R/RAMP3 receptor complex. ERM domain mutant of NHERF-1 blocks the inhibition of internalization of CL-R/RAMP3 complex seen with wild-type NHERF-1. HEK 293 cells were transfected with CL-R, RAMP3, and wild-type NHERF-1 or ERM domain mutant NHERF-1. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

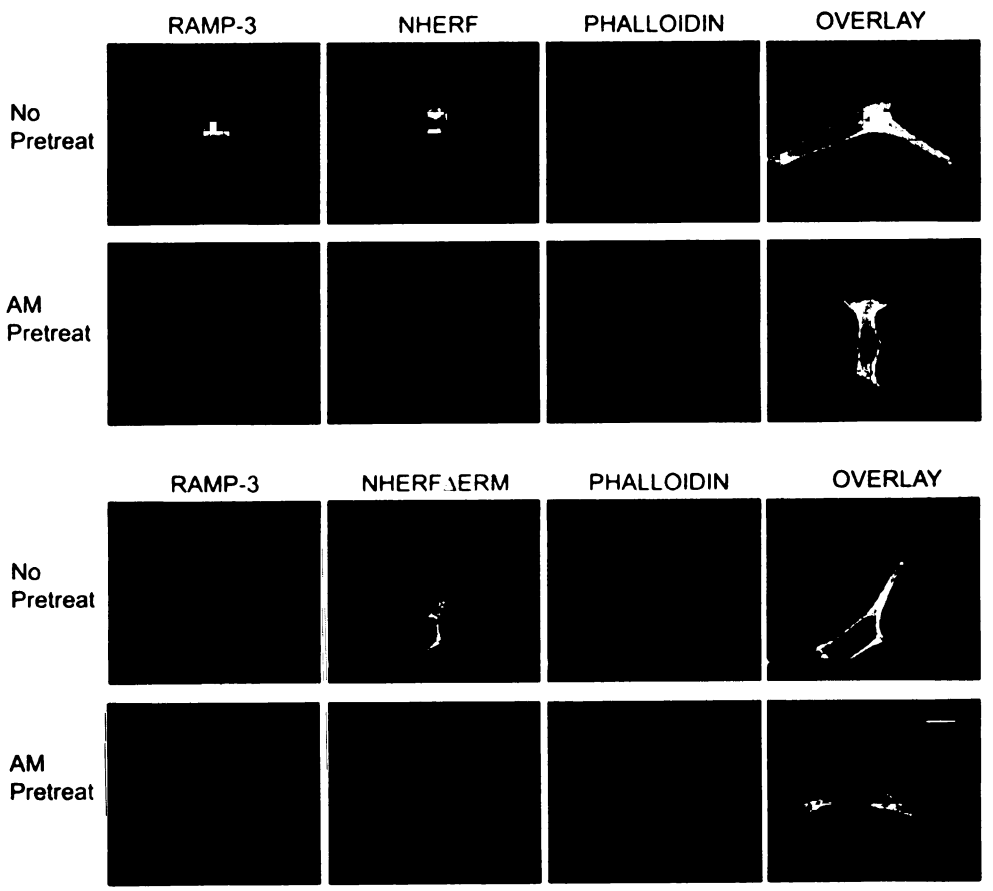

RAMP-3
NHERF
PHALLOIDIN
OVERLAY
No Pretreat
AM Pretreat
RAMP-3
NHERF ΔERM
PHALLOIDIN
OVERLAY
No Pretreat
AM Pretreat

Figure 23B: Mechanism of NHERF-1 inhibition of internalization of CL-R/RAMP3 receptor complex. Localization of RAMP3, NHERF-1 (or NHERF-1ΔERM), and actin cytoskeleton in HEK 293 cells during an internalization experiment. Untreated cells show RAMP3 and wild-type NHERF-1 distributed at the plasma membrane with NHERF-1 colocalizing with the actin cytoskeleton, and after AM pretreatment, all component remain in the same distribution. ERM domain mutant of NHERF-1 shows poor co-localization with the actin cytoskeleton in both untreated and AM-pretreated conditions, and therefore, shows internalization of RAMP3 with agonist pretreatment, HEK 293 cells were transfected with CL-R, RAMP3, and wild-type or ERM domain mutant NHERF-1. Experiments performed as described in Figure 18. Fixed cells were stained with anti-RAMP3 antibody (1:200) and anti-NHERF-1 antibody (1:250) with Cy5 anti-rabbit secondary antibody (1:400, in blue) and Cy3 anti-mouse secondary antibody (1:500, in red), respectively; actin cytoskeleton is stained with Alexa 488-phalloidin and is shown in green; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least twenty fields imaged from at least three experiments. Bar scales on all images represent 100μm.

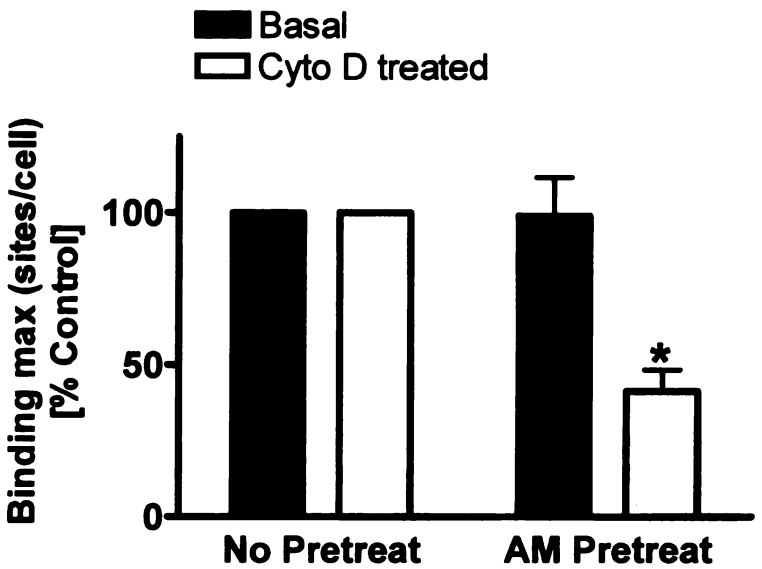


Figure 23C: Mechanism of NHERF-1 inhibition of internalization of CL-R/RAMP3 receptor complex. Cytochalasin D treatment blocks the inhibition of internalization of CL-R/RAMP3 complex. HEK 293 cells were transfected with CL-R, RAMP3, and wild-type NHERF-1. 48h post-transfection, cells were pretreated Cytochalasin D (10μM) for 15 min., then with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

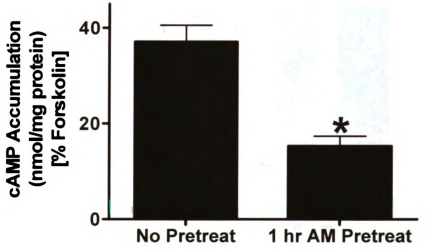


Figure 24A: Trafficking of AM-R in human proximal tubule cells (hPTE cells). In hPTE cells, AM-R signaling desensitizes with AM pretreatment. hPTE cells seeded in 24-well plates were pretreated with 10nm rAM for 1h were washed extensively to remove residual agonist and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in hPTE cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 4$ experiments.

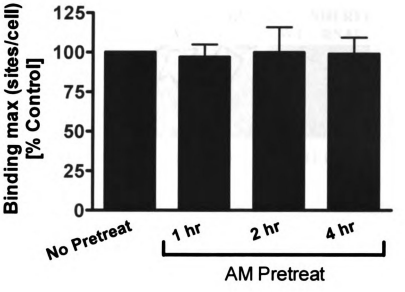


Figure 24B: Trafficking of AM-R in human proximal tubule cells (hPTE cells). AM-R fails to internalize when incubated in AM for varying time points. hPTE cells were grown as described in Figure 24A. Receptor internalization measured by whole-cell competition binding assays using ^{125}I-rAM as ligand (cold rAM served as the competitor) and number of binding sites/cell was estimated using the GRAPHPAD PRISM software. "No pretreat" represents samples at maximal radioligand binding that were not pre-incubated with agonist. "1,2, and 4h pretreat" represents samples pretreated with AM (10 nM) for according time period, washed as indicated in Methods section, and tested immediately after wash steps for radioligand binding. * $p \leq 0.05$; $n \geq 3$ experiments. In cells endogenously expressing AMR, internalization is inhibited, but desensitization in unaltered.

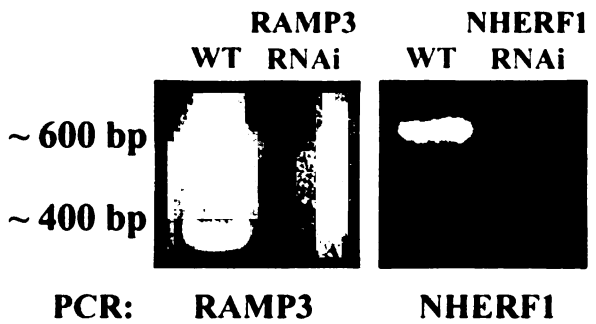


Figure 25A: Effect of RAMP3 and NHERF-1 RNA interference on internalization of AM2-R in human proximal tubule cells. RAMP3 and NHERF-1 RNA interference disrupts the inhibition of internalization of AM-R after agonist pretreatment. hPTE cells seeded in 48-well plates were transfected with d-siRNA of RAMP3 or NHERF-1 and incubated for 48 hr to allow RNA knockdown. mRNA analysis by Q-PCR showed dramatically decreased levels of RAMP3 and NHERF-1 mRNA in RAMP3 and NHERF-1 RNA interference samples (respectively) as compared to wild type, while *lacZ* knockdown had no effect on RAMP3 or NHERF-1 message levels (data not shown). RNA isolation and RT-PCR performed as described in Experimental Procedures section. n=3 experiments.

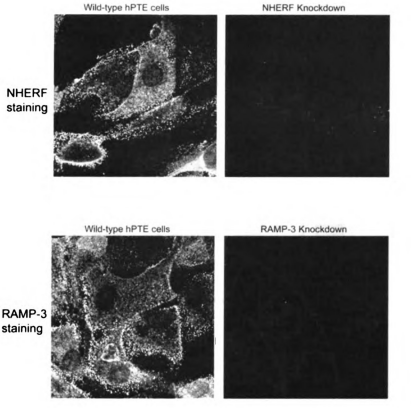


Figure 25B: Effect of RAMP3 and NHERF-1 RNA interference on internalization of AM2-R in human proximal tubule cells. RAMP3 and NHERF-1 RNA interference disrupts the inhibition of internalization of AM-R after agonist pretreatment. hPTE cells seeded in 48-well plates were transfected with d-siRNA of RAMP3 or NHERF-1 and incubated for 48 hr to allow RNA knockdown. Immunofluorescence microscopy of hPTEs with RAMP3 or NHERF-1 RNA interference demonstrated greatly decreased levels of RAMP3 and NHERF-1 protein expression in RAMP3 and NHERF-1 RNA knockdown cells, as compared to wild-type hPTE cells. *lacZ* knockdown had no effect on RAMP3 or NHERF-1 protein expression in hPTEs (data not shown). Cells prepared as described in Experiment procedures section for immunofluorescence microscopy. Fixed cells were stained with anti-RAMP3 antibody (1:200) or anti-NHERF-1 antibody (1:250) and detected with a Cy3 anti-rabbit secondary antibody (1:500). Images shown are representative of at least three experiments.

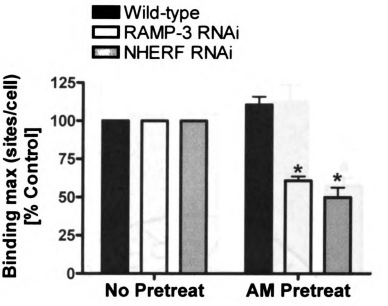


Figure 25C: Effect of RAMP3 and NHERF-1 RNA interference on internalization of AM2-R in human proximal tubule cells. RAMP3 and NHERF-1 RNA interference disrupts the inhibition of internalization of AM-R after agonist pretreatment. hPTE cells seeded in 48-well plates were transfected with d-siRNA of RAMP3 or NHERF-1 and incubated for 48 hr to allow RNA knockdown. AM-R is capable of internalization when RAMP3 or NHERF-1 are knocked down with RNA interference. hPTE cells were grown as described in Figure 24A. Receptor internalization measured by whole-cell competition binding assays using ^{125}I-rAM as ligand (cold rAM served as the competitor) and number of binding sites/cell was estimated using the GRAPHPAD PRISM software. "No pretreat" represents samples at maximal radioligand binding that were not pre-incubated with agonist. "1 pretreat" represents samples pretreated with AM (10 nM) for according time period, washed as indicated in Methods section, and tested immediately after wash steps for radioligand binding. * $p \leq 0.05$; $n \geq 3$ experiments.

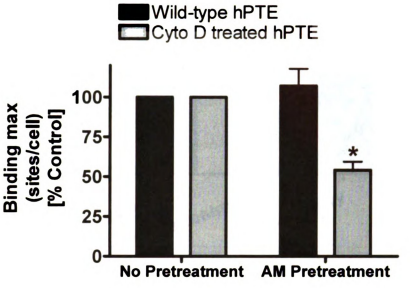


Figure 26: Mechanism of NHERF-1 inhibition of internalization of CL-R/RAMP3 receptor complex in hPTE cells. Cytochalasin D treatment blocks the inhibition of internalization of CL-R/RAMP3 complex. hPTE cells were grown as described in Figure 24A. hPTE cells were pretreated Cytochalasin D (10μM) for 15 min., then with AM (10 nM) for one hour, washed as described in Materials and Methods, and receptor internalization was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

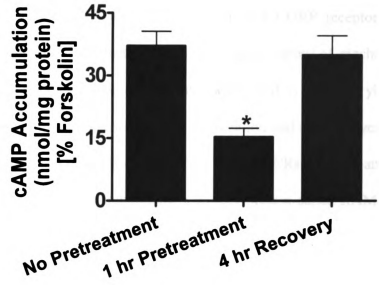


Figure 27: Trafficking of AM-R in human proximal tubule cells (hPTE cells). In hPTE cells, AM-R signaling desensitizes with AM pretreatment, but also resensitizes when then incubated in the absence of agonist. hPTE cells seeded in 24-well plates were pretreated with 10nm rAM for 1h were washed extensively to remove residual agonist, incubated for 4h in serum-free media in the absence of agonist and presence of 5μg/ml cycloheximide, and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in hPTE cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 4$ experiments.

5.4. Discussion

Like other GPCRs, the adrenomedullin and CGRP receptors mediate various physiological actions in different cell types using a variety of mechanisms [305, 306]. The receptor undergoes classical lifecycle wherein it is phosphorylated upon agonist stimulation, leading to desensitization, internalization and either recycling or degradation depending on the cell type. However, unlike other GPCRs, the AM and CGRP receptors are regulated by single transmembrane accessory protein called RAMPs (RAMP1-3). In addition to the original observation by Foord's group that RAMPs are necessary for the cell surface expression and specificity of CL-R to the ligands CGRP and AM, our observations reported recently demonstrated that RAMPs (particularly RAMP3) also has other roles that may regulate the lifecycle of the receptor complex. Specifically, RAMP3 interaction with NSF was found to be important for recycling the receptor after internalization. Absence of RAMP3 or inhibition of NSF resulted in receptor complex degradation [153]. The present study was undertaken to investigate other binding partners and additional roles for RAMP3 in CL-R/RAMP lifecycle. We show here using both heterologous expression, as well as endogenous systems, that RAMP3 interaction with NHERF-1 is essential for regulating the internalization of the CL-R/RAMP3 complex. In addition, similar to our NSF study, the PDZ motif in RAMP3 is critical for the interaction with NHERF-1. In particular, we have found that the Thr^{146} in RAMP3 is critical for this interaction. Whether RAMP3 is phosphorylated at this site by any of the kinases, and if this is essential for NHERF-1 interaction, is not known.

Several studies have shown NHERF-1 to play differing roles in various cellular processes, including receptor trafficking [257, 258]. NHERF-1 has been demonstrated to

bind the extreme COOH terminus of several G-protein coupled receptors (GPCRs), namely the β_2-adrenergic receptor, the κ-opioid receptor, and the P2Y purinergic receptor [154, 259]. Agonist exposure promotes NHERF-1 association with the β_2-adrenergic and the κ-opioid receptors. Unlike our study, NHERF-1 association with these receptors enhances the recycling of the receptors after agonist stimulation [240, 259]. The DSLL motif in the c-terminus of adrenergic receptor was found to the critical for NHERF-1 interaction. In the present study, we also found that the DTLL motif in RAMP3 is essential for the interaction with NHERF-1. Similar to the Thr in the DTLL motif, the Ser in the DSLL motif was found to be essential for the interaction with NHERF-1 [240]. In addition to GPCRs, NHERF-1 has also been shown to associate with the epidermal growth factor receptor (EGFR), a receptor tyrosine kinases (RTK). In this case, NHERF-1 association acts to stabilize the receptor at the plasma membrane and decrease the endocytosis rate of the receptor [217]. Using an endogenous system we have also shown here that NHERF-1 is essential to 'hold' the receptor-complex at the membrane, the absence of which leads to internalization of the receptor complex. While utilizing different mechanisms, collectively these data suggest that NHERF-1 interaction with both RTKs and G protein-coupled receptors and RAMP3 is mandatory to enhance the portion of receptors present at the cell surface.

Recent data suggests that RAMP3 can interact with receptors other than CL-R. It is important to determine if this novel role of RAMP3 in receptor trafficking is specific for CL-R or also for the other receptors RAMPs interact with, namely VPAC, PTH1- and 2-R, and glucagon receptors [286]. NHERF-1 and -2 have been reported to interact with the PTH1R in a scaffolding capacity and tether the receptor to phospholipase C-β (PLCβ)

and the actin cytoskeleton [301, 302]. In opossum kidney (OK) cells, NHERF-1 mediates PTH-stimulated entry of extracellular calcium by a mechanism that is apically localized, PLC-dependent, pertussis toxin-sensitive, and requires an intact actin cytoskeleton. NHERF1 was also shown in inhibit the activation-independent internalization of the PTH1R in kidney distal tubule cells (when stably expressed) [218]. Given the ability of RAMP2 and RAMP3 to interact with the PTH1R and PTH2R, respectively, RAMP3 may be interacting with NHERF-1 to play a trafficking role in these systems, as well.

In addition to AM, Roh et al. have reported that intermedin, a newly discovered peptide from the calcitonin gene peptide superfamily, can also bind the CL-R/RAMP3 complex [307]. It remains to be determined if this reported function for RAMP3 is specific for AM, or if another peptide like intermedin could yield similar results. In our studies of interaction of RAMP3 with NSF and NHERF-1, the PDZ motif of RAMP3 was found to be critical for the interaction. Whether this motif binds additional proteins in a cell type specific manner remains to be examined. One could hypothesize that these predicted cell-type specific interactions would lead to regulation of the various events in the receptor life cycle. In addition to RAMP3, RAMP1 and RAMP2 also regulate the expression of CL-R at the plasma membrane. Whether RAMP1 and 2 bind other proteins similar to RAMP3 also remains to be examined.

Conclusions: This study has shown that one of CL-R's heterodimeric partners, RAMP3, is capable of altering the trafficking of the receptor complex after agonist stimulation by interacting with NHERF-1 via its PDZ motif, and thus the actin cytoskeleton. We have

demonstrated here for the first time that NHERF-RAMP3 interaction dissociates receptor desensitization from internalization of the CL-R/RAMP complex. Additionally, this reveals a novel function for the RAMP accessory proteins in receptor trafficking and an additional difference between the AM1R and AM2R. With recent reports of RAMPs complexing and regulating GPCRs other than CL-R, future studies will focus on additional binding partners of RAMPs and how they regulate the various events in the GPCR life-cycle.

6. Novel function for receptor activity-modifying proteins in post-endocytic receptor trafficking.

6.1. Introduction

The recent discovery of receptor activity modifying proteins (RAMPs) has raised new possibilities for modes of regulation of G-protein coupled receptors (GPCRs). RAMPs were discovered as accessory proteins indispensable to the function of an orphan GPCR, now termed the calcitonin receptor-like receptor (CL-R)[275]. Three RAMP isoforms (1-3) have been identified as distinct gene products that yield single transmembrane-spanning proteins. RAMPs are required for the plasma membrane expression, as well as for determination of receptor phenotype for CL-R (selective ligand recognition)[275, 276]. Coexpression of RAMP-1 with CL-R yields a calcitonin gene-related peptide-1 (CGRP-1) receptor, while coexpression of RAMP-2 or -3 with CL-R produces adrenomedullin receptors, AM-1 and AM-2 receptors, respectively [277, 278]. AM and CGRP are multi-functional peptides with many overlapping functions, ranging from potent vasodilation to proliferation regulation to regulation of salt and water balance [279]. Differential expression of RAMP isoforms has been hypothesized to play a regulatory role in both physiological and pathophysiological disease states. Moreover, the recent identification of RAMP interactions with additional members of the Class II GPCR family and RAMP expression in cell lines lacking CL-R have raised the possibility of novel functions for RAMPs in GPCR regulation [286].

Upon activation, the CL-R/RAMP receptor complex causes cyclic AMP activation in most systems, irrespective of whether the ligand is AM or CGRP. In addition, the receptor complex undergoes desensitization and internalization (via clathrin-

mediated endocytosis) in response to a prolonged agonist stimulation [280]. Once internalized, the receptor complex either undergoes degradation or recycling, depending on the cell type. In HEK 293 cells the CL-R/RAMP complex has been shown to be targeted to the lysosomes for degradation, while in rat mesangial cells, the CL-R/RAMP receptor complex is sorted for dephosphorylation and resensitization (and presumably recycling) as a fully functional receptor [276, 285]. The mechanism that regulates the pathway to which the receptor complex is targeted after agonist-induced internalization remains unknown.

Factors influencing the sorting of receptors in the early endosomes are largely unknown, but some of the critical players are beginning to be identified for the GPCRs. It has been shown in other GPCR systems that interactions with PSD-95/Discs-large/ZO-1 homology (PDZ) domain proteins are responsible for altering the receptor-targeting after internalization [259, 267, 268]. The life cycle of the β2-adrenergic receptor (β2-AR) was reported to be altered in the presence of a protein termed N-ethylmaleimide sensitive factor (NSF) [268]. It has been shown that the β2AR interacts with NSF via a PDZ type I domain (-DSLL) at its extreme C-terminus. In addition, binding of NSF to the Glu2 subunits of the AMPA receptor was also demonstrated to be crucial for the recycling of the α-amino-3-hydroxy-5-methyl-isoxazolepropionate (AMPA) receptor [269, 270]. NSF is a hexameric ATPase that plays a chaperoning role for SNAREs in the majority of membrane fusion events in a cell, but when targeting membrane receptors for recycling, NSF acts independently of the SNARE complex to promote rapid resensitization of the receptors at the plasma membrane [261-263].

Similar to the C-terminus of β2-AR, human RAMP3 C-terminus has a type-I PDZ recognition sequence (-DTLL motif). CL-R, RAMP1 or RAMP2 do not, however, contain any PDZ motifs. We hypothesized that RAMP3, via its interaction with NSF, regulates the trafficking of the CL-R/RAMP3 complex. We show here that while CL-R/RAMP1 and CL-R/RAMP2 complexes do not interact with NSF, CL-R/RAMP3 complex interacts with NSF via the PDZ motif of RAMP3 [153]. Moreover, we demonstrate that over-expression of NSF in HEK-293 cells alters the life cycle of CL-R/RAMP3 complex from a degradative to recycling pathway via interactions of the PDZ motif of RAMP3 and NSF. These findings demonstrate that RAMP3, in addition to determining the receptor phenotype and allowing receptor membrane expression, is also significantly involved with the regulation of the turnover of the CL-R/RAMP complex.

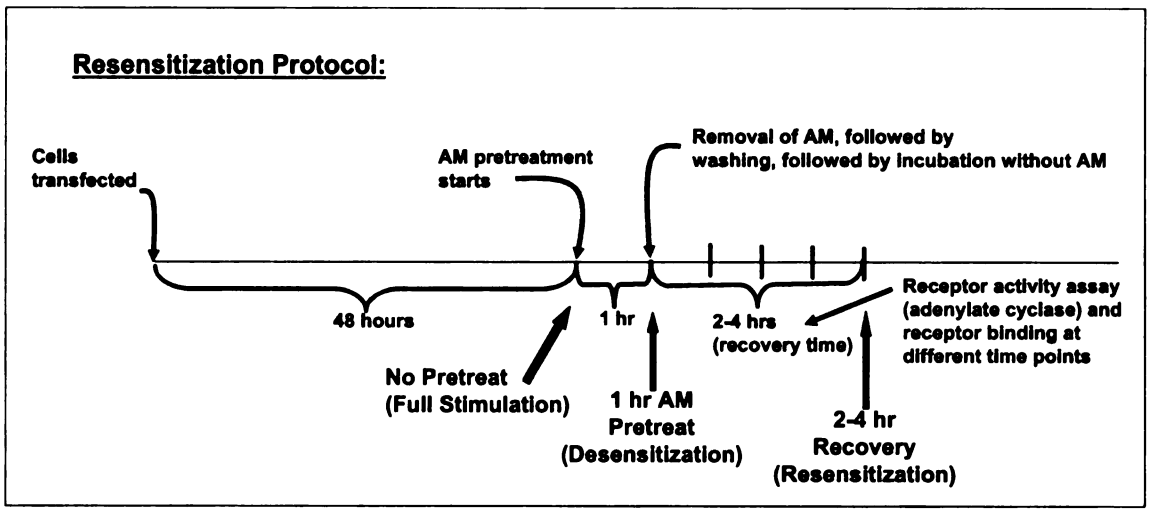
6.2. Materials and Methods

6.2.1. Materials: Adrenomedullin was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). 125I-labeled adrenomedullin was purchased from Amersham Biosciences Corp. (Piscataway, NY). N-ethylmaleimide was purchased from Sigma (St. Louis, MO). Cell culture media, fetal bovine serum, penicillin/streptomycin, trypsin-EDTA were purchased from GibcoBRL® (Grand Island, NY). RAMP3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and NSF antibody was from Calbiochem® (La Jolla, CA). Anti-mouse Cy3 and anti-rabbit Cy5 secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). All other reagents were of highest quality available.

6.2.2. Cell Culture and Transfection protocols: Rat mesangial cells were obtained from ATCC and are maintained in RMPI 1640 media containing 15% FBS and 1% penicillin-streptomycin. HEK-293T cells (obtained from ATCC) are maintained in DMEM low glucose media containing 10% FBS, 1% penicillin-streptomycin. Rat-2 fibroblast cells (obtained from ATCC) are maintained in DMEM high glucose media containing 10% FBS, 1% penicillin-streptomycin. Transfection of HEK293T and Rat-2 fibroblast cells was performed using Lipofectamine Plus protocol (Invitrogen Life Technologies, Carlsbad, CA). Cells were transfected with the DNA and Lipofectamine Plus as per manufacturer's protocol. Cells were collected for assays after 48 hours of transfection.

6.2.3. RAMP cloning and expression: Full length cDNA of human RAMPs1, 2 and 3 and bovine CL-R were described before [281, 282]. CL-R, cloned into N1-EGFP and also in pcDNA3.1 expression vectors, was used for transfection in HEK 293T cells.

6.2.4. Desensitization and Resensitization assays: 48 hours post-tranfection cells were pretreated with or without 10 nM AM in DMEM containing 0.2% BSA for indicated time periods (up to 4 hr). After agonist exposure, cells were washed three times with Dulbecco's phosphate buffered saline (dPBS, Gibco BRL) containing 0.2% BSA and either frozen for membrane preparation for adenylate cyclase assays or used immediately for intact-cell radioligand binding. For receptor resensitization assays, after agonist exposure, cells were washed and incubated for indicated time periods in DMEM containing 0.2% BSA and 5 μg/ml cycloheximide to allow receptor recovery.



6.2.5. Receptor binding: Homologous competition radioligand binding assays were performed as described by Aiyar et al and as established in our laboratory [283]. HEK 293T cells were transfected and approximately 200,000 cells/well seeded in poly-D-lysine pre-coated 24-well plates (BD Biosciences, Palo Alto, CA). 48 hours post-transfection cells were treated for desensitization or resensitization assays as described above. After agonist exposure, cells were washed three times with dPBS buffer containing 0.2% BSA then incubated with increasing concentrations (1 pM to 100 nM) of competing ligand (rAM) and 175-250 pM ^{125}I-rAM for 30 min at 37°C. After incubation, plates were washed three times with ice-cold assay buffer and the reactions were terminated by the addition of 2M NaOH. Cells were then harvested and associated radioligand activity is counted on a γ-counter. All binding assays were performed in duplicate, with each experiment repeated at least three times. Nonspecific binding was determined in the presence of 100 nM of unlabeled rAM. Data was analyzed by

LIGAND (assuming radioligand and competitor both bind reversibly to a single binding site; MacLigand, Version 4.97, NIH, Bethesda, MD) using the following equation:

$$B = \frac{B_{max} \bullet [\text{hot ligand}]}{K_D + [\text{hot ligand}] + [\text{cold ligand}]}$$

Where B represents specific binding, [hot ligand] the single concentration of [^{125}I]rAM studied, [cold ligand] the concentration of unlabeled rAM competing with the radiolabel for AM receptor binding, B_{max} the maximum number of binding sites and KD the equilibrium dissociation constant (the equation was solved where the "cold ligand" IC_{50} = [hot ligand] + K_D). Analysis of all binding data was performed by computer-assisted nonlinear least square fitting using GraphPad PRIZM version 4 (GraphPad Software, San Diego, CA). Binding sites/cell was calculated and data was expressed as percent of the control.

6.2.6. Adenylate cyclase assays: Cyclase activity was done as described before with slight modifications [282, 284]. Cells were harvested from P100 or P60 plates and homogenized in Tris HCl (10mM)/EDTA (10mM) buffer. Membranes were prepared by homogenization and centrifugation in Tris HCl (50mM)/MgCl (10mM) buffer. Final concentration of 20 μg of protein/assay tube was obtained. Membranes were incubated for 15 min at 30°C with appropriate concentrations of drugs (100nM AM, 10μM Forskolin) and assay mix containing ATP regeneration system and α^{32}P-ATP. After the reaction was stopped (with stop solution containing ^{3}H-cAMP) contents of the assay tubes were passed through Dowex and subsequently through alumina columns to separate the degradation products of ATP, by washing the dowex with water and alumina with

imidazole. Elution profile was done to determine the amount of water and imidazole needed to wash and elute the products. Product eluted from alumina column was counted for the presence of ^{3}H-cAMP and α^{32}P-cAMP in a β-counter. Each experiment was done in triplicates and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. Basal cyclase activity and forskolin stimulation did not show statistically significant differences between treatments.

6.2.7. cAMP accumulation assays: Rat mesangial cells were seeded on a 48-well plate (and Rat2 fibroblast cells on 24-well plates) until reaching 80-90% confluency, then incubated in serum-free media overnight before experiment. Resensitization experiments were carried out as described in Materials and Methods section, with cells pretreated with 10nM rAM and subsequently challenged for 10 min at 37°C with appropriate concentrations of drugs (100nM AM, 10μM Forskolin) in the presence of 200μM 3-isobutyl-1-methylxanthine. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in cells were calculated using a standard curve ranging from 10 to 10^{4} fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. Basal cyclase activity and forskolin stimulation did not show statistically significant differences between treatments.

6.2.8. RNA Interference analysis: Gene-specific d-siRNA for lacZ (control) and RAMP3 were generated and purified using BLOCK-iT Dicer RNAi kit from Invitrogen

(Carlsbad, CA). RMCs were transfected with d-siRNAs using Lipofectamine 2000 as per manufacturer's instructions (Invitrogen). 48 hours after transfection cells were frozen for mRNA analysis, or used for cAMP accumulation assays or immunofluorescence microscopy.

6.2.9. Quantitative PCR analysis: Total RNA was isolated from RMCs using Trizol reagent (GIBCO BRL). After sodium acetate-ethanol precipitation and several ethanol washes, RNA was used as a template in a quantitative PCR amplification procedure. Quantitative PCR analysis was carried out with the LUX (Light Upon Extension) fluorogenic primer method, following the protocol in the manufacturer's manual, as described by Nazarenko *et al* .

6.2.10. Mutagenesis procedure: Site-directed mutagenesis was performed using a PCR-based strategy that employs the *pfu* Turbo polymerase (Stratagene, La Jolla, CA). A pair of complementary oligonucleotides containing the appropriate point mutations in the sequence of RAMP or a premature stop codon at position 145 or 147 codon of RAMP-3 for deletion mutants were synthesized (Michigan State University Macromolecular structure facility). The PCR for the mutation was as follows: 94°C for 2 minutes; 30 cycles of 94°C for 30 sec., 50°C for 30 sec., 68°C for 8 min.; final cycle of 68°C for 8 minutes. PCR product was digested for 4 hours with DpnI enzyme (Invitrogen) and transformed in to DH5α cells. Mutations were confirmed by automated sequencing (Michigan State University Genomic Technology Support Facility).

6.2.11. Immunofluorescence microscopy: HEK 293 cells were transfected as described above and seeded at 24hr post-transfection onto collagen type I-coated coverslips. Resensitization assays were performed as described and reactions were stopped by fixing cells in 4% paraformaldehyde for 30min. at room temperature. Samples were permeablized with 0.1% v/v Triton X-100 in PBS and blocked overnight in 0.1% v/v Triton X-100 in PBS + 10% goat serum. Samples were incubated in primary antibody in blocking buffer for 2h at room temperature (NSF at 1:250 and RAMP3 at 1:200). Appropriate secondary antibodies were applied for 1h at room temperature (Goat anti-mouse Cy3 at 1:500 and Goat anti-rabbit Cy5 at 1:400). Coverslips were mounted in Shandon Permafluor mounting medium and slides stored at 2-8°C until analysis. Cells were visualized on a Zeiss 210 laser confocal microscope at a zoom of 2. Images presented are representative single optical sections of a z-series taken from at least twenty fields per experiment and at least three individual experiments. Images in this thesis/dissertation are presented in color.

6.2.12. Fusion protein overlays and western blotting: 10 μg of GST-fusion proteins were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose filters. Filters were blocked with 5% w/v fat-free milk powder in Tris-buffered saline with Tween 20 (TTBS: 20 mM Tris, pH 7.4, 500 mM NaCl, 0.1% v/v Tween 20) and incubated overnight at 4 °C in lysates of HEK293 cells with or without overexpression NSF. Blots were then washed three times with TTBS buffer and incubated with anti-NSF monoclonal antibody for 2 h at room temperature. After three washes with TTBS, filters were incubated for 1h with horseradish peroxidase-conjugated goat anti-mouse secondary

antibody (Gibco BRL®, Grand Island, NY), washed again with TTBS, soaked in Supersignal West Pico chemiluminescent substrate (Pierce) and exposed to x-ray film. Same protocol, with the exception of the overnight incubation with cell lysate, was followed for immunoblot analysis of RAMP3.

6.2.13. Statistics: Data are presented as mean ± S.E.M. Single group comparisons exercised a paired Student's t-test method. Statistical significance was set at $P<0.05$.

6.3. Results

6.3.1. Role of NSF in Resensitization of the CL-R/RAMP complex

Our lab has previously published that rat mesangial cells (RMCs) endogenously express the AM1R (CL-R + RAMP2) and the AM2R (CL-R + RAMP3) (8). This data was repeated and NSF expression was confirmed in the RMCs with RT-PCR and immunocytochemistry (data not shown). Our laboratory has also reported that pretreatment of rat mesangial cells (RMCs) with AM leads to an agonist-stimulated desensitization and internalization of the CL-R/RAMP complex. Phosphatase-dependent resensitization of AM responsiveness was also demonstrated after agonist-stimulated desensitization [285]. Measuring cAMP accumulation we repeated these results in this study (Figure 28). As a preliminary test to determine if NSF is involved in the resensitization of AM responsiveness, we used a pharmacological inhibitor of NSF, N-ethylmaleimide (NEM). In RMCs treated for 45 seconds with 50 μM NEM during the resensitization experiment, resensitization was blocked, as measured by cAMP

accumulation (Figure 28). NEM, however, did not affect basal cAMP accumulation or the desensitization response when compared to untreated cells, an important finding given the ability of NEM to interfere with Gα subunits (Figure 28). These results indicate that NSF plays a role in the sorting of the CL-R/RAMP complex following agonist-induced internalization in this endogenous CL-R/RAMP system where the receptor complex is recycled. To fully evaluate the molecular mechanisms of this observation, we used HEK 293 cells to examine the interaction of the CL-R/RAMP complex with NSF and the impact of this interaction on receptor trafficking.

In contrast to RMCs, HEK 293 cells express very low endogenous levels of RAMPs. Kuwasako et al. have demonstrated that in HEK 293 cells overexpressing the CL-R/RAMP complex, agonist-induced internalization leads to receptor trafficking to a degradation pathway [276]. In this study the internalized CL-R/RAMP complexes were colocalized with LAMP-1, a lysosomal marker, to show the targeting of the receptor for the degradation pathway. Utilizing adenylate cyclase activity assays, whole-cell ligand binding, and immunofluorescence microscopy we confirmed these findings (Figure 29A,B). Pretreatment of HEK 293 cells transfected with CL-R and RAMP3 with 10 nM AM for one hour resulted in desensitization of the adenylate cyclase response from 50% (of forskolin stimulation) in untreated cells to 28% in AM-treated cells (Figure 29A, left axis). Even after the removal of agonist and incubation with buffer alone for indicated times through 4h, the adenylate cyclase response remained desensitized (Figure 29A, left axis), indicating a lack of resensitization. Consistent findings were obtained with whole-cell binding and immunofluorescence microscopy experiments (Figure 29A, right axis, 29B).

To determine if NSF overexpression could alter the receptor trafficking in this cell system, NSF was co-transfected with CL-R and RAMP-3, and resensitization and recycling assays were performed. Resensitization and recycling were monitored by adenylate cyclase activity assays and whole-cell competition binding, respectively. In addition, visualization of the trafficking of the receptor complex was performed by immunofluorescence microscopy. In the absence of NSF, pretreatment with AM for one hour resulted in desensitization of the adenylate cyclase response and internalization of the receptor complex. Upon removal of agonist and incubation with buffer alone for 4h, the adenylate cyclase response remained desensitized and the receptor complex remained internalized, indicating a lack of resensitization (Figure 30A, B). In contrast, when NSF was co-transfected in the cells, although the desensitization response (i.e. response after 1h agonist treatment) was not altered, the cells now underwent time-dependent resensitization (i.e. response after 1, 2, or 4h agonist removal) in response to AM (Figure 30A). Consistent findings were obtained with whole-cell binding and immunofluorescence microscopy experiments (Figure 30B, 31). Time course experiments indicated the time course for complete resensitization and recycling of the CL-R/RAMP3 receptor complex to be 4h in HEK 293 cells, as measured by adenylate cyclase, whole-cell binding, and immunofluorescence microscopy experiments (Figure 31). All subsequent experiments in HEK 293 cells use the 4h time point to determine receptor complex recycling and resensitization. These results indicate that the presence of NSF alters the intracellular sorting of the CL-R/RAMP3 receptor complex after AM-stimulated endocytosis.

6.3.2. RAMP isoform-specific regulation of CL-R/RAMP receptor complex trafficking

To determine if this effect of NSF was specific for RAMP3, the additional RAMPs (RAMP-1 or -2) were tested for their ability to act with NSF to alter the receptor complex life cycle. Interestingly, in contrast to RAMP3, presence of NSF did not alter the resensitization response or recycling pattern of the CL-R/RAMP-1 or -2 receptor complexes. Both the activity and receptor number remained at desensitized levels in cells transfected with CL-R+RAMP1 or CL-R+RAMP2 (along with NSF) (Figure 32A-D). Both CL-R/RAMP1 and CL-R/RAMP2 complexes showed no difference in receptor expression levels at the plasma membrane (as measured with whole-cell binding) in untreated cells, as compared to CL-R/RAMP3 complex. These results indicate that RAMP3 must contain a molecular determinant distinct from the other RAMPs that allowed its interaction and activity with NSF.

6.3.3. Role of PDZ interactions in trafficking of the CL-R/RAMP3 complex

We hypothesized that the unique characteristic of RAMP3 that allowed its interaction with NSF to change receptor trafficking was a PDZ motif on its extreme C-terminus. In order to establish if this domain is critical for interaction of the CL-R/RAMP3 complex with NSF, the PDZ motif (-DTLL) on RAMP3 was deleted. Deletion of this domain did not affect basal adenylate cyclase activity or the desensitization response of the CL-R/RAMP3 complex in response to AM, even in the presence of NSF (Figure 32). In contrast, the deletion of the PDZ motif (-DTLL) significantly affected the resensitization and recycling of the CL-R/RAMP3 receptor

complex in the presence of NSF. Both radioligand binding and adenylate cyclase assays showed a loss of recycling and resensitization, respectively, of the receptor complex when RAMP3Δ145-8 was expressed in the presence of NSF, as compared to the wild-type RAMP3 with NSF (Figure 33A, B). Mutant RAMP3 (RAMP3Δ145-8) showed no difference in receptor expression levels at the plasma membrane (as measured with whole-cell binding) in untreated cells, as compared to wild-type CL-R/RAMP3 complex. Additionally, results were confirmed when a recycling assay with RAMP3Δ145-8 was performed with immunofluorescence microscopy (Figure 34).

To further test the hypothesis that the absence of the PDZ motif on the RAMP2 accounts for the lack of interaction of the CL-R/RAMP2 complex with NSF, and hence the inability of the CL-R/RAMP2 complex to follow a recycling pathway, the PDZ motif of RAMP3, the amino acids -DTLL, were substituted on the C-terminus of RAMP2, in exchange for its original four C-terminal amino acids (-EAQA). The RAMP2ΔDTLL mutant showed similar levels of adenylate cyclase activity and whole-cell radioligand binding without pretreatment and after desensitization, as compared to wild type RAMP2 in control experiments (Figure 35). The RAMP2ΔDTLL mutant also showed similar receptor expression levels at the plasma membrane (as measured with whole-cell binding) in untreated cells, as with the wild-type CL-R/RAMP2 complex. Resensitization assays (measured with adenylate cyclase activity) and recycling assays (measured by whole-cell binding) were performed in HEK 293 cells were transfected with CL-R, RAMP2ΔDTLL, and NSF, as described previously. Similar to the CL-R/RAMP3 complex, the CL-R- RAMP2ΔDTLL complex underwent resensitization and recycling in the presence of NSF, as assessed by adenylate cyclase and whole-cell

binding (Figure 35). These findings provide additional evidence that the PDZ motif on the RAMP3 is the site of interaction of the receptor complex with NSF, causing a change in receptor trafficking, from a degradative to a recycling pathway.

To further identify the critical amino acids in the PDZ recognition sequence which regulate the RAMP3/NSF interaction, point mutations of the amino acids of the RAMP3 PDZ motif to alanine were performed. The functional effects of the point mutations were analyzed with resensitization assays, measured by adenylate cyclase activity, and recycling assays, measured by whole-cell binding, as described before. Our results indicate that mutation of D145, T146, or L148 to alanine disrupted the RAMP3 interaction with NSF and inhibited the resensitization and recycling of the CL-R/mutant RAMP3 complex after AM-induced endocytosis in HEK 293 cells (Figure 36). Mutation of L147 to alanine had no effect on the resensitization or recycling of the receptor complex in the presence of NSF (Figure 36). Control experiments with the point mutations co-transfected with CL-R showed similar levels of adenylate cyclase activity and whole-cell binding as compared to wild-type RAMP3 without pretreatment and after desensitization. RAMP3 point mutants also showed no difference in receptor expression levels at the plasma membrane (as measured with whole-cell binding) in untreated cells, as compared to wild-type CL-R/RAMP3 complex.

To examine if the PDZ motif on RAMP3 is interacting with NSF, overlay assays were performed. This was accomplished using GST-RAMP3 fusion proteins in an overlay assay with cell lysates of HEK 293 cells overexpressing NSF. Control experiments run with GST protein showed no detectable bands when incubated with NSF lysates and probed with an NSF antibody (Figure 37A). Importantly, wild-type RAMP3

fusion proteins showed interaction with NSF in the cell lysates of HEK 293 cells overexpressing NSF in the overlay assay (Figure 37A). In addition, RAMP3Δ145-8 fusion proteins, lacking the PDZ motif on RAMP3, showed no detectable bands when incubated with NSF lysates and probed with an NSF antibody (Figure 37A). Lysates of HEK 293 cells not over-expressing NSF showed no detectable bands when run with GST-RAMP3 in the overlay assay and probed for NSF (Figure 37B). When blots used in the overlay assay were stripped and probed for RAMP3, a band was detected in the GST-RAMP3 lane in the exact location as in the overlay assay when probed for NSF (Figure 37C). This data demonstrates an interaction between RAMP3 and NSF via the PDZ motif on RAMP3, an interaction that is capable of regulating CL-R/RAMP3 (AM2R) complex trafficking.

6.3.4. RAMP3 and NSF regulation of receptor trafficking in unaltered cell lines

It was important to establish if our observations in the HEK 293 cells was transferable to unaltered cells lines. Reexamining the rat mesangial cells used in the first set of experiments, we employed RNA interference technology to knockdown RAMP3 expression. Having demonstrated the requirement of NSF in the cells for efficient receptor resensitization (Figure 28), the RAMP3 RNA interference experiment would determine if RAMP3 was also required for effective receptor resensitization. In both mRNA and protein expression studies, RAMP3 expression dramatically decreased, while control experiments using lacZ knockdown showed no significant alteration in RAMP3 expression when compared to wild-type cells (Figure 38A, B; data not shown). Resensitization assays were performed and cAMP accumulation was measured to

determine the effect of RAMP3 RNA interference on receptor resensitization in rat mesangial cells. RNA interference for RAMP3 showed similar levels of cAMP accumulation as compared to wild-type RMCs without pretreatment and after desensitization (Figure 38C). Strikingly, when allowed sufficient recovery time in the absence of agonist following desensitization, RMCs with RAMP3 RNA interference showed an inability to resensitize, unlike the wild-type RMCs (Figure 38C). This finding demonstrates that NSF and RAMP3 are both critical for receptor targeting for resensitization in rat mesangial cells, an unaltered cell line absent of the issues of overexpression.

As a further test of our proposed model, we employed a cell line that does not express RAMP3, but does express the AM1R (CL-R and RAMP2). We hypothesized that, with the absence of RAMP3, this cell line would fail to resensitize following agonist pretreatment, and that expression of RAMP3 in these cells would allow a switch in receptor targeting for resensitization. Rat2 fibroblast cells have been shown by Choksi *et al.* to lack RAMP3 expression, but do express the AM1R (CL-R and RAMP2) [308]. We repeated this finding and confirmed the expression of NSF in Rat2 fibroblast cells with RT-PCR and immunocytochemistry (data not shown). We performed resensitization assays with the Rat2 fibroblast cells and measured resensitization by cAMP accumulation. The Rat2 fibroblast cells exhibited a decrease in cAMP accumulation after agonist pretreatment and failed to resensitize, as predicted, when allowed to recover in the absence of agonist for four hours. Interestingly, consistent with our model, RAMP3 expression in the Rat2 cells showed no alteration in cAMP accumulation without pretreatment and after desensitization when compared to wild-type Rat2 cells, but now

showed resensitization when allowed recovery time in the absence of agonist (Figure 39). Furthermore, expression of the RAMP3 PDZ motif mutant in the Rat2 cells was unable to cause receptor resensitization, while showing similar levels of cAMP accumulation as compared to wild-type Rat2 cells without pretreatment and following desensitization (Figure 39). RAMP3 and RAMP3Δ145-8 showed similar levels of transfection efficiency in the Rat2 cells, as measured by immunocytochemistry (data not shown). This data further confirms the crucial role of RAMP3 in the targeting of the AM2R for resensitization/recycling after agonist-stimulated desensitization. Additionally, it suggests that differential RAMP expression in cells may determine the sorting of the AM-R from the endosomes after internalization.

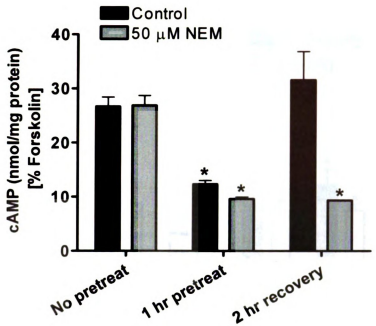


Figure 28: Effect of N-ethylmaleimide, an NSF inhibitor, on resensitization of cAMP accumulation in rat mesangial cells (RMCs). The 1h pretreatment time point shows desensitization of cAMP accumulation response in cells with or without treatment with NEM. The 2 hr recovery time point shows resensitization is inhibited in cells treated with NEM, as compared to untreated RMCs. This indicates a role for NSF in the resensitization observed in RMC cells. RMC cells seeded in 24-well plates were pretreated with 10nm rAM for 1h were washed extensively to remove residual agonist and treated with 50 μM NEM for 45 seconds. Following NEM treatment, cells were washed repeatedly and incubated in serum-free media with 5μg/ml cycloheximide for indicated times. After recovery time, RMC cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in rat mesangial cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

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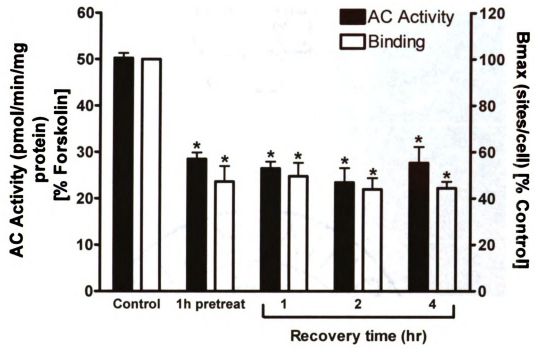

Figure 29A: The sorting of the AM2R (CL-R+RAMP3) in HEK 293 cells. CL-R/RAMP3 complex does not resensitize/recycle in HEK 293 cells after agonist-stimulated desensitization/internalization. HEK 293 cells transiently transfected with CL-R and RAMP3 were treated for one hour with AM (10 nM) and then washed and receptor resensitization (adenylate cyclase activity) and recycling (radioligand binding) were measured after indicated recovery times in the absence of agonist. Adenylate cyclase activity is shown on the left y-axis, expressed as percent maximal response (% forskolin stimulation), and radioligand binding is shown on the right y-axis, expressed as percent control. * $p \leq 0.05$; $n \geq 3$ experiments.

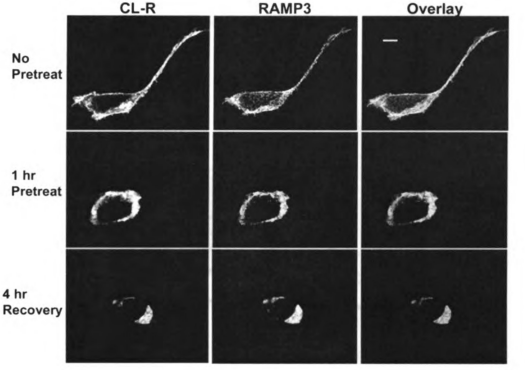

Figure 29B: The sorting of the AM2R (CL-R+RAMP3) in HEK 293 cells. Immunofluorescence microscopy shows failure of CL-R/RAMP3 receptor complex to recycle after agonist-stimulated internalization. HEK 293 cells transfected with CL-R-GFP and RAMP3 were pretreated with 10nM AM for 1h. After pretreatment with AM, cells were washed and incubated in serum-free media with 5μg/ml cycloheximide to allow receptor recycling for indicated times. Cells were fixed and components were visualized using anti-RAMP3 antibody (1:200) and detected with Cy5 secondary antibody (1:200), and CL-R is detected with an EGFP tag; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least twenty fields imaged per experiment from at least three experiments. Bar scales on all images represent 50μm.

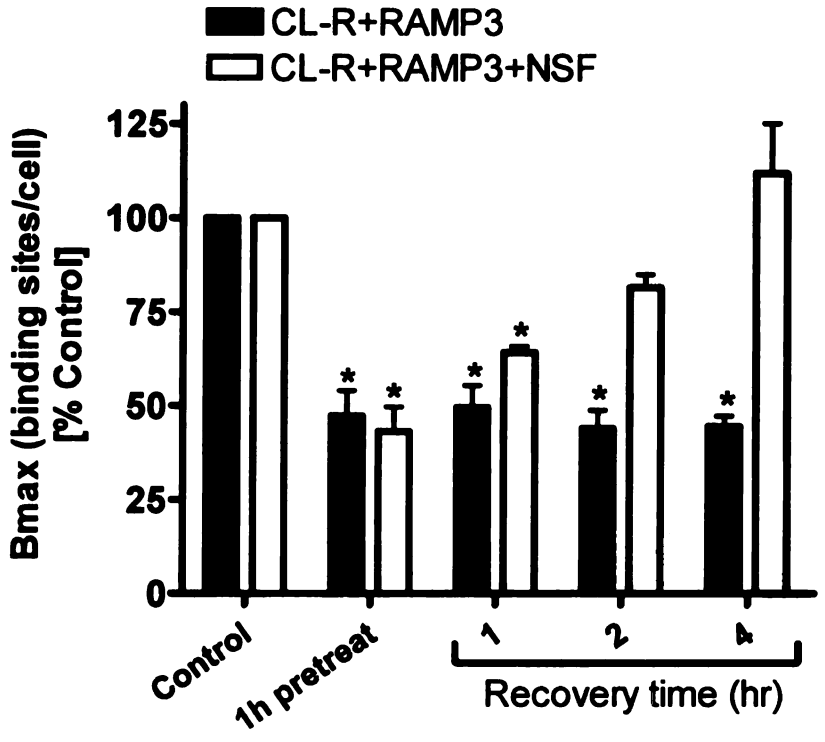

Figure 30A: The role of NSF in the sorting of the AM2R (CL-R+RAMP3) in HEK 293 cells. NSF causes recycling of AM2R in HEK 293 cells. HEK 293 cells transiently transfected with CL-R and RAMP3 with or without NSF. At 48h post-transfection, cells were treated for one hour with AM (10 nM) and then washed and receptor recycling was measured after indicated recovery times in the absence of agonist. Receptor recycling measured by whole-cell competition binding assays using ^{125}I-rAM as ligand (cold rAM served as the competitor) and number of binding sites/cell was estimated using the GRAPHPAD PRISM software. "No pretreat" represents samples at maximal radioligand binding that were not pre-incubated with agonist. "1h pretreat" represents samples pretreated with AM (10 nM) for one hour, washed as indicated in Methods section, and tested immediately after wash steps for radioligand binding. "1, 2, and 4 hr recovery" samples were pretreated with AM (10 nM) for one hour, washed, and allowed to recover for indicated times in media without agonist plus 5μg/ml cycloheximide, then analyzed for radioligand binding. NSF overexpression in cells expressing AM2R caused altered receptor trafficking from degradation to recycling pathway. * $p \leq 0.05$; $n \geq 3$ experiments.

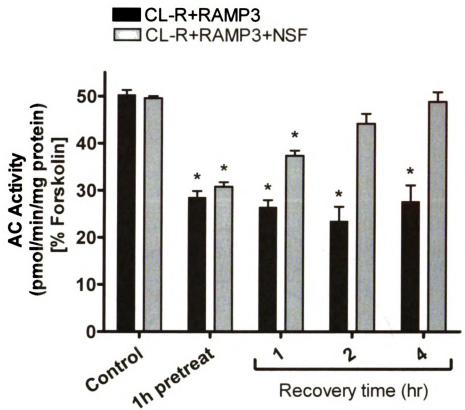

Figure 30B: The role of NSF in the sorting of the AM2R (CL-R+RAMP3) in HEK 293 cells. NSF causes recycling of AM2R in HEK 293 cells. NSF causes resensitization of AM2R in transfected HEK 293 cells. HEK 293 cells transfected and pretreated with agonist as described in 29*A*. After agonist pretreatments, membranes were extracted and AC activity in response to 100 nM AM was measured. NSF overexpression with AM2R allowed time-dependent receptor resensitization. Experiments performed in triplicates and data expressed as percent maximal stimulation (% Forskolin). * $p \leq 0.05$; $n \geq 4$.

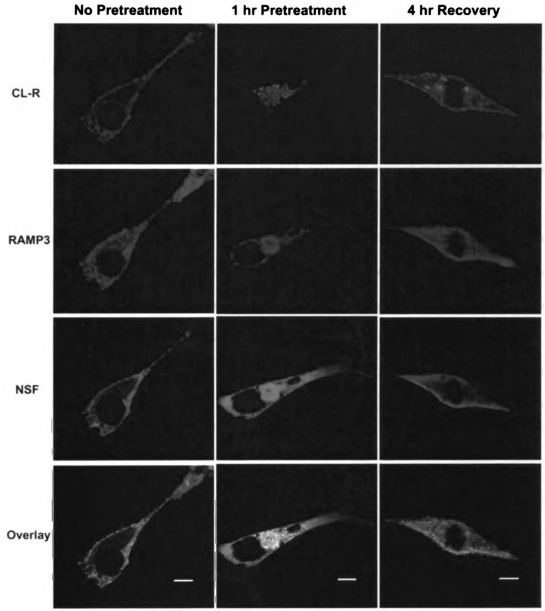
No Pretreatment
1 hr Pretreatment
4 hr Recovery
CL-R
RAMP3
NSF
Overlay

Figure 31: Localization of CL-R, RAMP3, and NSF in HEK 293 cells during a recycling experiment. After 1h AM pretreatment, CL-R and RAMP3 are internalized and show co-localization with NSF intracellularly. After 4h recovery time post-AM pretreatment, CL-R and RAMP3 show distribution at the plasma membrane of the cell, demonstrating recycling of the receptor complex. HEK 293 cells transfected with CL-R-GFP, RAMP3, and NSF were pretreated with 10nM AM for 1h. After pretreatment with AM, cells were washed and incubated in serum-free media with 5μg/ml cycloheximide to allow receptor recycling for indicated times. Note: "1 hr pretreatment" indicates time just after AM pretreatment and wash steps, with no recovery time. Cells were fixed and components were visualized using anti-RAMP3 antibody (1:200) and anti-NSF antibody (1:250) with Cy5 anti-rabbit secondary antibody (1:400, in blue) and Cy3 anti-mouse secondary antibody (1:500, in red), respectively; CL-R-GFP is detected with an EGFP tag and shown in green; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least twenty fields imaged per experiment from at least three experiments. Bar scales on all images represent 100μm.

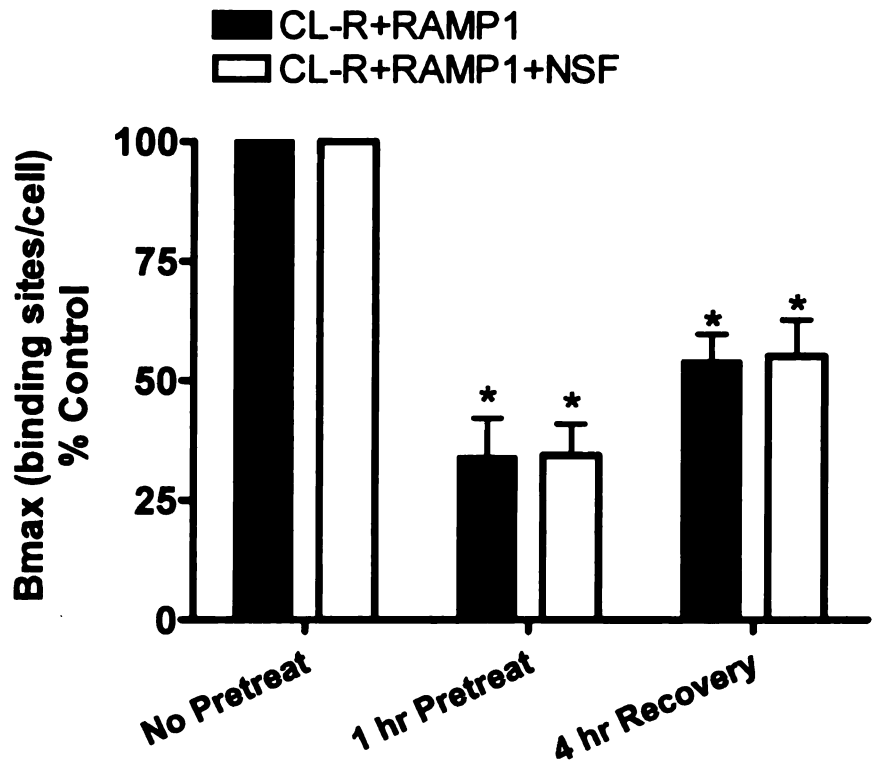

Figure 32A: The role of NSF in post-endocytic sorting of CGRP1 (CL-R+R1) and AM1 receptor. NSF overexpression does not alter the trafficking of the CGRP receptor after internalization. HEK 293 cells were transfected with CL-R and R1, with or without NSF. 48h post-transfection, cells were pretreated with CGRP (10 nM) for one hour, washed as described in Materials and Methods, allowed indicated recovery times in agonist-free media, and receptor recycling was measured with whole-cell competition binding using ^{125}I-rCGRP as the ligand and cold rCGRP as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

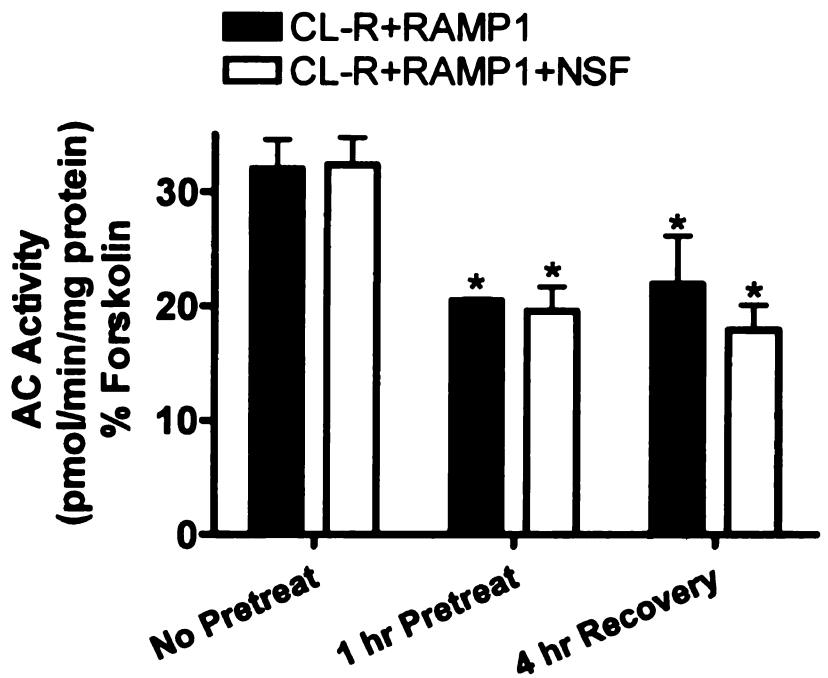

Figure 32B: The role of NSF in post-endocytic sorting of CGRP1 (CL-R+R1) and AM1 receptor. NSF overexpression does not cause CGRP receptor resensitization after agonist-stimulated endocytosis. HEK 293 cells were transfected and pretreated as in figure 29*A* and then membranes were extracted. Adenylate cyclase activity was measured in membranes stimulated with 100 nM CGRP for 15 min. Experiments performed in triplicates and expressed as percent maximal stimulation (% Forskolin). * $p \leq 0.05$; $n \geq 3$.

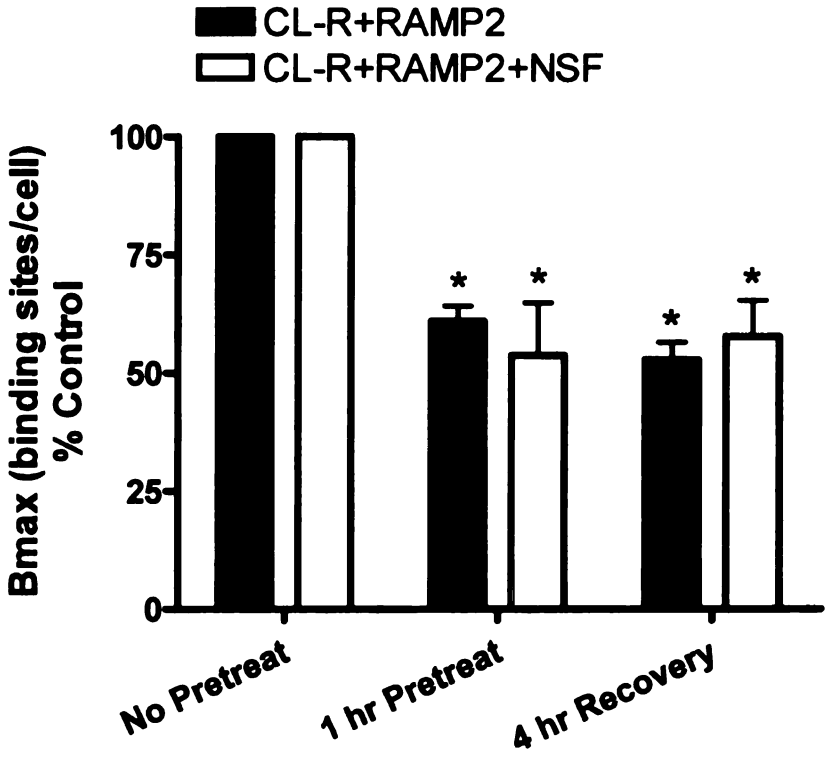

Figure 32C: The role of NSF in post-endocytic sorting of CGRP1 (CL-R+R1) and AM1 receptor. NSF overexpression does not cause recycling of AM1 receptor after internalization. HEK 293 cells were transfected with CL-R and R2, with or without NSF. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, allowed indicated recovery times in agonist-free media, and receptor recycling was measured with whole-cell competition binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 4$.

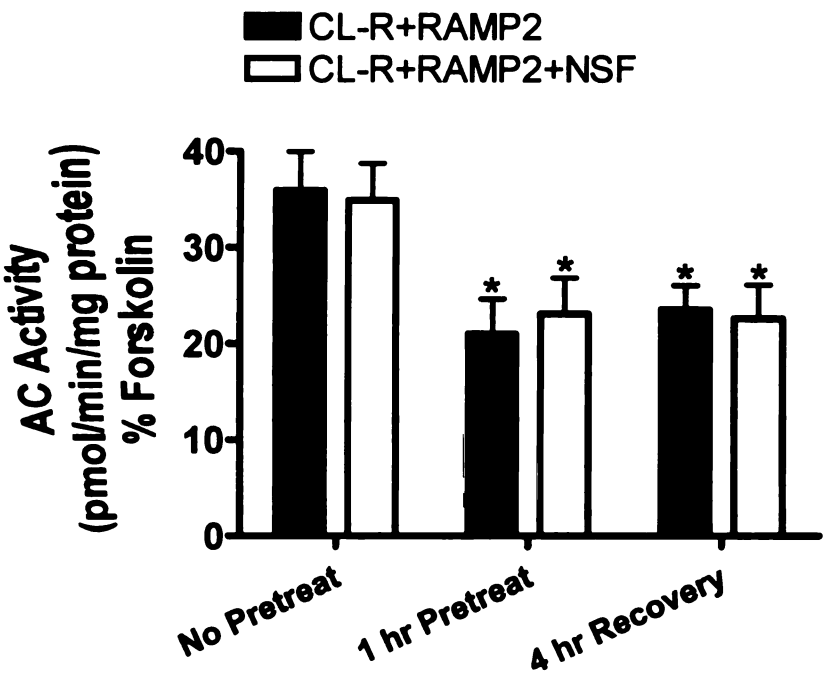

Figure 32D: The role of NSF in post-endocytic sorting of CGRP1 (CL-R+R1) and AM1 receptor. NSF overexpression does not cause AM1 receptor resensitization after agonist-induced internalization. HEK 293 cells were transfected and pretreated as in figure 29*A* and then membranes were extracted. Adenylate cyclase activity was measured in membranes stimulated with 100 nM AM for 15 min. Experiments performed in triplicates and expressed as percent maximal stimulation (% Forskolin). * $p \leq 0.05$; $n \geq 3$.

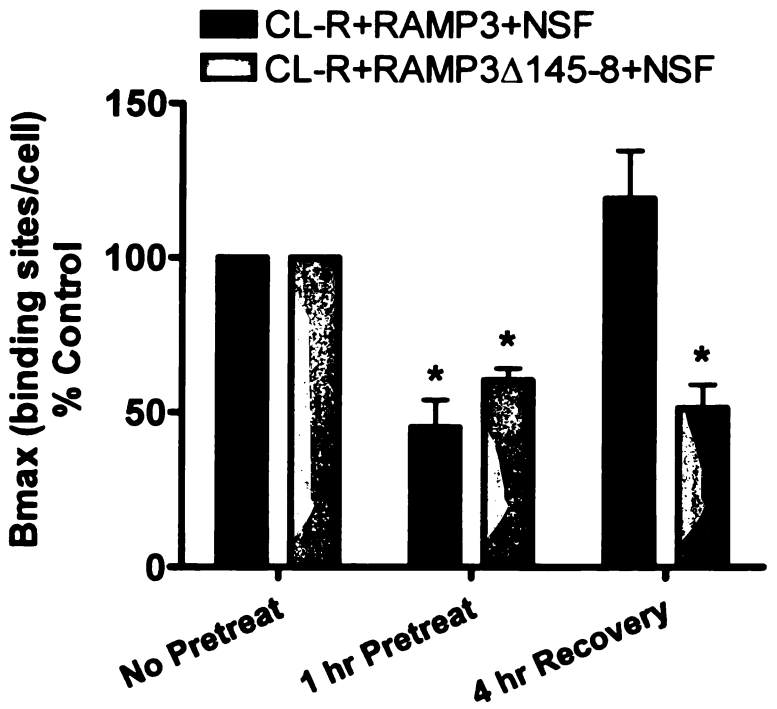

Figure 33*A*: The effect of RAMP3 PDZ motif deletion on the trafficking of the AM2R (CL-R+RAMP3). Deletion of RAMP3 PDZ motif blocked the recycling of the CL-R/RAMP3 complex when co-expressed with NSF. HEK 293 cells were co-transfected with CL-R, wild-type RAMP3 or RAMP3Δ145-8, and NSF. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, allowed 4h recovery times in agonist-free media, and receptor recycling was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

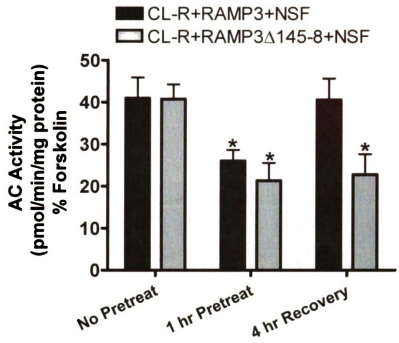

Figure 33B: The effect of RAMP3 PDZ motif deletion on the trafficking of the AM2R (CL-R+RAMP3). Deletion of RAMP3 PDZ motif blocked the resensitization of the CL-R/RAMP3 complex after agonist-stimulated desensitization when co-expressed with NSF. HEK 293 cells were transfected and pretreated as in figure 29*A* and then membranes were extracted as described in Materials and Methods section. Adenylate cyclase activity was measured in membranes stimulated with 100 nM AM for 15 min. Experiments performed in triplicates and expressed as percent maximal stimulation (% Forskolin). * $p \leq 0.05$; $n \geq 3$.

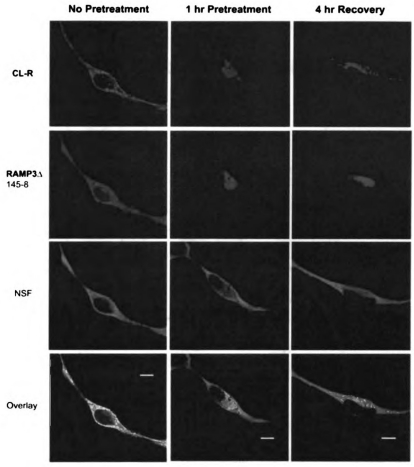

Figure 34: Localization of CL-R, RAMP3Δ145-8, and NSF in HEK 293 cells during a recycling experiment. Untreated and after one hour AM (10nM) pretreatment, CL-R and RAMP3Δ145-8 internalize similarly to the wild-type CL-R/RAMP3 complex. After a 4h recovery time in the absence of agonist, CL-R and RAMP3Δ145-8 fail to recycle to the plasma membrane. Experiments performed as described in Figure 31. Fixed cells were stained with anti-RAMP3 antibody (1:200) and anti-NSF antibody (1:250) with Cy5 anti-rabbit secondary antibody (1:400, in blue) and Cy3 anti-mouse secondary antibody (1:500, in red), respectively; CL-R-GFP is shown in green; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least twenty fields imaged from at least three experiments. Bar scales on all images represent 100μm.

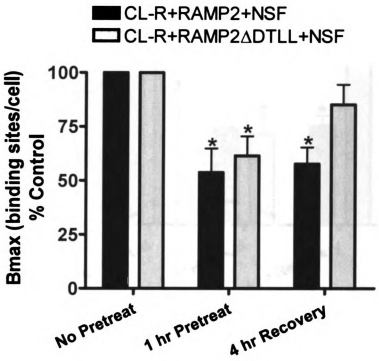

Figure 35A: Effect of PDZ motif substitution on the C-terminus of RAMP2 on recycling/resensitization of CL-R/RAMP complex. Substitution of PDZ motif (-DTLL) on the C-terminus of RAMP2 cause a change in receptor trafficking phenotype from degradation to recycling of the receptor complex. HEK 293 cells were co-transfected with CL-R, wild-type RAMP2 or RAMP2ΔDTLL, and NSF. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, allowed 4h recovery times in agonist-free media plus 5μg/ml cycloheximide, and receptor recycling was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 4$.

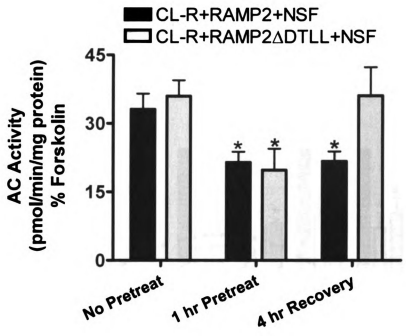

Figure 35B: Effect of PDZ motif substitution on the C-terminus of RAMP2 on recycling/resensitization of CL-R/RAMP complex. PDZ motif substituted on the C-terminal tail of RAMP2 cause resensitization of adenylate cyclase activity. HEK 293 cells were transfected and pretreated as in figure 29*A* and then membranes were extracted as described in Materials and Methods section. Adenylate cyclase activity was measured in membranes stimulated with 100 nM AM for 15 min. Experiments performed in triplicates and expressed as percent maximal stimulation (% Forskolin). * $p \leq 0.05$; $n \geq 4$.

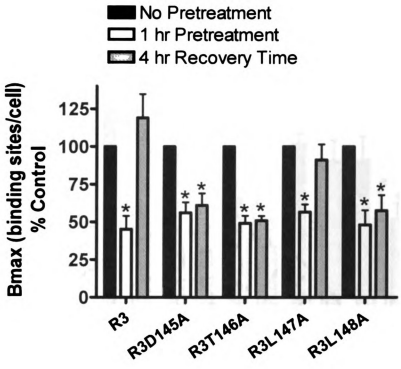

Figure 36.4: Effect of RAMP3 PDZ motif point mutations on the recycling/resensitization of the CL-R/RAMP3 complex in the presence of NSF. All point mutants within the PDZ motif on RAMP3, except R3L147A, blocked the recycling of the CL-R/RAMP3 complex seen when co-expressed with NSF. HEK 293 cells were co-transfected with CL-R, wild-type RAMP3 or RAMP3 point mutants, and NSF. 48h post-transfection, cells were pretreated with AM (10 nM) for one hour, washed as described in Materials and Methods, allowed 4h recovery times in agonist-free media, and receptor recycling was measured with whole-cell binding using ^{125}I-rAM as the ligand and cold rAM as the competitor. Number of binding sites/cell was estimated with GRAPHPAD PRISM software. * $p \leq 0.05$; $n \geq 3$.

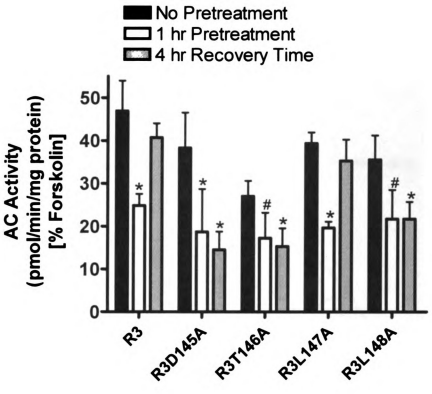

Figure 36B: Effect of RAMP3 PDZ motif point mutations on the recycling/resensitization of the CL-R/RAMP3 complex in the presence of NSF. Confirming the whole-cell binding data, all point mutations to the PDZ motif of RAMP3, except R3L147A, inhibited the resensitization of adenylate cyclase activity seen with wild-type RAMP3-CL-R complex and co-expression of NSF. HEK 293 cells were transfected and pretreated as in figure 29*A* and then membranes were extracted as described in Materials and Methods section. Adenylate cyclase activity was measured in membranes stimulated with 100 nM AM for 15 min. Experiments performed in triplicates and expressed as percent maximal stimulation (% Forskolin). * $p \leq 0.05$ two-tail; # $p \leq 0.05$ one-tail ; $n \geq 3$-4.

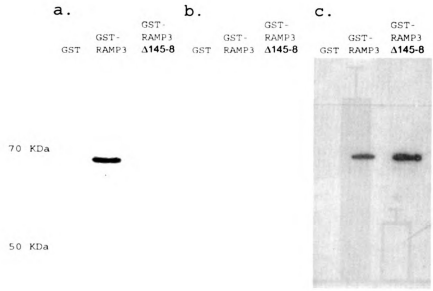

Figure 37: Interaction studies of RAMP3 with NSF. Fusion proteins of GST-RAMP3 demonstrate interaction with NSF in an overlay assay and western blot analysis. 10μg of GST, GST-RAMP3, and GST-RAMP3Δ145-8 protein was run and separated on an SDS-PAGE gel and transferred to a nitrocellulose filter. The nitrocellulose filter was incubated overnight in lysate of HEK293 cells transfected with or without NSF at 4°C. Filter was then washed and probed by immunoblot for NSF (1:250). Identical filters were probed by immunoblot for RAMP3 (1:400). *A)* GST, GST-RAMP3, and GST-RAMP3Δ145-8 overlay assay incubated in NSF-transfected HEK 293 lysates and probed for NSF. *B)* GST, GST-RAMP3, and GST-RAMP3Δ145-8 overlay assay incubated in non-transfected HEK 293 lysates and probed for NSF. *C)* Immunoblot of GST, GST-RAMP3, and GST-RAMP3Δ145-8 probed for RAMP3. Shown are representative blots of at least three experiments.

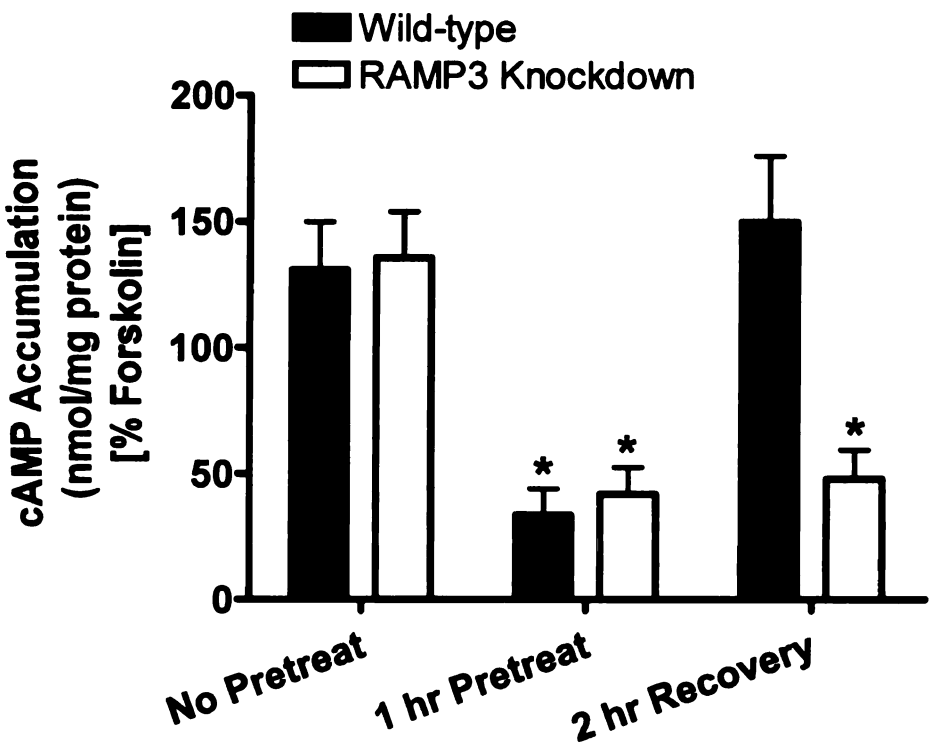

Figure 38A: Effect of RAMP3 RNA interference on resensitization of AM2-R in rat mesangial cells. RAMP3 RNA interference disrupts the ability of AM-R to resensitize after agonist pretreatment. RMC cells seeded in 24-well plates were transfected with d-siRNA of RAMP3 and incubated for 48 hr to allow RNA knockdown. Wild-type and d-siRNA transfected cells were pretreated with 10nm rAM for 1h and were washed extensively to remove residual agonist. Following washes, cells were incubated in serum-free media with 5 μg/ml cycloheximide for 2 hr. After recovery time, RMC cells were re-challenged with 100 nM rAM for 15 minutes and plates were frozen. cAMP levels were measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in rat mesangial cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated four times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 4$ experiments.

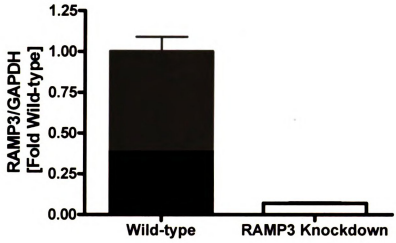

Figure 38B: Effect of RAMP3 RNA interference on resensitization of AM2-R in rat mesangial cells. RAMP3 RNA interference disrupts the ability of AM-R to resensitize after agonist pretreatment. RMC cells seeded in 24-well plates were transfected with d-siRNA of RAMP3 and incubated for 48 hr to allow RNA knockdown. mRNA analysis by RT-PCR showed dramatically decreased levels of RAMP3 mRNA in RAMP3 RNA interference sample as compared to wild type, while lacZ knockdown had no effect on RAMP3 message levels (data not shown). RNA isolation and Q-PCR performed as described in Experimental Procedures section. GAPDH expression measured for all samples for normalization. n=3 experiments.

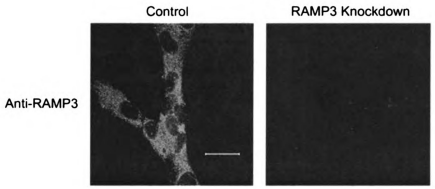

Figure 38C: Effect of RAMP3 RNA interference on resensitization of AM2-R in rat mesangial cells. RAMP3 RNA interference disrupts the ability of AM-R to resensitize after agonist pretreatment. RMC cells seeded in 24-well plates were transfected with d-siRNA of RAMP3 and incubated for 48 hr to allow RNA knockdown. Immunofluorescence microscopy of RMCs with RAMP3 RNA interference demonstrated greatly decreased levels of RAMP3 protein expression in RAMP3 RNA knockdown cells, as compared to wild-type RMC cells. LacZ knockdown had no effect on RAMP3 protein expression in RMCs (data not shown). Cells prepared as described in Experiment procedures section for immunofluorescence microscopy. Fixed cells were stained with anti-RAMP3 antibody (1:200) and detected with a Cy3 anti-rabbit secondary antibody (1:500, in red). Images shown are representative of at least three experiments. Bar scales on all images represent 20μm.

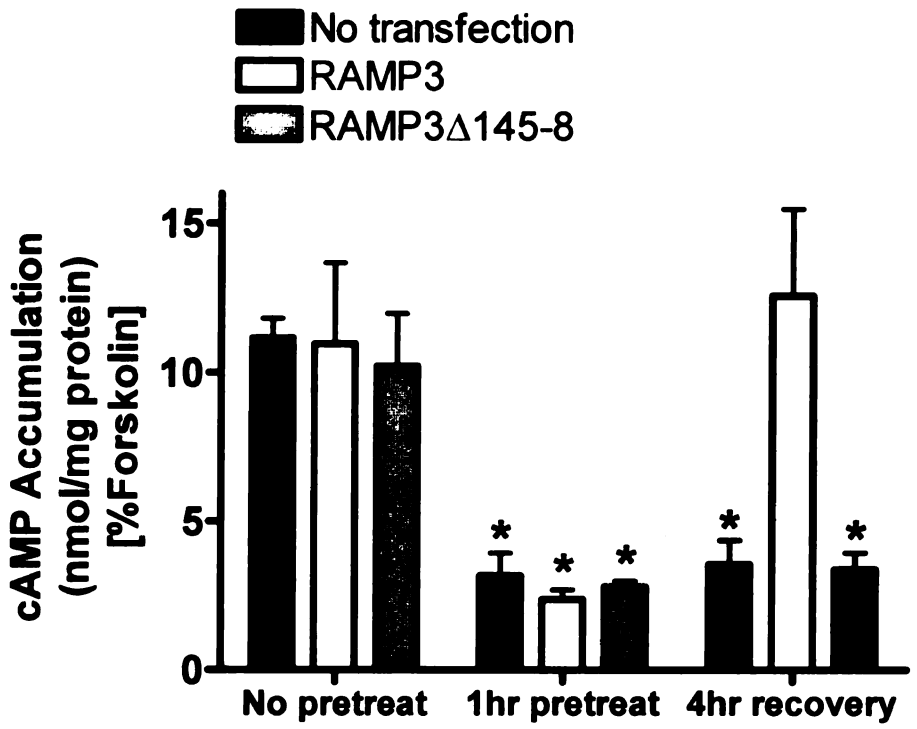

Figure 39: AM-R trafficking in Rat2 fibroblast cells. Rat2 fibroblast cells show a lack of receptor resensitization after agonist pretreatment, whereas when RAMP3 is expressed in Rat2 cells, receptor resensitization is now observed. Expression of PDZ motif mutant of RAMP3 in Rat2 cells is unable to alter the receptor trafficking. Rat2 cells seeded in 24-well plates were pretreated with 100 nm rAM for 1h and were washed extensively to remove residual agonist. Following washes, cells were incubated in serum-free media with 5μg/ml cycloheximide for indicated times. After recovery time, Rat2 cells were re-challenged with 1 mM rAM for 15 minutes and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in Rat2 fibroblast cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least 3 times. Data is expressed as percent maximal response, % forskolin. * $p \leq 0.05$; $n \geq 3$ experiments.

6.4. Discussion:

While many GPCRs utilize similar mechanisms for endocytosis, the functional consequences of endocytosis vary from receptor to receptor. Internalized receptors that are trafficked through a rapid recycling pathway are restored to the plasma membrane in a functional state to achieve resensitization. On the other hand, receptors that are internalized and targeted to late endosomes and lysosomes experience proteolytic degradation, thus promoting attenuation of receptor signaling and down-regulation of the receptor. The CL-R/RAMP system shows sorting of the receptor complex by both trafficking pathways in different cell lines. That is, in RMCs the CL-R/RAMP complex follows a post-endocytic recycling pathway, while in HEK 293 cells and Rat2 fibroblast cells the receptor complex undergoes degradation [276, 285]. Findings in this report demonstrate some of the cellular and molecular mechanisms responsible for the CL-R/RAMP receptor complex's trafficking. We have shown that the PDZ type I motif at the C-terminus of RAMP3 interacts with NSF to target the CL-R/RAMP3 complex for recycling after internalization. NSF is commonly known to interact with αSNAP (soluble NSF attachment protein) and SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins to form the 20S particle, a complex that plays a critical role in intracellular membrane fusion and exocytosis [261-263]. While other labs have demonstrated involvement of NSF with the trafficking of receptors, including the β_2AR and AMPA receptors, this study is the first demonstration of NSF acting in concert with a heterodimeric partner of a receptor to alter the trafficking of the GPCR [268, 269].

Targeting of the receptor complex after agonist-stimulated internalization demonstrates a novel role for RAMP3. This new function for RAMP3 may explain the

altered RAMP expression patterns in certain animal models of disease. RAMP3 expression has been shown to be increased during the transition from left ventricular hypertrophy to heart failure in Dahl salt-sensitive and deoxycorticosterone acetate (DOCA)-salt spontaneously hypertensive rats and in the myocardium of rats with chronic heart failure, where recycling of the receptor complex would be advantageous for continued ligand responsiveness [309-311]. In fact, in various cardiorenal disease states where AM is protective, circulating plasma levels of AM have been shown to be increased. For example, in chronic glomerulonephritis, type I diabetes, and type II diabetes plasma AM levels are elevated [293-295]. In addition, AM delivery through adenoviral injection has been shown to decrease cardiac hypertrophy and renal damage in rat models of hypertension and improves cardiac function and prevents renal damage in streptozotocin-induced diabetic rats [296-300]. If indeed, AM is exerting protective effects against these diseases, then overcoming desensitization of the receptor complex would be important. It is of interest to determine if NSF expression or localization is altered in these disease states as well. Furthermore, studies identifying additional molecular mechanisms of CL-R/RAMP trafficking may be valuable for therapeutic targeting.

While not only discovering a novel function for the RAMPs in post-endocytic trafficking, these findings also demonstrate a functional difference between the AM1R (CL-R+RAMP2) and AM2R (CL-R+RAMP3) receptor complexes, in spite of very similar second messenger systems and the physiological responses thus far identified. This study demonstrates the first difference between the RAMP2 and RAMP3 isoforms in the trafficking of the AM1R and AM2R. We suggest that this novel difference in

AM1 and AM2 receptors may lead to either or both of the following two consequences: 1) there could be yet discovered roles of AM that differentially act through AM1 and AM2R because of this novel role of RAMP3 or 2) in disease conditions, expression of RAMP3 may be preferentially stimulated to effect recycling of CL-R/RAMP3 complex to affect physiological consequences of AM. This could result in a beneficial or harmful effect depending on the system in question (cardiorenal diseases vs. cancer, respectively).

It is important to address the possibility of a species-specific effect, as the prototypic class I PDZ motif is present only in the human RAMP3 and not other species thus far sequenced. The human RAMP3 contains the classical PDZ class I motif (-DTLL), while the two rodent species sequenced (rat and mouse) have a·C-terminal amino acid sequence of –DRLL. The resensitization experiments in the rat mesangial cells demonstrate the importance of the rat RAMP3 for efficient receptor targeting for resensitization. Furthermore, the resensitization experiments in the Rat2 fibroblast cells illustrate the ability of human RAMP3 to alter receptor targeting in a rat cell line to promote receptor resensitization/recycling after agonist pretreatment. Taken together, these data suggest that the lack of complete conservation between species of the C-terminal PDZ motif on RAMP3 does not alter our proposed model.

Finally, recent data suggests that RAMP3 can interact with receptors other than CL-R. It is important to determine if this novel role of RAMP3 in receptor trafficking is specific for CL-R or also for the other receptors RAMPs interact with, namely VPAC, PTH1- and 2-R, and glucagon receptors [286]. In addition to AM, Roh et al. have reported that intermedin, a newly discovered peptide from the calcitonin gene peptide superfamily, can also bind the CL-R/RAMP3 complex [307]. If this reported function for

RAMP3 is specific for AM, or if another peptide like intermedin could cause this phenomenon, remains to be tested.

This study has shown that CL-R's heterodimeric partner, RAMP3, is capable of altering the trafficking of the receptor complex after endocytosis by interacting with NSF via its PDZ domain. This demonstrates a novel function for the RAMP accessory proteins and the first difference between the AM1R and AM2R. With recent reports of RAMPs complexing and regulating additional GPCRs, these findings may reveal a more widespread form of regulation of the GPCR life-cycle.

7. Summary and Conclusions

7.1. Specific aims, major hypotheses, and results of the study

The major aim of this study was to investigate the role of the different RAMP isoforms in the regulation of the AM receptor complex life-cycle. It was hypothesized that through post-translational modifications and protein-protein interactions, the three RAMP isoforms differentially regulate the trafficking and signaling of the AM receptor. Hence, as summarized below, the following specific aims are addressed and the various hypotheses within these aims were tested, and the corresponding experimental results were obtained:

Specific Aim #1: Phosphorylation of amino acid residues of AM receptor complex is involved in regulation of receptor desensitization and internalization.

Hypothesis 1: Specific serine/threonine residues in the C-terminus or 3rd intracellular loop of CL-R are required for AM-R desensitization and internalization.

Hypothesis 2: Specific serine/threonine residues in the intracellular domain of RAMPs are required for desensitization and internalization of AM-R.

Hypothesis 3: Based on the isoform of RAMP associated with CL-R, different amino acid residues are important for receptor phosporylation, desensitization, and internalization.

Hypothesis 4: Different protein kinases regulate the phosphorylation of CL-R in presence of different RAMP isoforms.

Results:

a. Serine/threonine amino acid residues (Ser 421 for AM1 receptor and Thr 423 for AM2 receptor) on the C-terminal tail of CL-R are required for CL-R/RAMP complex desensitization and internalization.
b. Serine and threonine residues in the third intracellular loop of CL-R or intracellular domain of RAMPs are not required for receptor desensitization and internalization.
c. Protein kinase A inhibitor blocks only CL-R/RAMP2 desensitization and internalization, while a PKC inhibitor blocks only CL-R/RAMP3 desensitization and internalization, suggesting different kinases are involved in the phosphorylation of CL-R when different RAMP isoforms are associated.

Specific Aim #2: Protein-protein interactions of RAMPs with other proteins are involved in the regulation of agonist-stimulated internalization of the AM receptor.

Hypothesis 1: RAMP3 (but not RAMP1 or RAMP2) interacts with NHERF-1 to inhibit agonist-induced receptor internalization.

Hypothesis 2: RAMP3 interacts with NHERF-1 via a PDZ recognition motif on RAMP3 and a type I PDZ domain on NHERF-1.

Hypothesis 3: NHERF-1 inhibits internalization of the AM2 receptor (CL-R+RAMP3) via interactions with MERM proteins in the actin cytoskeleton.

Results:

a. NHERF-1 specifically interacts with RAMP3 and causes an inhibition of agonist-induced receptor internalization, without affecting receptor desensitization.
b. RAMP3 interacts with NHERF-1 via a PDZ recognition motif on the extreme C-terminus of RAMP3 and a type I PDZ domain on the N-terminus of NHERF-1.
c. NHERF inhibits internalization of AM2 receptor through interactions with the MERM actin cytoskeletal proteins via a C-terminal MERM domain on NHERF-1.

Specific Aim #3: Protein-protein interaction of the RAMPs with various other proteins is involved in the regulation of AM receptor trafficking from the sorting endosome.

Hypothesis 1: RAMP3, but not RAMP1 or RAMP2, interacts with NSF to target the receptor complex for recycling pathway after agonist-stimulated internalization.

Hypothesis 2: RAMP3 interacts with NSF via a PDZ recognition motif on RAMP3.

Results:

a. RAMP3 (but not RAMP1 or RAMP2) interacts with NSF to target AM receptor complex for a recycling pathway after agonist-induced internalization.

b. RAMP3 was determined to interact with NSF via a PDZ recognition motif on the extreme C-terminus of RAMP3.

7.2. Limitations of the study.

1. The primary limitation of this study is that all experiments were performed in cell culture system, as opposed to using a whole-animal model system. The practicality of defining detailed molecular mechanisms required the use of cell culture systems.
2. The cell culture systems utilized that were derived from the rat may not necessarily reflect the same regulatory mechanisms in human cells. The rat cell lines were chosen because of the specific RAMP isoform expression levels. Obtaining and maintaining human cell lines, both from the standpoint of cost and labor, was not practical.
3. All changes in receptor signaling were elicited by exogenous administration of AM. Concentrations of AM used were within the established circulating levels of AM, yet it remains unknown what concentrations mesangial cells and proximal tubule cells are exposed to *in vivo*. Until physiological and pathological concentrations are determined in the vicinity of these two cell types, no arguments can be made regarding this issue.
4. Changes in receptor trafficking were characterized using cAMP accumulation as the endpoint. Measuring the role of receptor trafficking

changes in regulation of a more physiological parameter (such as proliferation or migration) may provide additional information.

5. Chemical and pharmacological inhibitors were used to examine the roles of NSF and protein kinases. Given the limited specificity of chemical and pharmacological inhibitors, the results from these studies should be interpreted with some degree of caution. Nonetheless, it should be noted that the concentrations used in the studies have been published by others and characterized as specific within that range. In addition, where possible, supplementary controls were tested.

7.3. Positive outcomes of the study.

This thesis research sought to examine differences in AM receptor signaling, dictated by the co-expression of the different RAMP isoforms. The three individual studies within the project characterized roles for the RAMP isoforms in differentially regulating the post-translational modifications and protein-protein interactions that regulate AM receptor complex signaling.

While phosphorylation of CL-R had been demonstrated with prolonged agonist exposure, this study was the first to report specific amino acids of the receptor critical for desensitization and internalization. In addition, it was not previously known that association of CL-R with different RAMP isoforms generated different phosphorylation patterns on the receptor, regardless of exposure to the same ligand (AM). It could be envisioned that this differential regulation of the receptor, directed by the RAMP isoforms, allows further protein-protein interactions that regulate receptor signaling and

trafficking. Future research investigating the interaction of differentially phosphorylated AM receptor complexes with different arrestin molecules could clarify a role for phosphorylation in the targeting of the receptor complex after agonist stimulation.

The last two studies defined a role for the RAMPs in protein-protein interactions that regulate the trafficking and life-cycle of the receptor complex. Interactions of NHERF-1 and NSF with RAMP3 have been demonstrated, in different ways, to promote AM receptor signaling. The major finding of this thesis research that could potentially contribute to the greater field of receptor biology is the concept of RAMP expression dictating the receptor fate after agonist-stimulated desensitization. In light of the recent report of RAMPs' interaction with other GPCRs, our observations may be applicable to additional receptor systems. Future studies investigating the role of RAMPs in the trafficking of other GPCRs could help establish this idea of more widespread receptor regulation. Given the documented expression of RAMPs in tissues devoid of CL-R expression, the possibility of additional regulatory roles for the RAMPs has been proposed.

This thesis research has defined novel roles for the RAMP proteins in regulation of the AM receptor signaling and endocytic trafficking. Considering the established beneficial and harmful effects of AM signaling in different physiological systems (cardiorenal disease vs. cancer, respectively), it is important to have specific mechanisms to promote or repress AM signaling. In review of this study, we believe differential RAMP isoform expression provides a form of this indispensable receptor regulation.

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