THE ROLE OF BETA-ARRESTIN IN REGULATING THE MUSCARINIC ACETYLCHOLINE TYPE II RECEPTOR

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THE ROLE OF BETA-ARRESTIN IN REGULATING THE MUSCARINIC ACETYLCHOLINE TYPE II RECEPTOR

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DEDICATION

This thesis is dedicated in memory of my mother Diane Louise Jones.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

$AT_{1A}R$	Angiotensin II Type 1A receptor
AP-2	Adaptor protein-2
ARF	ADP-ribosylation factor
β_2-AR	beta 2-adrenergic receptor
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary deoxyribonucleic acid
COPD	Chronic obstructive pulmonary disease
COS-7	African Green Monkey Kidney Fibroblast
DAG	1, 2-diacylglycerol
DNA	Deoxyribonucleic acid
Dyn	Dynamin
EEA-1	Early endosome autoantigen-1
EGFR	Epidermal growth factor receptor
Eps15	Epidermal growth factor receptor pathway substrate 15
ERK	Extracellular regulated kinase
ESCRT	Endosomal sorting complex required for transport
GASP	GPCR-associated sorting protein
GDP	Guanosine 5'- diphosphate
GFP	Green fluorescent protein
GPCR	G protein coupled receptor
GRK	GPCR regulatory kinase

GTP	Guanine 5'-triphosphate
HA	Hemagglutinin
HeLa	Cervical cancer cells
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
IP_3	Inositol 1, 4, 5-trisphosphate
JNK	c-jun N-terminal kinases
LPA	Lysophosphatidic acid
LPA_1	Lysophosphatidic acid receptor 1
mAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen activated protein kinase
Mdm-2	Murine double-minute 2
MEF	Mouse embryonic fibroblasts
MEF KO1	β-arrestin 1 knockout cells
MEF KO2	β-arrestin 2 knockout cells
MEF K01/2	β-arrestin 1 and 2 knockout cells
MVB	Multivesicular body
NMS	*N*-methylscopolamine
NSF	*N*-ethylmaleimide-sensitive fusion protein
PAF	Platelet activating factor receptor
PAR2	Protease-activated receptor 2
PBS	Phosphate buffered saline
PI3-K	Phosphatidylinositol 3-kinase

PIP2	Phosphotidyl inositol-3,4-bisphosphate
PKA	cAMP dependent protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
RASMC	Rat aortic smooth muscle cell
RGS	Regulator of G protein signaling proteins
SNX-1	Sorting nexin-1
TfnR	Transferrin receptor
UIM	Ubiquitin interacting motif
V_2R	Vasopressin 2 receptor
WT	Wild type cells

SUMMARY

The focus of my work is to elucidate the role of β-arrestin in regulating the M_2 mAChR which is currently unclear in the literature. In particular, we examined the involvement of β-arrestin in mediating internalization and down-regulation of stimulated M_2 mAChRs.

Muscarinic acetylcholine receptors belong to the superfamily of GPCRs that are commonly expressed in a variety of tissues and are classified into five known subtypes (M_1-M_5). Of these subtypes, the M_2 mAChRs are expressed predominantly in the heart and brain where their stimulation leads to the regulation of myocardial contractility and neurotransmission. Given the critical role of M_2 mAChRs in cardiovascular and neurotransmitter homeostasis, the mechanism behind M_2 mAChR regulation is important to investigate. Since β-arrestin 1 and 2 regulate the activity of many other GPCRs, we sought to identify their roles in regulating M_2 mAChR activity. To achieve this goal we utilized mouse embryonic fibroblasts (MEF) derived from β-arrestin knockout mice lacking one or both isoforms as well as exogenous expression of wild type and β-arrestin mutants. In the first study, M_2 mAChRs transiently expressed in wild type MEF cells underwent agonist-induced internalization and were subsequently sorted into intracellular compartments. In contrast, stimulated M_2 mAChRs failed to undergo internalization and sorting into intracellular compartments in MEF β-arrestin double knockout cells (MEF KO1/2). In MEF KO1/2 cells, expression of either β-arrestin 1 or 2 isoforms resulted in rescue of agonist-promoted internalization. Stimulation of M_2 mAChRs led to a stable co-localization with GFP-tagged β-arrestin within endocytic structures in multiple cell

lines; the compartment to which β-arrestin localized was determined to be the early endosome. Agonist-promoted internalization of M_2 mAChRs was moderately rescued in MEF β-arrestin 1 and 2 double knockout cells expressing exogenous arrestin mutants that were selectively defective in interactions with clathrin (β-arrestin 2 ΔLIELD), AP-2 (β-arrestin 2-F391A), or both clathrin/AP-2. Expression of a truncated carboxy-terminal region of β-arrestin 1 (319-418) and Eps 15 mutants, potent inhibitors of clathrin-mediated endocytosis, completely abrogated agonist-promoted internalization of M_2 mAChRs in wild type MEF cells. Once internalized, post-endocytic trafficking of the M_2 mAChR appeared to be regulated by both Rab5 and Rab7 GTPases suggesting that the receptor is sorted from the early endosome to the lysosome for degradation. In summary, this study demonstrates that agonist-induced internalization of M_2 mAChR is β-arrestin- and clathrin-dependent, and that the receptor stably co-localizes with β-arrestin in early endosomal vesicles suggesting it behaves as a class B receptor.

Given that stimulated M_2 mAChRs remain stably associated with β-arrestin in the cytosol (class B receptor), we sought to identify its function in regulating the post-endocytic trafficking (down-regulation) of the M_2 mAChR. In the second study, we investigated the role of ubiquitination of β-arrestin in the agonist-promoted lysosomal sorting and subsequent degradation of the M_2 mAChRs. MEF cells lacking both isoforms of β-arrestin (MEF KO1/2) were unable to down-regulate M_2 mAChRs whereas MEF cells from single knockout mice (MEF KO1 or KO2) retained the ability to do so. Expression of β-arrestin 1 or 2 in MEF KO1/2 cells rescued down-regulation. In wild type MEF cells, both M_2 mAChR and β-arrestin exhibited basal ubiquitination that was increased following agonist stimulation. Receptor degradation appeared to be regulated

by the ubiquitination status of β-arrestin 2, since expression of a chimeric form fused to ubiquitin increased both constitutive and agonist-promoted down-regulation, whereas expression of a β-arrestin 2 mutant lacking putative ubiquitination sites, β-arrestin $2^{K18R, K107R, K108R, K207R, K296R}$, significantly blocked receptor degradation. Expression of the β-arrestin $2^{K11R, K12R}$ mutant, in contrast, did not prevent degradation of M_2 mAChRs while both mutants had no affect on agonist-promoted internalization. Further localization studies in MEF KO1/2 cells revealed that β-arrestin $2^{K18R, K107R, K108R, K207R, K296R}$ mutant did not disrupt stable arrestin/receptor complexes in the cytosol but did block delivery of M_2 mAChRs to the late endosome/lysosome. Pretreatment of cells with lactacystin, which inhibits proteasome-dependent recycling of ubiquitin, blocked down-regulation without affecting internalization or the ubiquitination state of the M_2 mAChR while ubiquitination of β-arrestin 2 diminished significantly. These results support a role for ubiquitinated β-arrestin in mediating M_2 mAChR degradation in the lysosome. Stable association with β-arrestin, stable agonist promoted ubiquitination of β-arrestin, and agonist-promoted down-regulation of the receptor supports the notion that M_2 mAChR belongs to the class B. We further propose a mechanism for differential ubiquitination of β-arrestin in regulating agonist-promoted degradation of the M_2 mAChR. Colletively, these studies give us new insight on the function of β-arrestin in regulating the activity of the M_2 mAChR, a class B receptor.

CHAPTER 1

INTRODUCTION

G protein-coupled receptors

Activation and Regulation

G protein-coupled receptors (GPCRs) belong to the largest family of membrane bound-receptors with over 600 genes identified to date, accounting for 2% of the human genome [1, 2]. GPCRs are seven transmembrane proteins that reside embedded in the plasma membrane and are involved in mediating a broad range of biological processes including sensory perception, neurotransmission, cellular metabolism, cellular growth and differentiation, and immune responses [3]. The localization of GPCRs on the cell surface allows them to detect extracellular stimuli (e.g., hormones, light, odorant, growth factors, neurotransmitters) and to respond to those stimuli by triggering an intracellular signaling cascade, which culminates in a cellular response such as apoptosis, differentiation, growth, and secretion. Because GPCRs are involved in many physiological and pathophysiological processes, understanding on a cellular and molecular level how GPCR signaling processes are regulated is of great importance. Indeed, over 30% of the pharmaceutical therapeutics on the market today are targeted to modulating the activity of GPCRs [4].

GPCRs exert these physiological responses by coupling to a heterotrimeric G protein comprised of α-, β-, γ- subunits. On activation, GPCRs promote the exchange of GDP for GTP on the Gα subunit. This in turn leads to a liberation of G protein

complexes into Gα-GTP and Gβγ subunits that are then free to interact and modulate downstream enzymes and effectors including adenylyl cyclase, phosphatidylinositol kinases, nonreceptor tyrosine kinases, ion channels, and phospholipases [5]. Changing levels of second messengers via G protein activation leads to amplification of a signal transduction cascade which culiminates in a cellular response. GPCRs are defined by their ability to activate a G protein signaling pathway(s). These pathways include $G\alpha_i$, $G\alpha_q$, $G\alpha_s$, $G\alpha_{12/13}$ subfamilies that inhibit adenylyl cyclase, stimulate phospholipase C, stimulate adenylyl cyclase, and activate Rho pathways, respectively (Fig. 1) [6, 7]. The signal transduction cascade is terminated following hydrolysis of GTP bound to the Gα by regulator of G protein signaling proteins (RGS) [8]. The heterotrimeric complex can reform and reassociate with an unstimulated GPCR.

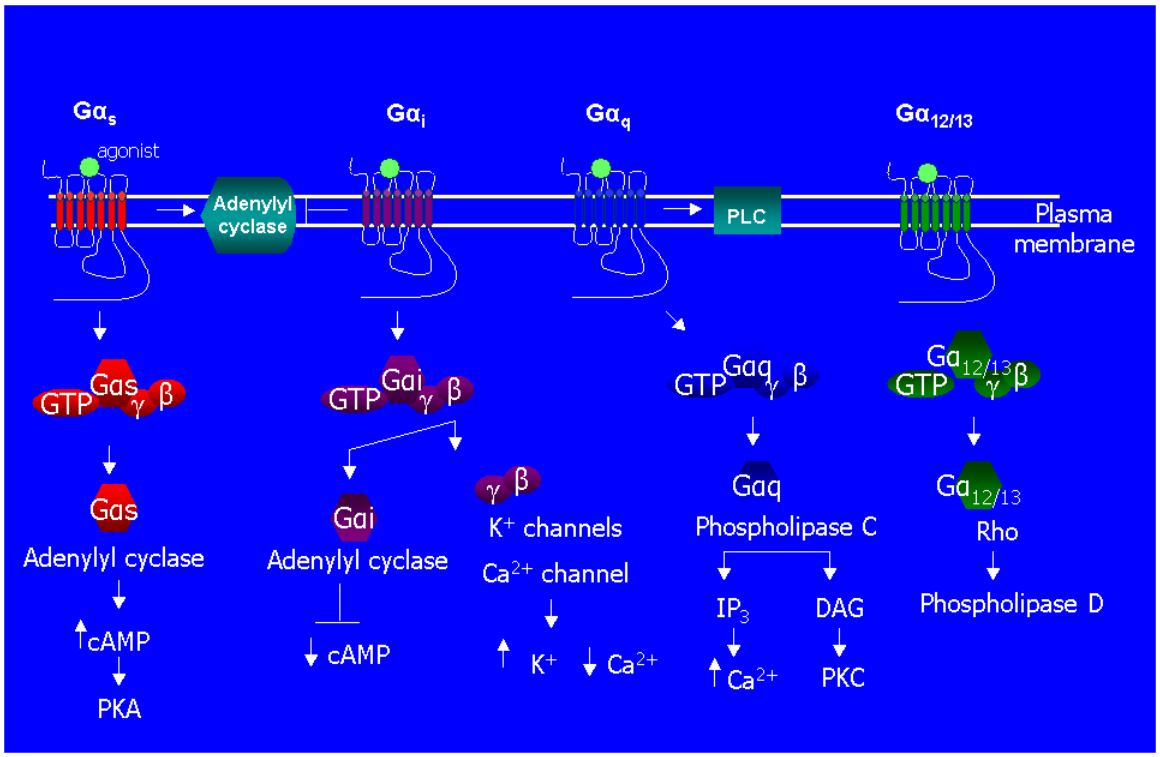

Fig. 1. Signaling pathways exhibited by GPCR mediated G protein activation. Agonist-induced activation of GPCRs stimulates or inhibits effector proteins by functionally coupling to a heterotrimeric G protein. Upon GPCR activation, the G protein exchanges GDP for GTP on the Gα subunit. Both the α subunit and βγ-complex can interact with effectors to evoke a cellular response. PKA, protein kinase A; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; PLC, phospholipase C.

To monitor the broad array of signaling events that can occur following GPCR activation, complex regulatory mechanisms are in place to tightly control the responsiveness of the receptor [9]. To ensure a controlled signaling event, GPCRs undergo three modes of regulation: desensitization (1), which is typically accompanied by internalization (2), and recycling or down-regulation (3). Desensitization is a complex event that involves agonist-dependent phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs) followed by binding of a cytosolic adaptor protein known as β-arrestin to phosphorylated receptors. β-arrestin association uncouples the receptor from its cognate G protein by sterically blocking further interaction, thereby attenuating receptor-mediated G protein activation [10-12]. This desensitization process is typically followed by internalization or endocytosis whereby the receptor is removed from the cell surface. Receptor internalization can serve to resensitize deactivated receptors or to target the receptor for degradation, a process known as down-regulation. Resensitization promotes "reactivation" of receptors by dephosphorylating them in endocytic vesicles in preparation for recycling them back to the plasma membrane as functional receptors whereas down-regulation serves to diminish the sensitivity of a cell to stimuli by decreasing the total number of functional receptors presumably by proteasomal or lysosomal mediated degradation [13, 14]. Typically, chronic stimulation of receptors leads to down-regulation [9, 15].

Mechanisms of Desensitization, Internalization, and Down-regulation

The best-characterized mechanism of desensitization and subsequent agonist-induced endocytosis stems from work performed on the β_2-adrenergic receptor (β_2-AR). Desensitization of ligand occupied β_2-AR is mediated by GRK2 whereby specific serine/theronine residues in the cytoplasmic tail are phosphorylated. Phosphorylation of β_2-AR facilitates its interaction with β-arrestin. β-arrestins contribute to β_2-AR desensitization by physically disrupting receptor/G protein association and can further serve as an adaptor protein to target the receptor to pre-formed clathrin coated pits at the plasma membrane [12, 16, 17]. Redistribution of β_2-AR into clathrin coated pits and subsequent scission from the plasma membrane into primary endocytic vesicles via the GTPase dynamin forms the basis for receptor removal from the plasma membrane (Fig. 2).

Although many GPCRs utilize the same clathrin-mediated pathway as the β_2-AR, other receptors are internalized through an alternative endocytic pathway. Additional internalization routes include caveolae or non-clathrin, non-caveolae mediated pathways, which differ in size and composition of the vesicle coat [18]. Caveolae are flask shaped invaginations that are rich in caveolin proteins, glycosphingolipids and cholesterol [19]. Many receptor systems are targeted to and internalize via caveolae which further require dynamin activity [20-23]. However, β-arrestin does not contribute to this endocytic route [24]. Clathrin- and caveolae-independent endocytosis remains to be fully characterized; however, reports indicate that Arf6 GTPase facilitates internalization independent of dynamin function [25-27].

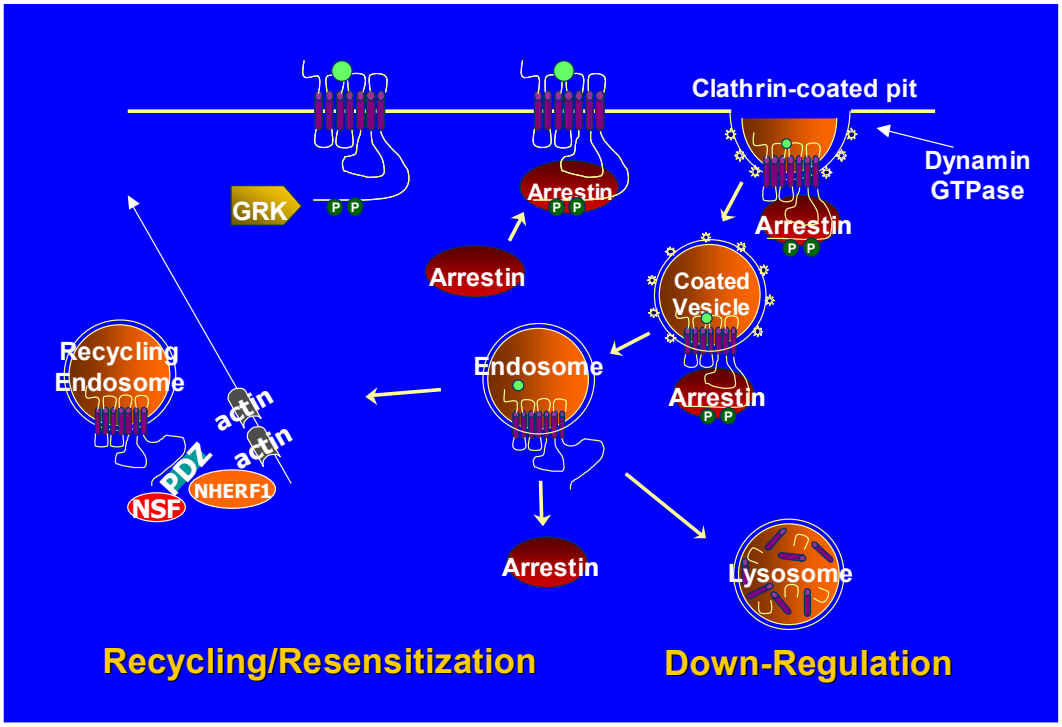

Fig. 2. Model summarizing internalization and endocytic sorting of the β_2-adrenergic receptor (β_2-AR). Upon agonist addition, β_2-AR is phosphorylated at specific serine/threonine residues in the cytoplasmic tail by GRK2 resulting in β-arrestin translocation and binding to the receptor. β-arrestin directs the receptor to clathrin-coated pits prior to clathrin- and dynamin-mediated endocytosis. Delivery of GPCRs to the early endosome promotes receptor dephosphorylation, ligand removal, and β-arrestin disassociation. The resensitized receptor than enters the recycling pathway via a PDZ domain-mediated interaction with proteins NSF and/or NHERF which can interact with the actin cytoskeleton.

The ADP-ribosylation factor (Arf6 GTPase) belongs to a family of Ras-related, GTP binding proteins that affect trafficking between the cell surface and endosomes [28]. Activation of Arf6 has been shown to alter the lipid composition of membranes and to promote actin remodeling [29, 30]. Interestingly, Arf6 GTPase appears to participate in all three endocytic processes thereby regulating a variety of GPCRs including the β_2-AR [27, 31]. Other players involved in the non-clathrin, non-caveolae endocytic pathway remain to be elucidated (Fig. 3).

The functional consequence of agonist-induced endocytosis has been shown to include either resensitization and/or down-regulation. Many internalized GPCRs are sorted to recycling endosomes whereby endosome specific phosphatases dephosphorylate the receptor. Dephosphorylated receptors are then recycled back to the cell surface as functional receptors [10, 32, 33]. Specific sequences in the C-terminal domain of GPCRs have been determined to be required for efficient recycling. The β_2-AR contains a PDZ binding domain that binds and interacts with *N*-ethylmaleimide-sensitive fusion protein (NSF) and Na^+/H^+ -exchanger regulator factor 1 (NHERF1) that serve to regulate sorting to recycling compartments (Fig. 2) [34, 35]. The μ-opioid receptor lacks these interacting motifs but presumably contains another signal for recycling [36]. The N-formyl peptide receptor requires the presence of β-arrestin proteins for efficient recycling through an unknown mechanism [37].

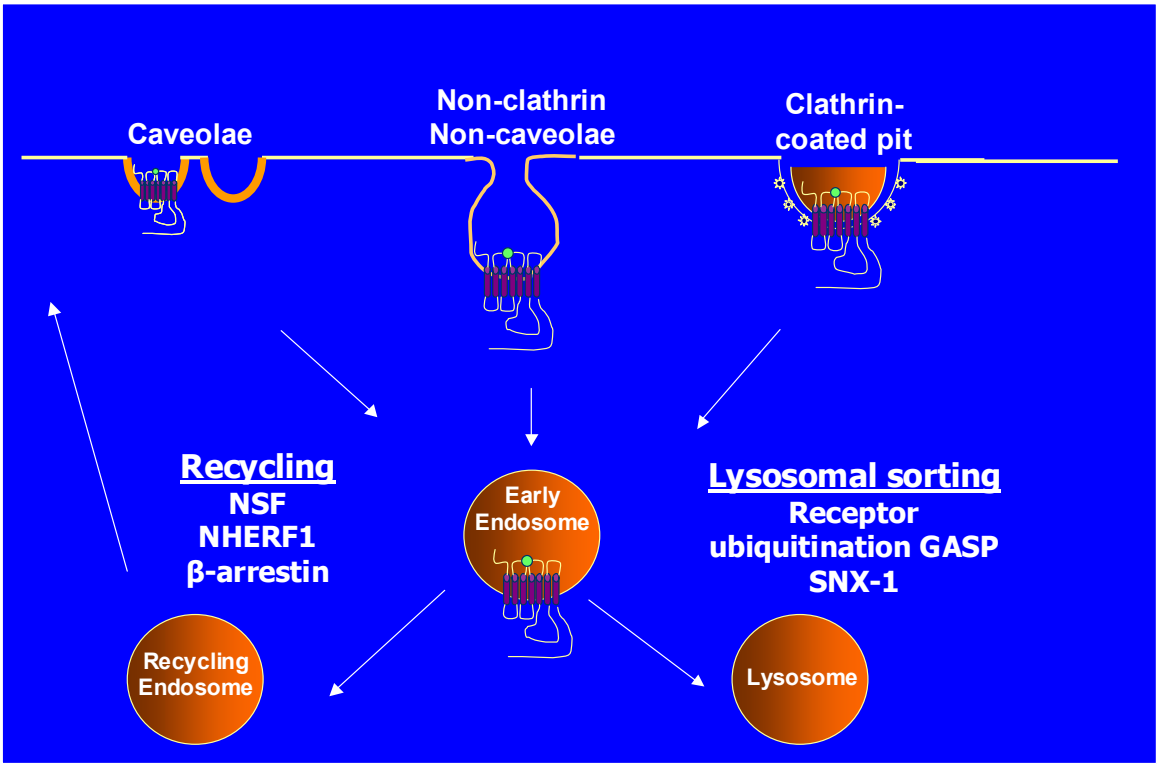

Fig. 3. Multiple internalization routes and subsequent post-endocytic trafficking of GPCRs. Agonist-induced endocytosis of GPCRs can lead to internalization through multiple pathways including caveolae, clathrin, and non-clathrin/non-caveolae pathways. Once internalized from the cell surface, GPCRs can be recycled back to the cell membrane or be targeted for lysosomal degradation dependent on protein interactions. Receptors such as the β_2-AR interact with NHERF and NSF proteins that regulate β_2-AR sorting to recycling compartments. Other GPCRs such as the N-formyl peptide receptor require β-arrestin for efficient recycling. In contrast, receptors sorted to the lysosome may interact with GASP or SNX-1 proteins to mediate transport. Other signals such as receptor ubiquitination can also be a major player in lysosomal targeting.

In contrast, many GPCRs that are acutely or chronically stimulated can be rapidly internalized and delivered to lysosomes for degradation [14, 38-40]. The sorting mechanism for lysosomal delivery is still not well understood; however, recent efforts have also identified several interacting proteins and sorting motifs that specify this process. It has been shown that some GPCRs can undergo ubiquitination, which serves as a signal for entry into the degradative pathway. Presumbably, the ubiquitin moiety(ies) interacts with sorting machinery on endosomes to shuttle the receptor to the lysosome. Other interacting proteins can also facilitate movement of GPCRs along the degradative pathway. Lysosomal sorting of some GPCRs requires that the C-terminal domain of the GPCR binds to GPCR-associating sorting protein (GASP) or sorting nexin-1 (SNX-1) proteins [41-43]. Interestingly, normally recycled receptors can enter the degradative pathway upon chronic stimulation (Fig. 3). The mechanism behind this "traffick switch" appears to be dependent upon the concentration of agonist. For example, exposure of the neurokinin-1 receptor (NK_1R) to low concentrations of agonist induces minimal phosphorylation, transient association with β-arrestin, and fast recycling. Higher concentrations of agonist lead to extensive phosphorylation, prolonged interactions with β-arrestin and eventual degradation. Thus, the extent of NK_1R phosphorylation, dependent upon concentration of agonist, can determine whether the receptor is recycled or degraded [44-47]. Like the NK_1R, the cannabinoid 1 receptor switches to the degradative pathway following chronic stimulation. The authors suggested that high doses of agonist lead to stronger association of GASP with the C-terminal tail of the cannabinoid 1 receptor [43].

GPCR Post-Endocytic Trafficking

Recycling and degradative pathways utilized by receptors are managed by proteins known to control membrane trafficking along these endocytic routes. Some of the key regulators of post-endocytic trafficking are the Rab GTPase members, Ras-like GTP binding proteins. Activation of Rab5 GTPase plays a key role in early endosomal localization of GPCRs by mediating fusion of primary endocytic vesicles with the early endosome [48-50]. Rab5 GTPase has also been implicated in mediating agonist-induced endocytosis of some GPCRs via direct or indirect interactions with the receptor [48, 51]. Once receptors enter the early endosome, receptors are then delivered to recycling and/or degradative pathways depending on the signals that specify their transport (mentioned previously).

Receptors destined for the recycling compartment enter early endosomal extensions that bud off into recycling compartments. Delivery of cargo back to the cell surface is controlled by activation of Rab4 and Rab11 GTPases [50, 52, 53]. In contrast, receptors that enter the degradative pathway remain in the vesicular portion of the early endosome, which then invaginates into the lumen forming specialized vesicles [54, 55]. These compartments are known as mulitvesicular bodies (MVB) and/or late endosomes. Cargo within the mulitvesicular bodies can then fuse with the lysosome for degradation [56]. Fusion of late endocytic vesicles with the lysosome appears to involve activation of Rab7 GTPase (Fig. 4) [55, 57-60].

Recent studies indicate that sorting along the lysosomal pathway requires the cargo to be ubiquitinated. Ubiquitinated proteins are thought to interact with microdomains within the vesicular region of the early endosome thereby preventing entry

into the recycling pathway [61]. These microdomains are thought to contain a scaffolding protein known as HRS (hepatocyte growth factor-regulated tyrosine kinase substrate) that interacts with ubiquitinated cargo via a UIM (ubiquitin interacting motif) and subunits of the mammalian ESCRT (endosomal sorting complex required for transport) [56, 61-63]. It has been demonstrated that the sorting of ubiquitinated cargo to the lysosome for degradation is mediated by HRS since a mutant form of HRS lacking UIM blocks trafficking of epidermal growth factor receptor (EGFR) and E-caderin to the lysosome [56, 64, 65]. Interestingly, it has been reported that delivery of cargo to the lysosome requires its deubiquitination at the late endosome/MVB. Alwan *et al.* reported that deubiquitination of the EGFR by the 26S proteasome occurs prior to its lyososomal degradation [66, 67].

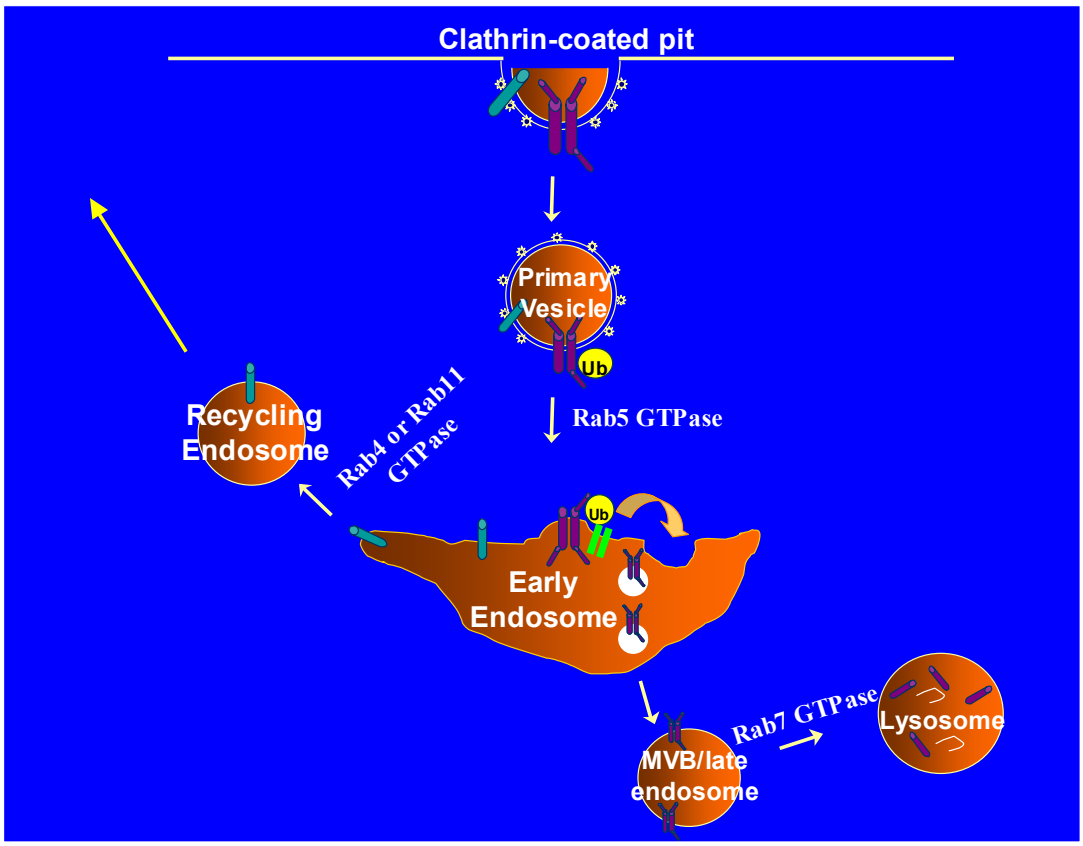

Fig. 4. Schematic of lysosomal and recycling sorting of receptors by Rab GTPases and ubiquitination. Following ligand activation, receptors are internalized into primary endocytic vesicles that fuse with the early endosome mediated by Rab5 GTPase. Dependent on the receptor's association with interacting proteins, a sorting decision is made to either recycle the receptor (blue) or down-regulate the receptor (purple). Delivery of receptor to the plasma membrane via recycling compartments is mediated by Rab4 and Rab11 GTPases. Receptors destined for the lysosome are sorted into MVB/late endosome. One such pathway involves ubiquitinated receptor that can interact with hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) at specific microdomains of the early endosome (green). Maturation of MVB/late endosome leads to fusion with the lysosome, a process mediated by Rab7 GTPase.

Mechanism of GPCR Regulation by β-arrestin

Two widely coexpressed isoforms of nonvisual β-arrestin (β-arrestin-1, β-arrestin-2) are now known to regulate nearly all GPCRs studied to date [9]. As mentioned previously, β-arrestins are major players involved in mediating GPCR desensitization and internalization but have since been implicated in GPCR post-endocytic trafficking and G protein independent signaling. The diverse roles they play have enormous implications on the functionality of GPCR-mediated signaling.

The classical role of nonvisual β-arrestins is to selectively bind to phosphorylated activated forms of GPCRs to attenuate receptor-mediated signaling at the cell surface. β-arrestin bound to GPCRs can then promote direct interaction with the clathrin heavy chain and the heterotetrameric AP-2 adaptor complex, essential components of clathrin-coated pits [16, 68]. β-arrestin-GPCR complexes are subsequently removed from the plasma membrane via clathrin-mediated endocytosis, which further requires dynamin GTPase activity. Thus, β-arrestins can mediate agonist-induced endocytosis by directly interacting with the endocytic machinery.

β-arrestin's ability to attenuate receptor mediated signaling at the cell surface by functioning at both the desensitization and internalization level would support the notion that cell-signaling cascades are arrested. However, elaborate studies have now revealed that β-arrestins can further serve as scaffolds to recruit signaling components for receptor mediated signaling independent of G proteins at the site of endosomes. Some of the β-arrestin binding partners that may link GPCRs to intracellular signaling pathways include nonreceptor tyrosine kinases such as the c-Src family, c-Jun amino-terminal kinase (JNK) and ERK-1 or -2 mitogen-activated protein kinase (MAPK). Some evidence indicates

that spatially restricting Src to the cytoplasm can modulate the endocytic proteins directly involved in the regulation of GPCRs. Src has been shown to activate dynamin GTPase and mediate GRK2 down-regulation thereby providing a feedback mechanism to enhance and prevent further GPCR endocytosis, respectively [69, 70]. Interactions of β-arrestin with JNK and ERK1/2 appear to participate in distinct cellular processes. One apparent role for intracellular β-arrestin-ERK1/2 complexes is the reorganization of the cytoskeleton to drive chemotaxis [71-75]. It has been proposed that β-arrestin/MAPK complexes can facilitate phosphorylation of proteins involved in chemotaxis since these complexes are found at the leading edge during protease-activated receptor 2 (PAR2) induced cell migration [72, 76]. The targets for MAPK phosphorylation, however, are currently unknown. Moreover, β-arrestin signaling complexes further appear to facilitate proliferation and protect cells from apoptosis [77, 78]. Although the role for β-arrestin in other biological functions is unknown, the implications that GPCR-β-arrestin complexes participate in subcellular localization of signaling cascades suggests that more biological activities dependent on the GPCR are involved.

Recent reports also suggest that β-arrestins can function at post-endocytic stages to govern receptor trafficking. It has been shown that receptors show differential affinities for β-arrestin and therefore they are classified into two groups. Class A receptors, including the β_2-AR, α_{1b}-adrenergic receptor, and endothelin A receptor, bind to β-arrestin 2 with higher affinity than β-arrestin 1 [79, 80]. These receptors are thought to recruit β-arrestin to the plasma membrane whereby translocation to clathrin-coated pits occurs. Following clathrin-coated pit localization and subsequent internalization, β-arrestin disassociates from the receptor. Hence receptors enter early endosomes devoid

of β-arrestin and are subsequently resensitized and rapidly recycled [81]. In contrast, class B receptors, including the vasopressin V2 receptor (V_2R), angiotensin II type 1A receptor ($AT_{1A}R$), and neurotensin receptor 1, bind to both β-arrestin isoforms with equal affinity [82]. These receptors stably associate with β-arrestin so that β-arrestin/receptor complexes remain intact and are internalized together into early endosomes. This interaction can be maintained for prolonged periods of time. This stable association may dictate the kinetics of receptor recycling since $AT_{1A}R$ and V_2R recycle very slowly (Fig. 5) [83, 84]. Whether or not stable β-arrestin/receptor complexes contribute to receptor down-regulation is currently unknown.

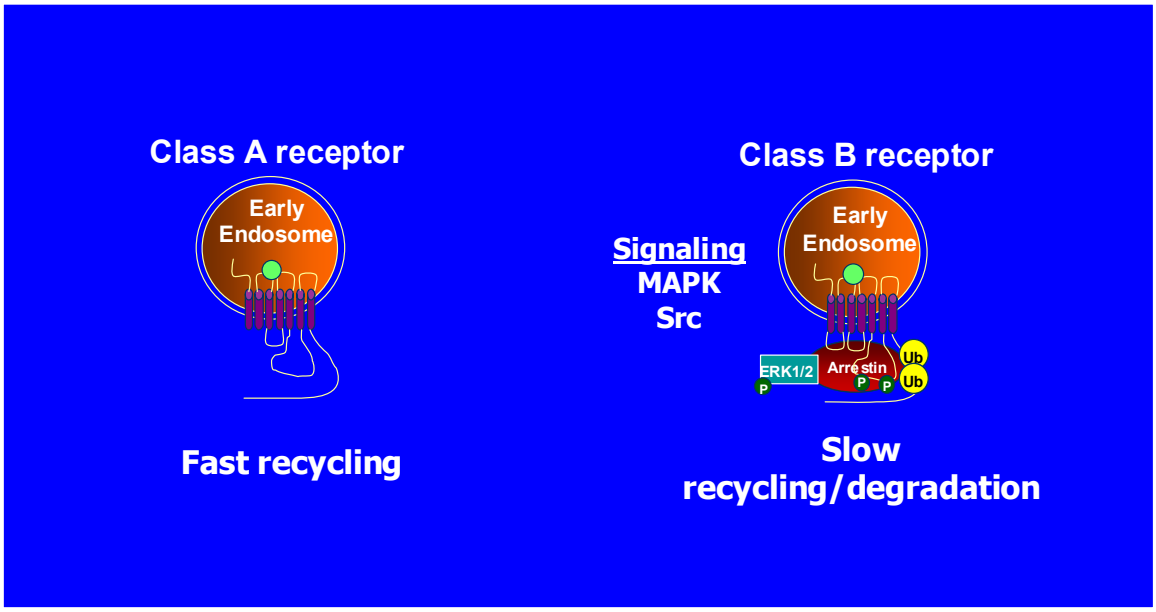

Fig. 5. Role of β-arrestin in the intracellular trafficking and signaling of GPCRs. Class A GPCRs exhibit transient interactions with β-arrestin such that the receptor enters the early endosome devoid of β-arrestin. These GPCRs undergo resensitization and are subsequently rapidly recycled to the plasma membrane. Class B GPCRs remain stably bound with β-arrestin at early endosomal compartments. These receptors are either slowly recycled or targeted for degradation by the lysosome. Stable β-arrestin association correlates with sustained ubiquitination patterns. β-arrestin can also serve as a scaffold in GPCR signal transduction to c-Src or MAPK family proteins in the cytosol.

The mechanism behind stable β-arrestin binding to GPCRs appears to involve ubiquitination. Upon receptor activation, β-arrestin undergoes ubiquitination, which serves to facilitate receptor endocytosis. The ubiquitination state of β-arrestin directly correlates with the binding pattern of GPCRs. Thus, β-arrestin when bound to class A receptors shows a transient ubiquitination pattern while β-arrestin association with class B receptors reveals sustained ubiquitination [85, 86]. For example, Shenoy and coworkers showed that expression of a β-arrestin 2-ubiquitin chimera converted the class A β_2-AR receptor into a class B receptor. The data infer that liberation of β-arrestin 2 from β_2-AR requires its deubiquitination [87].

GPCR Ubiquitination

Ubiquitination is a highly conserved process that involves the covalent attachment of an ~8-kDa polypeptide to ε-amino groups of lysine residues in target proteins. Ubiquitin attachment is achieved through a series of enzymatic events involving classes of enzymes known as ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s) [88]. Polyubiquitination of substrate proteins has long been known as a potent targeting signal for degradation in the 26S proteasome; however, recognition by the proteasome requires more than 4 ubiquitin moieties appended to one lysine residue [89]. Shorter ubiquitin chains known as monoubiquitination or multi-monoubiquitination are now shown to regulate a wide variety of cellular process, including virus budding, histone activity, DNA repair, endocytosis, and lysosomal degradation [90-93].

It has been shown that several cell-surface receptors are labeled with a single ubiquitin polypeptide, monoubiquitination, in response to agonist. This process is sufficient to target these receptors to endocytic vesicles [94-97]. In particular, the Ste2p receptor, a GPCR equivalent in yeast, undergoes ligand-mediated monoubiquitination in the C-terminal tail that is required for internalization [98]. Interestingly, mutation of a lysine residue within the SINNDAKSS motif abolishes ubiquitination and dramatically reduces internalization. Alternatively, internalization of the growth-hormone-receptor in mammalian cells is mediated by the ubiquitin-proteasome system although ubiquitination of the receptor itself is not necessary [99]. These data suggest that ubiquitin moieties may bind adaptor complexes involved in endocytosis directly or that ubiquitination of some associating protein is required for receptor internalization. In fact, Eps15 and epsin, adaptor proteins that interact directly with AP-2 and clathrin, respectively, possess a ubiquitin interacting motif (UIM) that recognizes ubiquitin and promotes monoubiquitination of the adaptor proteins themselves [100]. Thus, these molecules act as ubiquitin receptors to link monoubiquitinated receptors with the endocytic machinery of clathrin-coated pits [101]. Moreover, β-arrestin 2 has recently been shown to recruit E3 ubiquitin ligase Mdm2 upon stimulation of the β_2-AR, which then catalyzes ubiquitination of β-arrestin 2. Concurrently, β_2-AR undergoes multi-monoubiquitination in a β-arrestin dependent fashion by an unknown ubiquitin ligase. Interestingly, ubiquitination of β-arrestin 2 is essential for rapid receptor internalization whereas β_2-AR ubiquitination is required for efficient receptor degradation [85].

Indeed, ubiquitin modification of membrane bound proteins including some GPCRs may be important for shuttling receptors to the lysosome for degradation rather

than serving as an endocytosis signal [85, 102]. The PAR2, platelet activating factor (PAF), β_2-AR, neurokinin receptor, and chemokine receptor 4 all appear to undergo agonist-mediated multi-monoubiquitination which serves to function as a sorting signal downstream of internalization by directing receptors to the lysosome for degradation [47, 85, 102-104]. Lysosomal degradation of the V_2R requires polyubiquitination at lysine 268 [86]. Mutant forms of these receptors that fail to become ubiquitinated lose the ability to undergo lysosomal/proteasomal-mediated degradation while internalization remains unaltered. β_2-AR and V_2R both require the β-arrestin 2 isoform for receptor ubiquitination since receptor ubiquitination is abolished in mouse embryonic fibroblasts lacking the β-arrestin 2 isoform [85, 86]. The authors speculate that β-arrestin serves as a platform for the recruitment of an as yet unidentified E3 ligase.

Muscarinic Acetylcholine Type 2 Receptor: M_2 mAChR

Physiological and Pathophysiological Processes of M_2 mAChR

Members of the muscarinic acetylcholine receptor family (M_1-M_5) are known to be involved in mediating the effects of the neurotransmitter, acetylcholine, in the central and peripheral nervous systems [105]. M_1, M_3, and M_5 are selectively coupled to the G_q proteins while M_2 and M_4 are preferentially linked to G_i proteins [106-108]. The M_2 mAChR subtype is found throughout the parasympathetic nervous system where it controls pulmonary and urinary function, movement, pain, body temperature, neurotransmission, and cardiac function [108-110].

Interest in the M_2 mAChR subtype resides in the fact that this receptor plays a fundamental role in the peripheral and central nervous systems. Peripheral M_2 mAChR

actions reduce the rate and force of contraction in the heart and facilitate smooth muscle cell contractility [108, 111-114]. These physiologic effects occur when vagal nerve endings release acetylcholine to the target tissues expressing M_2 mAChRs. Signaling through the G_i-protein results in adenylyl cyclase inhibition and opening of K^+ channels [115]. A reduction in heart rate can be ascribed to an influx of potassium ions that leads to hyperpolarization thereby opposing the pacemaker current. Concurrently, decreases in cAMP levels reduce calcium channel activation via a reduction in cAMP dependent protein kinase (PKA) activity (Fig. 6). Lower intracellular calcium levels lead to a lower force of heart contraction. Chronic stimulation of M_2 mAChR can lead to the development of congestive heart failure as shown in patients afflicted with chronic Chagas's disease and cardiomyopathy [116-118].

In airway, urinary, and stomach smooth muscle, however, decreased PKA activation contributes to contractility while $G_{\alpha i}$ activation appears to stimulate Rho, which can mediate actin polymerization and contraction [119, 120]. Alterations in M_2 mAChR mediated signaling have been implicated in disorders of smooth muscle function, including chronic obstructive pulmonary disease (COPD) and urinary incontinence [112, 121, 122].

To decrease the magnitude of central and peripheral tissue responses, acetylcholine activation of M_2 mAChR present on presynaptic nerve endings inhibits further acetylcholine release [109, 123]. This feedback control mechanism also known as autoinhibition appears to involve liberated $G_{\beta\gamma}$ subunits that interact with voltage-operated calcium channels to inhibit calcium influx which is involved in neurotransmitter release [108, 124, 125]. Blockade or loss of pulmonary M_2 mAChR autoreceptors has

been implicated in COPD and asthma [126-130]. Indeed, elevated acetylcholine levels are found in asthmatic patients [131]. Therefore, excessive acetylcholine levels must be controlled via presynaptic M_2 mAChR autoinhibition to avoid bronchoconstriction mediated by the M_3 mAChR subtype in the target tissue, airway smooth muscle [112, 130].

This autoinhibition of presynaptic M_2 mAChR (autoreceptors) appears to be of particular importance in the central nervous system since maintenance of acetylcholine levels is critically important to memory, learning, and locomotor control [123, 132-134]. Cholinergic transmission of acetylcholine to postsynaptic neurons forms the basis for cognition particularly in the hippocampus, striatum, and cortex [135, 136]. However, proper receptor density must be maintained since excessive acetylcholine levels reported in M_2 mAChR knockout mice lacking presynaptic M_2 mAChR autoreceptors severely impaired cognition [137]. Indeed, dementia of the Alzheimer's type appears to be a result of lower levels of M_2 mAChR autoreceptors probably due to loss of cholinergic neurons [138]. To help with cognitive defects, Alzheimer's patients are administered acetylcholinesterate inhibitors to prolong acetylcholine levels in the synapse [139, 140]. In contrast, others have reported that blockade of M_2 mAChR autoreceptors can enhance acetylcholine release as well as other neurotransmitters (GABA and glutamate) which contributes to enhanced memory and learning [133, 138]. Irrespective of the mechanism by which cognition occurs, dysregulation of M_2 mAChR autoreceptors may contribute to the pathogenesis of many diseases including age-associated memory loss, Alzheimer's and Parkinson's disease [141, 142].

Given the complex nature of M_2 mAChR function in numerous physiological and pathophysiological processes, understanding the mechanisms involved in regulating its activity at a cellular level is of high clinical relevance. It is feasible that the pathway involved in modulating M_2 mAChR cell surface levels can provide alternative therapeutic targets. This is of particular importance since treatment of M_2 mAChR related illnesses has been limited because subtype specific agonists or antagonists are lacking which lead to unwanted side effects via activation of various mAChR subtypes [143, 144]. The development of pharmacological agents selectively targeted to M_2 mAChR or proteins involved in its regulation could potentially treat incontinence, pain management, dementia, psychosis, and COPD more effectively.

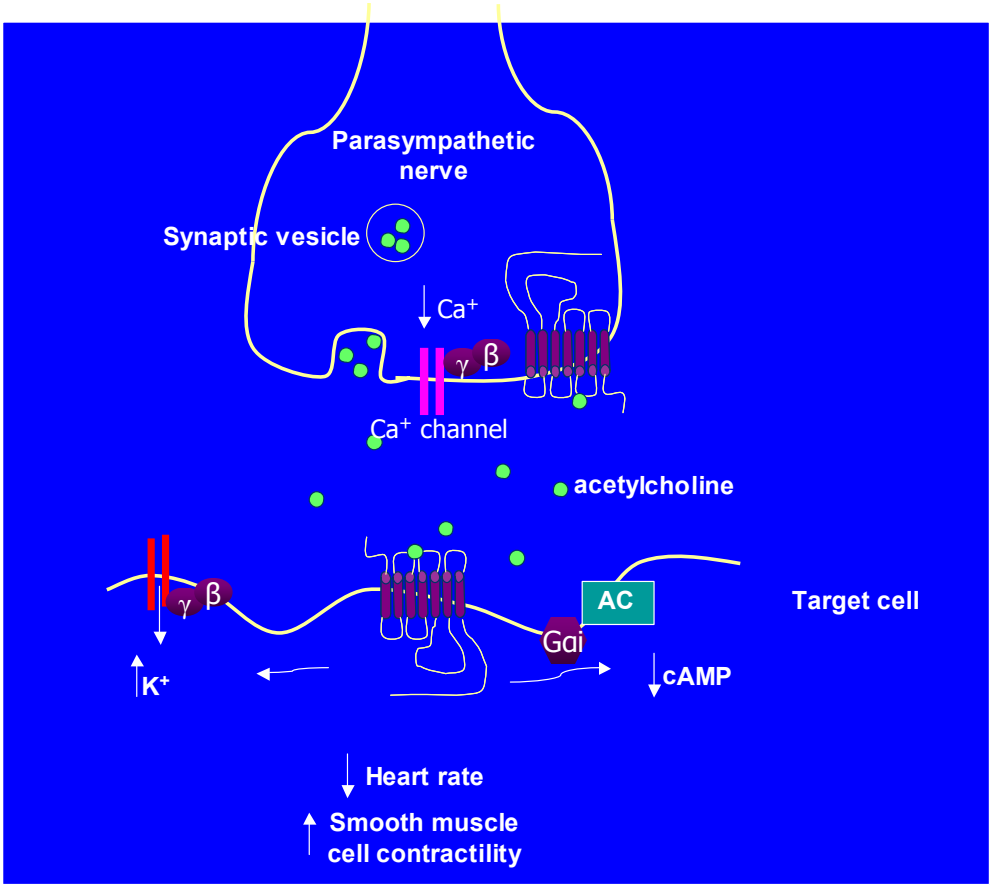

Fig. 6. The role of M_2 mAChR activation in presynaptic neurons and target cells. In the target cell, M_2 mAChR activation leads to inhibition of adenylyl cyclase (AC) by the Gαi subunit resulting in a decrease in cAMP levels while the βγ subunit opens K^+ channels. The physiological consequence includes a decrease in heart rate and an increase in smooth muscle cell contractility. Activation of M_2 mAChR autoreceptors on presynaptic terminals leads to an inhibition of high voltage-activated calcium channels which decreases Ca^{2+} levels and blocks release of acetylcholine.

Regulation of M_2 mAChR

As mentioned previously, M_2 mAChRs belong to the superfamily of GPCRs and are therefore highly regulated. The molecular events regulating M_2 mAChR mediated signaling have been characterized to a limited degree. Desensitization of the M_2 mAChR occurs following phosphorylation at serine/threonine residues in the central part of the third intracellular loop by GRK2 and other members of the GRK family [145-148]. GRK2 mediated phosphorylation appears to be sufficient for M_2 mAChR desensitization and internalization [145, 148, 149]. Whether β-arrestin proteins are involved in this process is somewhat obscure. Evidence suggests that β-arrestin 1 and 2 can bind to M_2 mAChR *in vivo* and *in vitro* implicating a role in desensitization and subsequent internalization [150-154]. However, reports in the literature have been unclear and contradictory and as such the internalization pathway utilized by M_2 mAChR remains contentious.

It has been shown that an atypical endocytic pathway independent of β-arrestin, clathrin, and dynamin proteins regulates M_2 mAChR [24, 155-157]. These studies performed in HEK 293 cells utilized β-arrestin dominant negative mutant proteins that lack interactions with AP-2, clathrin, and/or the phosphorylated form of the receptor. Moreover, an additional study introduced a dominant negative form of dynamin GTPase, a potent inhibitor of clathrin mediated endocytosis [158]. While these mutants have been reported to severely abrogate agonist-induced endocytosis of the prototypic β_2-AR as well as M_1, M_3, and M_4 mAChR subtytpes, they had no affect on M_2 mAChR internalization [24, 153, 156-158]. Lack of β-arrestin and dynamin involvement suggests

that activated M_2 mAChRs may interact with an as yet unidentified arrestin-like molecule to mediate endocytosis. Indeed, Wu and coworkers demonstrated that a peptide sequence from the M_2 mAChR third intracellular loop does not bind with β-arrestin from an enriched brain fraction while that peptide fragment of the M_3 mAChR is able to do so [159]. However, it has also been reported that the phosphorylated form of M_2 mAChR binds with high affinity to β-arrestin proteins [150-154]. Thus, it is plausible that β-arrestin proteins may facilitate desensitization and internalization in one cell line while not in another. In agreement with this statement, others have reported that M_2 mAChRs translocate to caveolae, a process that does not require β-arrestin, in response to receptor stimulation in cardiac myocytes where they are internalized in a dynamin-dependent manner [160, 161]. Additionally, others have reported a role for Arf6 GTPase in regulating agonist-mediated endocytosis in the HeLa cell line [26, 27].

Although a mechanism for β-arrestin interaction with M_2 mAChR is unclear, even less is known about the post-endocytic trafficking events exhibited by the M_2 mAChR, a key component to receptor regulation. Previous reports suggested that internalized M_2 mAChRs recycle to the cell surface at a very slow rate without any appreciable down-regulation [149, 162]. In contrast, others have shown significant agonist-induced down-regulation of the M_2 mAChR as well as receptor delivery to multivesicular bodies in neurons *in vivo* [163, 164]. Based on these observations, it is conceivable that M_2 mAChR behaves as a class B receptor and that β-arrestin may indeed participate in the post-endocytic trafficking of the M_2 mAChR.

Taken together, these observations raise the possibility that regulation of the M_2 mAChR may involve an atypical pathway for internalization distinct from other classical

endocytic mechanisms but may require β-arrestin at stages later in the trafficking pathway. Therefore, this study is designed to clarify the role of β-arrestin proteins in regulating the M_2 mAChR at the internalization and post-endocytic level. To examine the importance of β-arrestin in regulating M_2 mAChR activity, studies were conducted in mouse embryonic fibroblasts derived from β-arrestin knockout mice. Because the M_2 mAChR appears to be regulated differently from other mAChR subtypes, elucidation of this pathway may assist in the development of therapeutic agents that are specific to the M_2 mAChR subtype, a problem with mAChR selective ligands [165].

PART 1

AGONIST MEDIATED INTERNALIZATION OF THE MUSCARINIC ACETYLCHOLINE TYPE II RECEPTOR IS BETA-ARRESTIN DEPENDENT

CHAPTER 2

BETA-ARRESTIN INVOLVEMENT IN MUSCARINIC ACETYLCHOLINE TYPE II RECEPTOR INTERNALIZATION

INTRODUCTION

Muscarinic acetylcholine receptors belong to the superfamily of G-protein coupled receptors (GPCRs) that are commonly expressed in a variety of tissues and are classified into five known subtypes (M_1 -M_5 mAChR). M_1, M_3, and M_5 mAChRs are selectively coupled to G_q proteins while M_2 and M_4 mAChRs are linked to G_i/G_0 proteins [143, 166]. M_2 mAChRs are the primary muscarinic subtype in the heart where their stimulation leads to the regulation of myocardial contractility [108]. As with other GPCRs, M_2 mAChR activity is tightly regulated by desensitization and internalization. These regulatory mechanisms are typically associated with receptor phosphorylation followed by either recycling or down-regulation [148, 149, 167-170].

Desensitization is a complex process that involves agonist-dependent phosphorylation at specific serine/threonine residues by G-protein-coupled receptor kinases (GRKs) followed by β-arrestin binding. Two widely expressed isoforms of β-arrestin (1 and 2) are known to be involved in uncoupling receptors from their cognate G-proteins thereby attenuating receptor signaling [12, 68]. Typically, agonist-induced phosphorylation facilitates receptor internalization, which serves to either resensitize or down-regulate desensitized receptors [79]. β-arrestins have been shown to facilitate internalization by directly interacting with the β_2 subunit of the clathrin-AP2 (adaptor

protein 2) complex and clathrin itself [16, 68]. Thus, β-arrestins can induce receptor sequestration by directly interacting with the endocytic machinery. Many receptors such as the prototypic β_2-adrenergic receptor (β_2-AR) internalize in a clathrin and β-arrestin dependent fashion. Hence, β-arrestin facilitates clathrin-mediated endocytosis [16, 68].

In addition to desensitization and internalization, β-arrestins are known to play a role in other cellular processes that include intracellular trafficking and signaling [79]. Association of β-arrestin with agonist-occupied receptors has been shown to initiate intracellular signaling by functioning as an assembly site for signaling components such as Src, JNK3, and ERK1/2 [171-174]. Therefore, β-arrestin-receptor complexes can lead to cytosolic retention and activation of signaling molecules following receptor-mediated signaling at the cell surface. The physiological roles of this process include decreasing cell proliferation and regulating cytoskeletal rearrangements by spatially restricting ERK activation to the cytosol [71, 173]. Recent reports have also suggested that β-arrestins can function at post-endocytic stages to regulate receptor sorting. It has been shown that receptors exhibit differential affinities for β-arrestin and therefore are classified into two groups [82]. Class A receptors (including β_2-AR and dopamine receptors) are thought to interact with β-arrestin at the plasma membrane but immediately disassociate following localization to clathrin-coated pits. Hence receptors enter early endosomes devoid of β-arrestin and are typically resensitized and rapidly recycled [84]. In contrast, class B receptors (vasopressin-V_2R, angiotensin-$AT_{1A}R$, and neurotensin receptors) stably associate with β-arrestin so that β-arrestin/receptor complexes remain intact and are internalized into juxtanuclear endosomal compartments [83]. This interaction can persist for prolonged periods of time. This stable association may dictate the kinetics of receptor

recycling since $AT_{1A}R$ and V_2R recycle very slowly [83, 84]. A functional consequence of β-arrestin association may also be to facilitate receptor down-regulation.

The role of β-arrestins in regulating the trafficking of M_2 mAChRs has been contradictory and unclear. Reports have demonstrated that phosphorylation by GRK2 on serine/threonine residues in the third intracellular loop of M_2 mAChRs recruits β-arrestin and leads to receptor desensitization and subsequent internalization [148]. Whether β-arrestin is involved directly in agonist-promoted endocytosis of M_2 mAChRs remains unclear. Indeed over-expression of β-arrestin has been reported to increase agonist-promoted internalization of M_2 mAChRs but not of M_1 or M_3 mAChRs [156]. Furthermore, Claing and coworkers have shown that M_2 mAChRs internalize in a dynamin- and β-arrestin-insensitive manner when expressed in HEK293 cells [24]. Others have reported that the Arf6 GTPase (ADP-ribosylation factor 6) facilitates M_2 mAChR entry into primary vesicles, which fuse with clathrin-derived early endosomes [26, 27]. These data do not necessarily rule out β-arrestin as a regulator in agonist-promoted endocytosis of M_2 mAChRs. Therefore, to clarify whether agonist-promoted internalization of M_2 mAChRs is arrestin dependent, we utilized mouse embryonic fibroblasts (MEFs) derived from β-arrestin null mice that lack expression of one or both isoforms (β-arrestin 1 and 2) and their wild type littermates as control cells [80]. Here we report that agonist-promoted internalization of M_2 mAChRs is β-arrestin dependent and M_2 mAChRs form stable complexes with β-arrestin at the early endosome. Furthermore, we demonstrate that agonist-promoted internalization of M_2 mAChRs is clathrin-dependent in MEF cells while clathrin independent mechanisms appear to function in HeLa cells. Subsequent post-endocytic trafficking of M_2 mAChR is regulated by both

Rab5 and Rab7 GTPases. Taken together, these results suggest that β-arrestin and Rab GTPases play important roles in regulating M_2 mAChR activity.

CHAPTER 3

MATERIALS AND METHODS

Materials

[^{3}H]-*N*-methylscopolamine (NMS) (81-84 Ci/mmol) was purchased from Amersham Corp. (Buckinghamshire, England). Dulbecco's Modified Eagle's Medium (DMEM), F-10, penicillin/streptomycin, fetal bovine serum, restriction enzymes and LipofectAMINE 2000 were purchased from Invitrogen (Carlsbad, CA). EX-GEN was purchased from Fermentas (Hanover, MD). The anti-FLAG M2 monoclonal antibody and mouse anti-M1 FLAG antibody were purchased from Sigma-Aldrich (St. Louis, MO); mouse antibodies against β-arrestin 1 and 2 were purchased from Santa Cruz (Santa Cruz, CA). The anti-HA.11 monoclonal antibody was purchased from Covance Research Product (Berkley, California). Secondary HRP-conjugated antibodies were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). Alexa 594-conjugated goat anti-mouse and Alexa 488-conjugated goat anti-rabbit were purchased from Molecular Probes (Eugene, OR). Lysophosphatidic acid (1-oleoyl-2-hydroxy-sn-glycerol-3-phosphate; LPA) was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Carbachol, atropine, isoproterenol, and all other reagents were purchased from Sigma-Aldrich. Dr. Neil Nathanson (University of Washington) kindly provided the construct expressing the porcine FLAG-tagged M_2 mAChR [175]. FLAG-tagged LPA_1 was a kind gift of Junken Aoki (University of Tokyo, Japan) [176]. HA-tagged M_1, M_3, M_4, and M_5 mAChRs and HA-tagged β_2AR were purchased from UMR

cDNA Resource Center (University of Missouri). Arrestin mutants, β-arrestin 2-ΔLIELD, β-arrestin 2-F391A, β-arrestin 2 ΔLIELD /F391A, and truncated carboxyl-terminal region of β-arrestin 1 (319-418) were kindly provided by Dr. Jeffrey Benovic (Thomas Jefferson University) [177, 178]. The MEF wild type, β-arrestin 1 and 2 single knockouts, β-arrestin 1 and 2 double knockout cells, and constructs for FLAG-tagged β-arrestin 1 and 2 were kindly provided by Dr. Robert Lefkowitz (Duke University Medical Center) [80]. Constructs encoding β-arrestin 2-GFP and β-arrestin 1-GFP were generous gifts from Dr. Stefano Marullo and have been previously described [179]. The plasmids encoding GFP-tagged Rab7 and Rab5 wild type and mutant forms were provided by Dr. Bo van Deurs and Dr. Stephen Fergusson, respectively [48, 59]. Eps15 constructs were generous gifts from Alexandre Benmerah [180].

Cell Culture and Transient Transfection

HeLa, MEF wild-type, MEF single and double β-arrestin knockout, rat aortic smooth muscle cells (RASMCs), and COS-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 I.U. /ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO_2. For immunocytochemistry, HeLa cells were grown on glass coverslips at a density of 120,000 cells/well in six-well dishes and transfected with EX-GEN or LipofectAMINE 2000 according to the manufacturer's protocol using 1 μg of DNA/well. For ligand binding assays, MEF cells were plated at 80,000 cells/well in 24 well plates and transfected with EX-GEN or LipofectAMINE 2000 according to the manufacturer's protocol using 1 μg of DNA/well.

Radioligand Binding Assay

Receptor internalization was determined by measuring the binding of the membrane impermeable muscarinic antagonist [^{3}H]-*N*-methylscopolamine ([^{3}H]-NMS) to intact cells as previously described [181]. Briefly, 24-42 h after transfection, MEF cells cultured in 24-well plates were treated or not treated with 1 mM carbachol for 60 min at 37°C. Cultures were washed twice with 1 ml of ice-cold PBS, and labelled with 720 fmol of [^{3}H]-NMS in 1 ml PBS for 4 h at 4°C. Non-specific binding was determined as the bound radioactivity in the presence of 1 μM atropine. Labelled cells were washed two times with 1 ml of ice-cold PBS, solubilized in 0.5 ml of 1% Triton X-100 and combined with 3.5 ml of scintillation fluid followed by measurement of radioactivity. Receptor internalization is defined as percent of surface M_2 mAChRs not accessible to [^{3}H]-NMS at each time relative to non-carbachol-treated cells.

Immunoblotting

Western blot analysis was performed on cells cultured in 6-well plates. The cells were solubilized in 0.5 ml of lysis buffer containing: 50 mM HEPES (pH 7.5), 0.5% (v/v) Nonidet P-40, 250 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride and 1 μg/ml of protease inhibitors leupeptin, aprotinin, pepstatin A, and 100 μM benzamidine. The protein concentration was determined using the Bradford assay method. Fifty μg of cell lysates were subjected to 4-20% SDS-PAGE. After transfer, the nitrocellulose membrane was blocked and then probed with anti-FLAG monoclonal antibody. Immunoreactive bands were visualized by enhanced chemiluminescence after adding HRP-conjugated anti-mouse antibody. After

stripping with 0.1M glycine (pH 2.5), the membrane was re-probed with anti-β-actin using a detection kit from Oncogene (Cambridge, MA).

Indirect Immunofluorescence

24 h following transfection, cells were treated as described in the figure legends, fixed in 4% formaldehyde in PBS for 5 minutes, and rinsed with 10% adult calf serum and 0.02% azide in PBS (PBS/serum). Fixed cells were incubated with primary antibodies diluted in PBS/serum containing 0.2% saponin for 45 minutes, and then washed with PBS/serum (3 x 5 min.). The cells were then incubated with fluorescently labelled secondary antibodies in PBS-serum and 0.2% saponin for 45 minutes, washed with PBS/serum (3 x 5 min.) and once with PBS, and mounted on glass slides. Images were acquired using a Zeiss LSM 510 scanning confocal microscope or an Olympus BX40 epifluorescence microscope equipped with a 60x Plan pro lens, and photomicrographs were prepared using an Olympus MagnaFire SP digital camera (Olympus America, Inc.). All images were processed with Adobe Photoshop 7.0 software.

RNA Isolation and RT-PCR

Total cellular RNA from MEF cells, cortex and cerebellum of 2-3 week old Sprague Dawley rat pups was isolated using TriZol according to the manufacturer's instructions. A 50 µl reaction solution containing 1 µg total RNA was reverse-transcribed, and PCR was performed using gene-specific primers and the Qiagen One-step RT-PCR kit. Gene specific primers and amplification reactions were as follows: Rat M_1 mAChR (175 bp amplified product): CCTCTGCTGCCGCTGTTG (sense) and

GGTGGGTGCCTGTGCTTCA (antisense); Rat M_2 mAChR (686 bp amplified product): CACGAAACCTC TGA CCTACCC (sense) and TCTGACCCGACGACCCAACTA (antisense); Rat M_4 mAChR (587 bp amplified product): TGGGTCTTGTCCTTTGT GCTC (sense) and TTCATTGCCTGTCTGCTT TGTTA (antisense); Rat β-actin (764 bp amplified product): TTGTAACCAACTGGGACGATATGG (sense) and GATCTT GATCT TCATGGT GCTAGG (antisense). Cycling parameters were 30 minutes at 50°C for reverse transcription followed by 1 minute 95°C hot start followed by 28 cycles at 95°C for 1 minute, 62°C for 1 minute, and 72°C for 45 seconds and a final cycle at 72°C for 7 minutes.

CHAPTER 4

RESULTS

Endogenous Expression of mAChR Subtypes in MEF Cells

To determine whether the MEF cells used in this study expressed endogenous mAChRs, we performed RT-PCR aimed at detecting mRNA encoding M_1, M_2 and M_4 mAChR subtypes. As positive controls, we used postnatal rat cerebellum tissue for M_2 mAChR mRNA and postnatal rat cortical tissue for M_1 and M_4 mAChR mRNA. RT-PCR analysis clearly demonstrated that MEF wild type as well as MEF double knockout cells (MEF KO1/2) did not express mRNA encoding M_1, M_2, or M_4 mAChR subtypes (Fig. 7). Accordingly, radioligand-binding assays also confirmed that MEF wild type as well as MEF KO1/2 did not express mAChRs at any detectable level (*data not shown*). Therefore, we concluded that MEF cells do not express endogenous mAChRs.

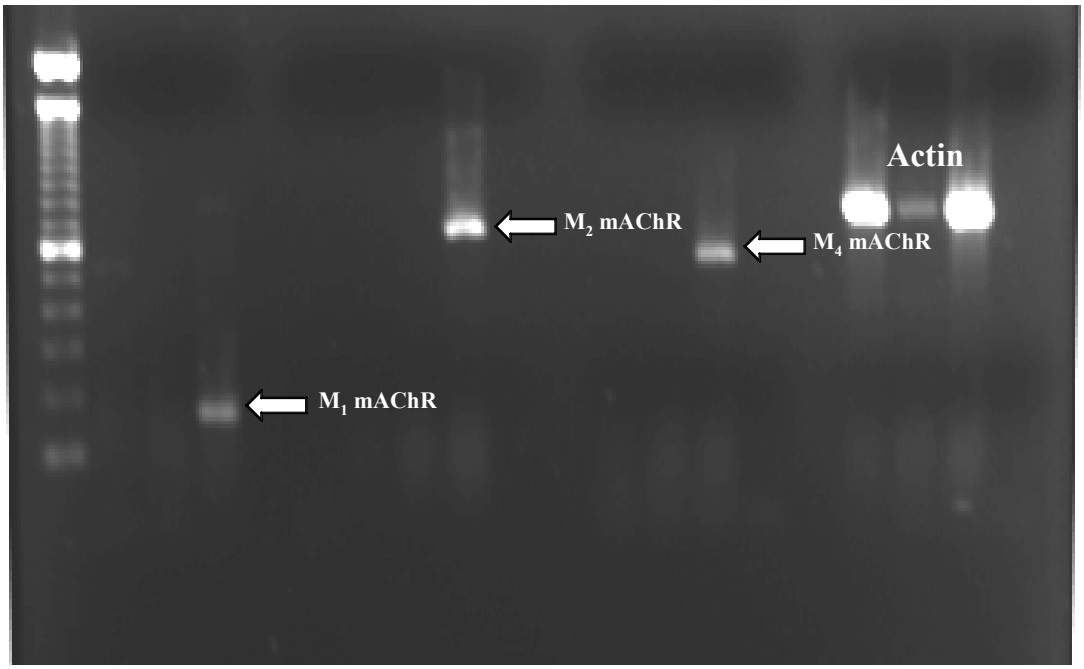

Fig. 7. Mouse embryonic fibroblasts (MEF) cells do not express mRNA encoding M_1, M_2 or M_4 mAChR subtypes. A representative gel showing lack of mAChR expression in MEF wild type and KO1/2 cells. Lanes consisted of 100 bp ladder (lane 1), wild type MEF cells (lanes 2, 5, 8 and 11), MEF β-arrestin KO1/2 (lanes 3, 6, 9, and 12), rat cortex (lanes 4 and 10 and 13), and rat cerebellum (lane 7). The white arrows point to M_1, M_2 and M_4 mAChR PCR product from cDNA as positive controls. The electrophoresis gel shown is a representative of at least 3 independent experiments.

Internalization of M_2 mAChR, LPA_1, and β_2-AR is β-arrestin Dependent

To examine whether ectopically expressed M_2 mAChRs undergo agonist-promoted internalization in MEF cells, we transiently transfected MEF wild type and corresponding β-arrestin null cells with a plasmid encoding a FLAG-tagged porcine M_2 mAChR. Following 24 h transfection, MEF wild type, MEF KO1, MEF KO2, and MEF KO1/2 cells were stimulated with 1 mM carbachol for 1 h at 37ºC. The number of receptors remaining at the cell surface was measured using a saturating concentration of the hydrophilic ligand [^{3}H]-NMS. Approximately 40% of surface M_2 mAChRs were internalized in wild type MEF cells while M_2 mAChRs in MEF KO1 and MEF KO2 cells were internalized by 33% and 42%, respectively. In contrast, M_2 mAChRs were not internalized in MEF KO1/2 cells (Fig. 8A). These results demonstrated that exogenously expressed M_2 mAChRs undergo agonist-promoted internalization in MEF wild type cells and either β-arrestin isoform was sufficient for sequestration. To further evaluate where M_2 mAChRs were localized, we used confocal immunofluorescence microscopy in MEF wild type or MEF KO1/2 cells transiently expressing a FLAG-tagged M_2 mAChR in the absence or presence of 1 mM carbachol. As indicated in Figure 8B, diffuse cell surface localization of M_2 mAChRs was observed prior to carbachol addition in both MEF phenotypes, although a portion of receptors are localized intracellularly, possibly as Endoplasmic Reticulum or Golgi vesicular pools. Upon addition of agonist, M_2 mAChRs transiently expressed in MEF wild type cells redistributed into discrete intracellular vesicles dispersed throughout the cell while M_2 mAChRs expressed in MEF KO1/2 cells remained primarily at the cell surface (Fig. 8B).

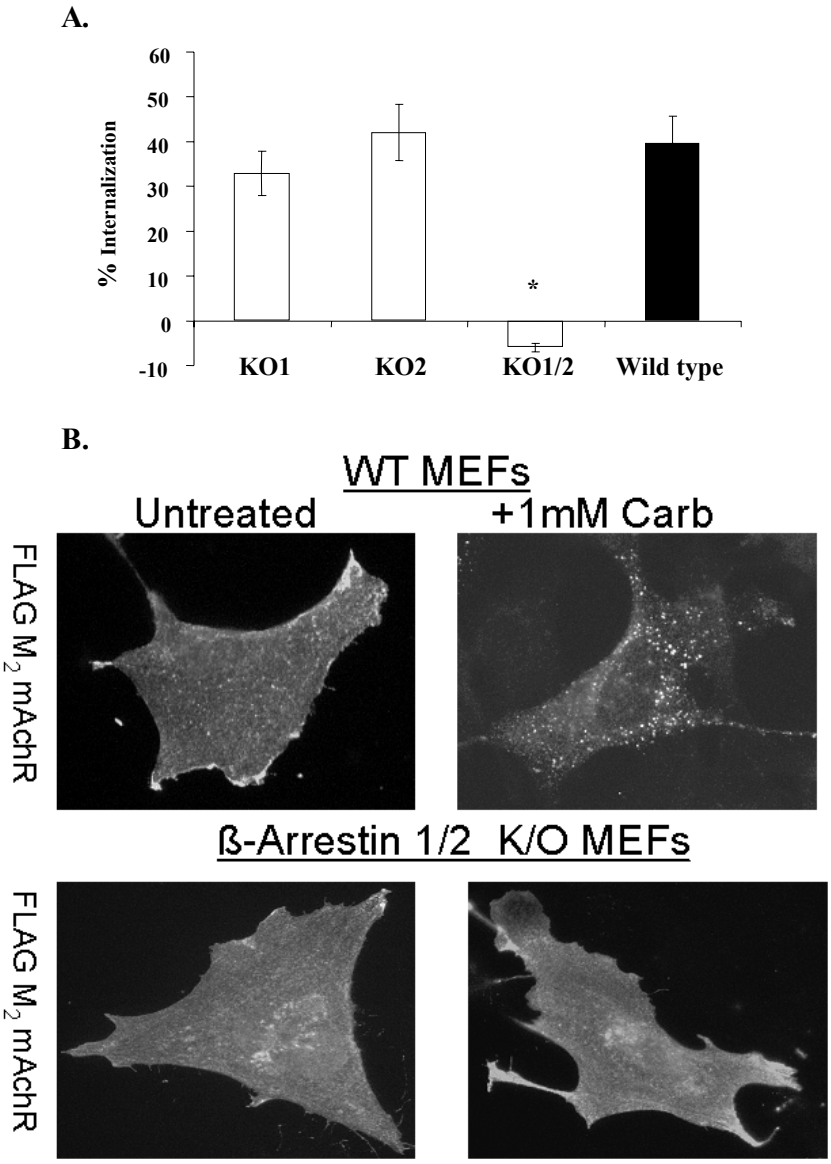

Fig. 8. Agonist-promoted internalization of M_2 mAChR in MEFs is β-arrestin–dependent. (A) Approximately 24 h following transfection with FLAG-tagged M_2 mAChR, MEF KO1, MEF KO2, MEF KO1/2, and MEF wild type cells were stimulated with 1 mM carbachol for 1 h and agonist-promoted internalization was determined using [^{3}H]-NMS. Data are presented as the mean ± standard error from 3 separate experiments with each experiment consisting of 8 to 11 independent determinations. Statistical test was performed using ANOVA with the post hoc Bonferroni/Dunn test (asterisk indicates * $p<0.001$). (B) MEF wild type and MEF KO1/2 cells were transfected as described above and then incubated in the presence or absence of 1 mM carbachol for 30 minutes prior to indirect immunofluorescence to assess FLAG-tagged receptor localization. Images were acquired at 40X. This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

To further evaluate β-arrestin's function in mediating GPCR endocytosis, we compared agonist-promoted internalization of the β_2-AR and LPA_1 receptor in these cell lines. MEF wild type and MEF KO1/2 cells were transiently transfected with plasmids encoding either FLAG-tagged LPA_1 or HA-tagged β_2-AR receptor and subsequently treated with and without LPA or isoproterenol, respectively. Stimulated and unstimulated β_2-AR and LPA_1 receptors remained at the cell surface in MEF KO1/2 cells while addition of agonist for 30 minutes led to the redistribution of receptors into discrete punctate vesicles in wild type MEF cells (Fig. 9A and B). The diffuse pattern shown in MEF KO1/2 and MEF wild type cells represents surface plasma membrane localization since the absence of detergent leads to an identical staining pattern as seen in untreated cells (*data not shown*). The FLAG- or HA-tag is located at the N-terminus of these receptors and is accessible to exogenously added antibody even in the absence of detergent. These findings further indicate that β-arrestins are important in the agonist-induced endocytosis of M_2 mAChRs and other GPCRs.

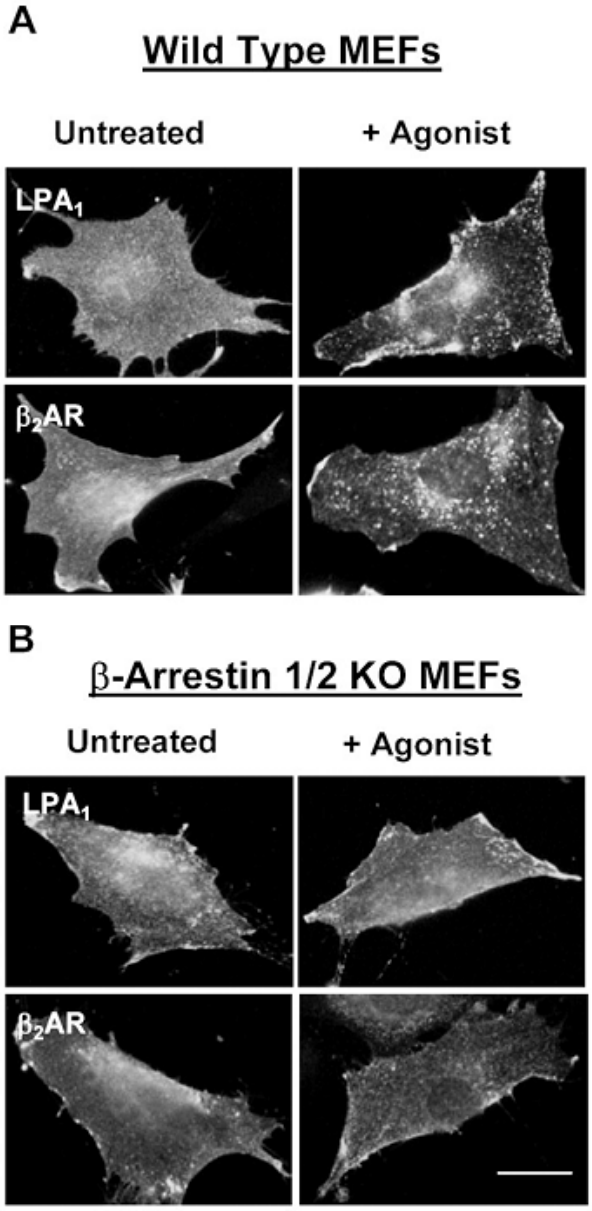

Fig. 9. Agonist-promoted internalization of FLAG-tagged LPA_1 and HA-tagged β_2-AR in MEF cells is β-arrestin–dependent. (A) Approximately 24 h following transfection with either plasmid encoding for FLAG-tagged LPA_1 or HA-tagged β_2-AR, MEF wild type cells were stimulated with or without 10 μM LPA or 20 μM isoproterenol, respectively. Cells were fixed and processed for indirect immunofluorescence localization of the epitope tagged receptors. (B) MEF KO1/2 cells were transfected as described above and then incubated in the presence or absence of agonist for 30 minutes. Images were acquired at 40X. Bar, 10 μM. This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

Given that agonist-promoted internalization of the M_2 mAChR does not occur in MEF KO1/2 cells, we were interested in determining whether β-arrestin can rescue this endocytic defect. To examine this question we analyzed agonist-promoted internalization in MEF KO1/2 cells co-expressing M_2 mAChR and FLAG-tagged β-arrestin 1 and/or 2 (Fig. 10A). Western blotting analysis confirmed that FLAG-tagged β-arrestins were expressed (Fig. 10B). Cells were treated with 1 mM carbachol for 1 h and the extent of receptor internalization was assessed using [^{3}H]-NMS. MEF KO1/2 cells reintroduced with β-arrestin 1, β-arrestin 2, or both isoforms exhibited M_2 mAChR uptake similarly (Fig. 10A). These data suggest that not only is agonist-promoted internalization of M_2 mAChR β-arrestin-dependent but also there is no selectivity between β-arrestin isoforms (Fig. 8A and 10A).

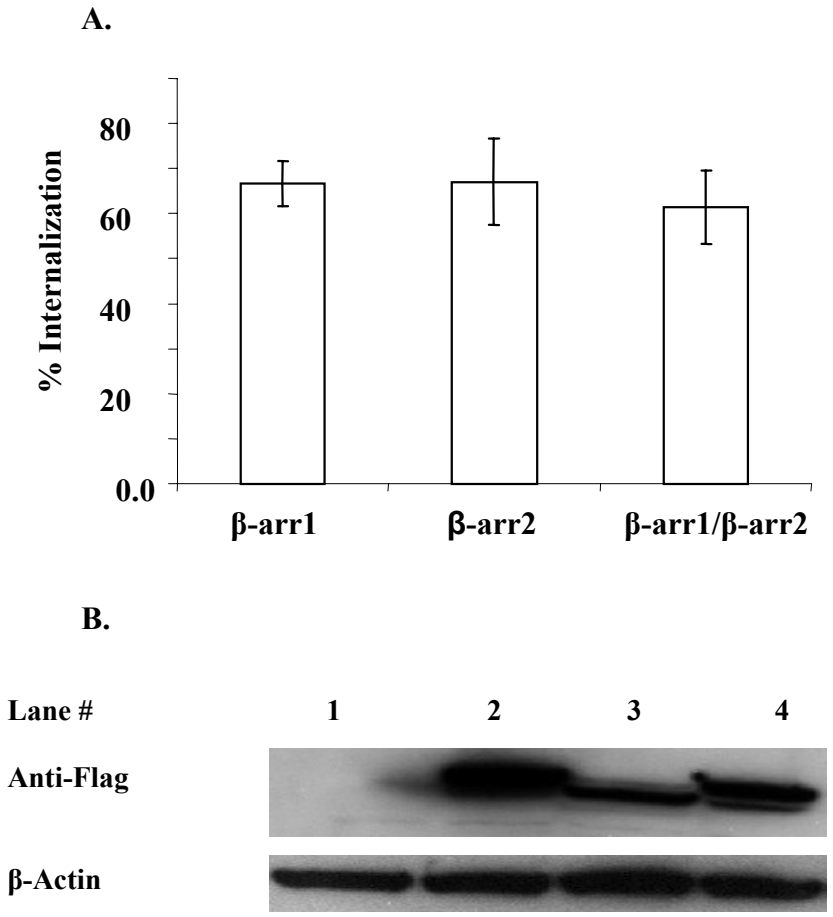

Fig. 10. Expression of β-arrestin 1 or 2 rescued agonist-promoted internalization of M_2 mAChRs in MEF KO1/2 cells. Approximately 24 h following co-transfection with constructs encoding M_2 mAChR and β-arrestin, cells were stimulated with 1 mM carbachol for 1 h. (A) Agonist-promoted internalization was determined as described in Methods. Data are presented as the mean ± standard deviation of 5 independent experiments consisting of 8-11 determinants. (B) A representative immunoblot of FLAG-tagged β-arrestin and internal protein control β-actin is shown. Lanes consisted of: non-transfected MEF KO1/2 (1), MEF KO1/2 expressing β-arrestin 1 (2), MEF KO1/2 expressing β-arrestin 2 (3), and MEF KO1/2 expressing β-arrestin 1 and 2 (4). Western blot shown is a representative of at least 3 independent experiments.

Agonist-Promoted Internalization of the M_2 mAChR is Clathrin-Dependent in MEF Cells

Having demonstrated that β-arrestin is required for internalization, we wanted to know if its function in mediating this process requires clathrin and AP-2 binding. Previously, sequestration of M_1, M_3, and M_4 mAChRs was shown to be both β-arrestin and clathrin-dependent [24, 182]. In contrast, sequesteration of M_2 mAChR was reported to be largely β-arrestin and clathrin-independent [153, 156]. To address whether the β-arrestin-dependent internalization we observed in MEFs was independent of clathrin, we expressed in MEF KO1/2 cells β-arrestin mutants that were selectively defective in interaction with clathrin (β-arrestin 2 ΔLIELD), AP-2 (β-arrestin 2-F391A), or both clathrin/AP-2 (β-arrestin 2 ΔLIELD/F391A) [183]. Expression of either the β-arrestin 2 ΔLIELD or β-arrestin 2-F391A mutant rescued agonist-promoted M_2 mAChR internalization in MEF KO1/2 cells (Fig. 11). However, internalization was only moderately rescued by transient expression of a β-arrestin 2 mutant defective in both clathrin and AP-2 interaction (Fig. 11). These results indicate that β-arrestin-dependent internalization of M_2 mAChR may include a component that is independent of interactions between clathrin and AP-2.

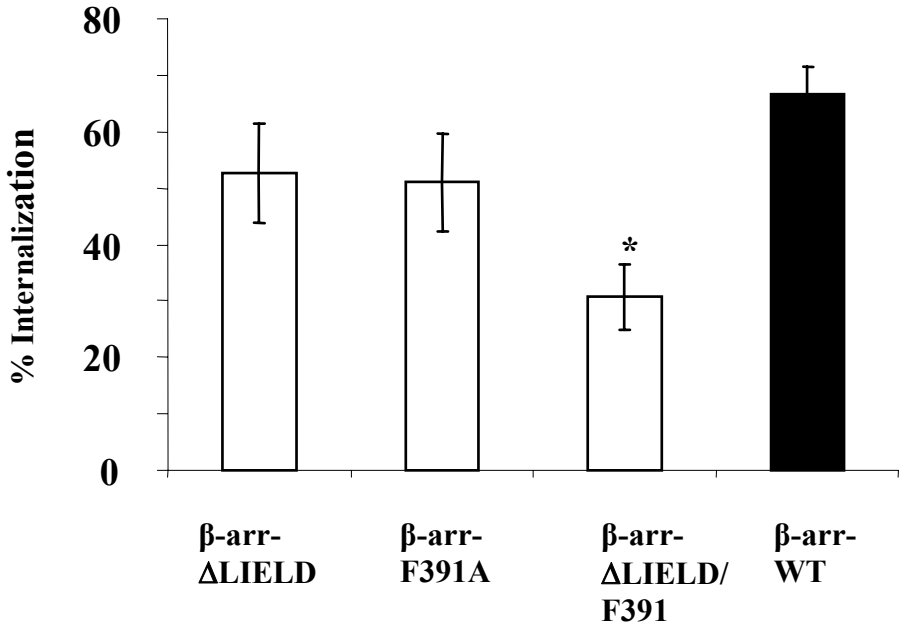

Fig. 11. Expression of β-arrestin mutants deficient in clathrin and/or AP-2 binding interaction partially supports agonist-promoted internalization of M_2 mAChRs in MEF KO 1/2 cells. Approximately 24 hr following co-transfection with FLAG-M_2 mAChR and β-arrestin 2 clathrin (ΔLIELD), AP-2 (F391A), or clathrin and AP-2 (ΔLIELD/ F391A) mutants, MEF KO1/2 cells were stimulated with 1 mM carbachol for 1 h and agonist-promoted internalization was determined as described in Methods. Data are presented as mean ± standard deviation from 4 independent experiments consisting of 8-11 determinants.

In contrast, recent studies by Santini and co-workers [178] showed that agonist-mediated activation of the β_2-AR was still capable of inducing recruitment into clathrin coated pits in cells expressing mutant β-arrestin proteins that were defective in binding with clathrin or AP-2, albeit to a reduced degree. To address this question further we utilized a more potent inhibitor of clathrin-mediated endocytosis, a peptide inhibitor known as β-arrestin 1 (319-418). Expression of this truncated COOH-terminal region of β–arrestin 1 (319-418), which is constitutively localized to preformed clathrin-coated pits but lacks receptor binding thereby blocking endogenous clathrin-binding sites, completely inhibited the β_2-AR mediated clustering of clathrin coated pits [177]. Therefore, we conducted experiments with the truncated β–arrestin 1 (319-418) to determine whether agonist-promoted internalization of the M_2 mAChR in MEFwt cells would be affected. Transient expression of the truncated β–arrestin 1 (319-418) completely inhibited the agonist-promoted internalization of the M_2 mAChR in MEF wild type cells (Fig. 12).

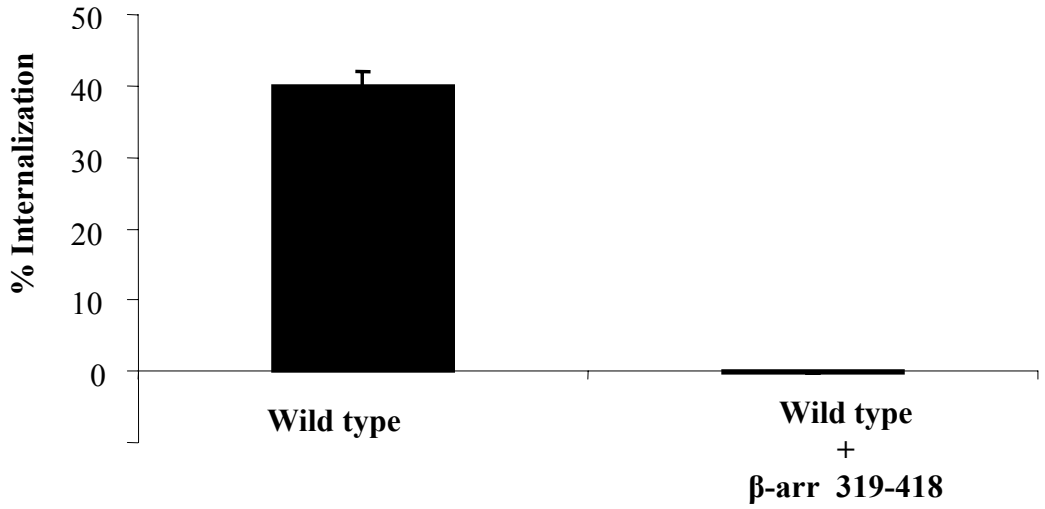

Fig. 12. Expression of truncated carboxyl-terminal region of β-arrestin 1 (319-418) completely blocked agonist promoted M_2 mAChR internalization in MEFwt cells. Approximately 24 hr following transfection with the β-arrestin 1 C-terminal domain peptide (319-418), wild type MEF cells were stimulated with 1 mM carbachol for 1 h and agonist-promoted internalization of receptor was determined using [^{3}H]-NMS. Data are presented as the mean ± standard error from 3 separate experiments with each experiment consisting of 8 to 11 independent determinations. Statistical test was performed using ANOVA with the post hoc Bonferroni/Dunn test (asterisk indicates * $p<0.001$).

Eps15 is known to constitutively associate with AP-2 (adaptor protein-2) that plays a critical role in both the organization and function of plasma membrane coated pits by directly binding to clathrin [184]. To further confirm that activated M_2 mAChRs undergo clathrin-mediated endocytosis, we co-expressed potent clathrin inhibitors known as DIII and EΔ95/295 Eps15 mutants in MEF wild type cells and assessed subcellular localization following receptor stimulation. Both DIII and EΔ95/295 Eps15 mutants are known to potently inhibit clathrin-mediated pathways by outcompeting endogenous Eps15 for AP-2 binding sites, or lacks EH domains required for proper localization with clathrin pits, respectively [185]. The DIII Δ2 mutant lacks AP-2 binding sites and therefore serves as a control. Here we show that MEF wild type cells transiently expressing M_2 mAChR and DIII or EΔ95/295 Eps15 mutants lacked the ability to undergo agonist-mediated endocytosis (Fig. 13). Expression of DIII Δ2 had no affect on M_2 mAChR uptake. Taken together, it could be argued that the agonist-promoted internalization of M_2 mAChR involved a clathrin-dependent pathway in MEF wild type cells.

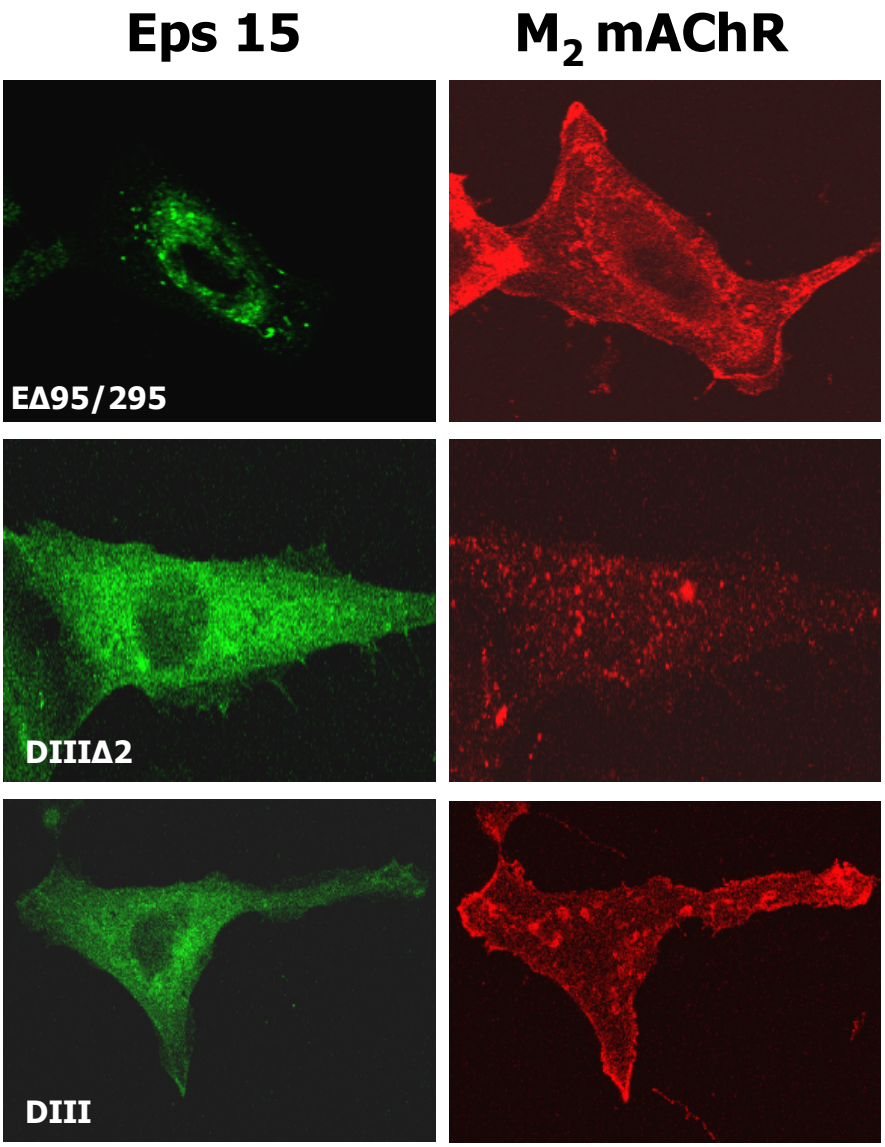

Fig. 13. Eps15 mutants block agonist promoted endocytosis of the M_2 mAChR in MEF wild type cells. MEF cells were transiently transfected with HA-tagged M_2 mAChR and GFP-tagged DIII, DIIIΔ2, or EΔ95/295 Eps15 mutants. Cells were treated with 1 mM carbachol for 30 minutes at 37^0C and subsequently processed for indirect immunofluorescence microscopy. Eps15 constructs (green) and M_2 mAChR (red) were visualized by confocal microscopy. This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

Agonist-Promoted Internalization of the M_2 mAChR is Clathrin-Independent in HeLa Cells

As mentioned previously, it has been shown by others that activated M_2 mAChRs internalized into various cell lines via a poorly understood non-clathrin mediated pathway [145, 153, 155-157, 175]. Reports have also indicated a possible role for caveolae in mediating agonist-induced internalization [160]. To determine whether stimulated M_2 mAChRs exogenously expressed in the HeLa cell line internalize in a clathrin-dependent manner as shown with the MEF wild type cells, we introduced potent inhibitors of clathrin endocytosis, DIII and EΔ95/295 Eps15, and of both clathrin- and caveolae-mediated endocytosis, dynamin K44A. Dynamin regulates the budding of vesicles from the plasma membrane through c-src mediated activation. Dynamin K44A significantly inhibits clathrin- and caveolae-mediated endocytosis, but it does not affect detachment of uncoated vesicles. Although M_2 mAChR exhibits sensitivity to other dynamin mutants (DynK535M), this particular mutant does not prevent its internalization [158]. We found that dynamin and Eps15 dominant negative mutants had no significant effect on the extent of M_2 mAChR internalization as shown by the presence of discrete vesicles by confocal microscopy in stimulated cells and by [^{3}H]-NMS binding (Fig. 14A and B). These results may indicate that clathrin may not be important in agonist-promoted internalization of the receptor in the HeLa cell line. This suggests that exogenous expression of M_2 mAChR in different cell types may exhibit different pathways of endocytosis.

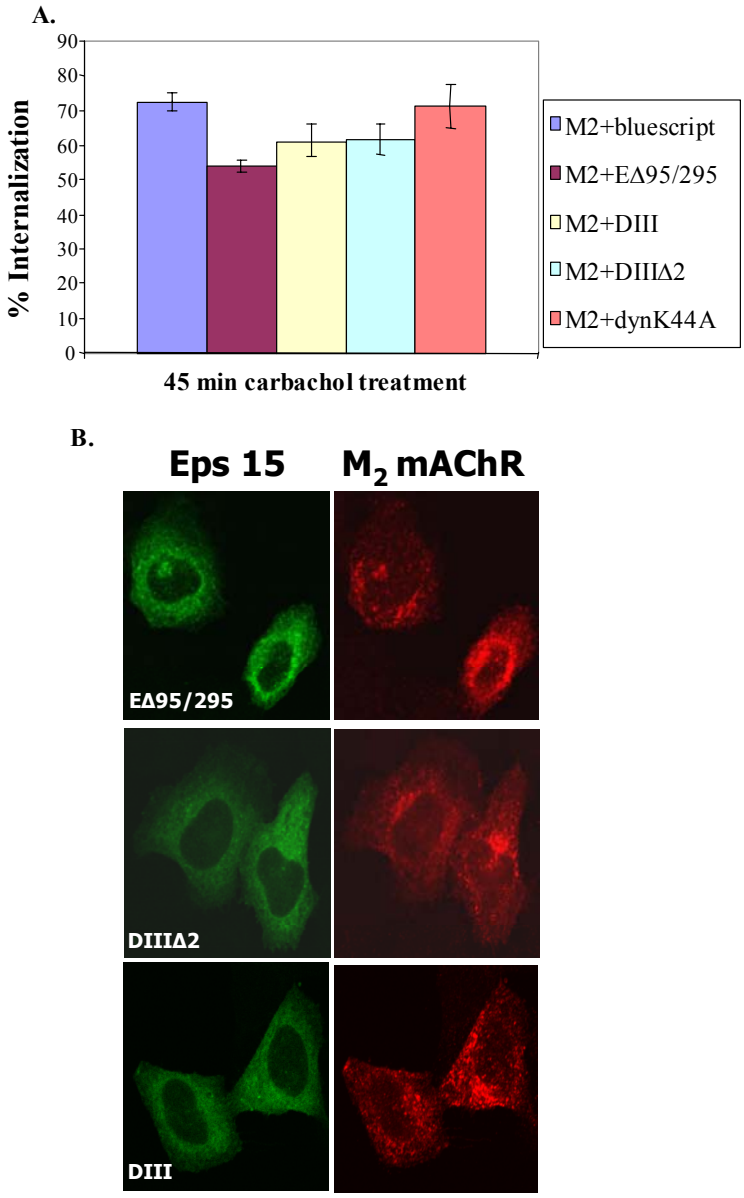

Fig. 14. Potent inhibitors of clathrin-mediated endocytosis do not significantly affect agonist-promoted internalization of the M_2 mAChR in HeLa cells. (A) HeLa cells transiently expressing M_2 mAChR with Dynamin K44A or Eps15 mutants were treated with 1 mM carbachol for 30 minutes at 37°C. Cells were exposed to [^{3}H]-NMS to assess receptor internalization. (B) HeLa cells co-expressing Eps15 constructs and HA-tagged M_2 mAChR were incubated in presence of 1 mM carbachol for 30 minutes. Eps15 mutants (green) and receptor (red) were visualized by immunofluorescence microscopy as described in Materials and Methods. This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

Interaction between M_2 mAChR and β-arrestin

Having demonstrated that β-arrestins are essential in mediating internalization of the M_2 mAChR, we were interested in determining if M_2 mAChR behaves as a class A or class B receptor in the MEF cells. To address this question, we introduced GFP-tagged β-arrestin 1, 2, or both isoforms with FLAG-tagged M_2 mAChRs into MEF KO1/2 and wild type cells and assessed their localization by immunofluorescence microscopy. Internalized M_2 mAChRs remained associated with β–arrestin 1-GFP (*data not shown*) or β–arrestin 2-GFP (Fig. 15) in intracellular compartments following 30 minutes stimulation with 1 mM carbachol. This phenomenon suggests that M_2 mAChR behaves as a class B receptor.

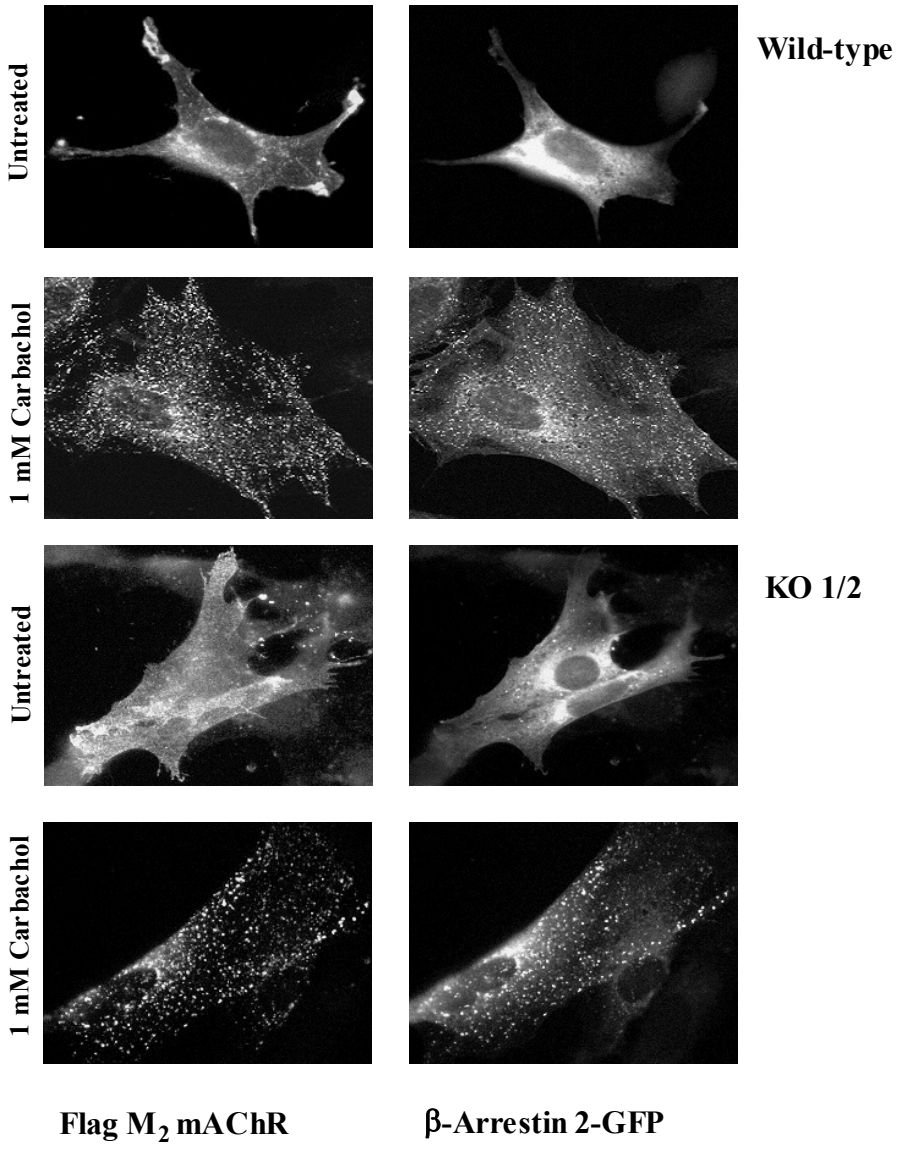

Fig. 15. Stimulation of M_2 mAChRs leads to stable co-localization of β-arrestin 2-GFP at intracellular sites. MEF wild type or KO1/2 cells were transiently co-transfected with the human FLAG-tagged M_2 mAChR and β-arrestin 2-GFP constructs. Following 30 minutes of 1 mM carbachol stimulation, cells were fixed and processed for indirect immunofluorescence. Localization of β-arrestin 2-GFP and M_2 mAChR was visualized by confocal microscopy. This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

To determine if this phenomenon occurs in other cell types we expressed M_2 mAChRs in HeLa, COS-7, and rat aortic smooth muscle cells (RASMCs). As observed in MEF KO1/2 and wild-type cells, stimulated M_2 mAChRs remained co-localized with β–arrestin 2-GFP in HeLa, COS-7, and RASMCs (Fig. 16).

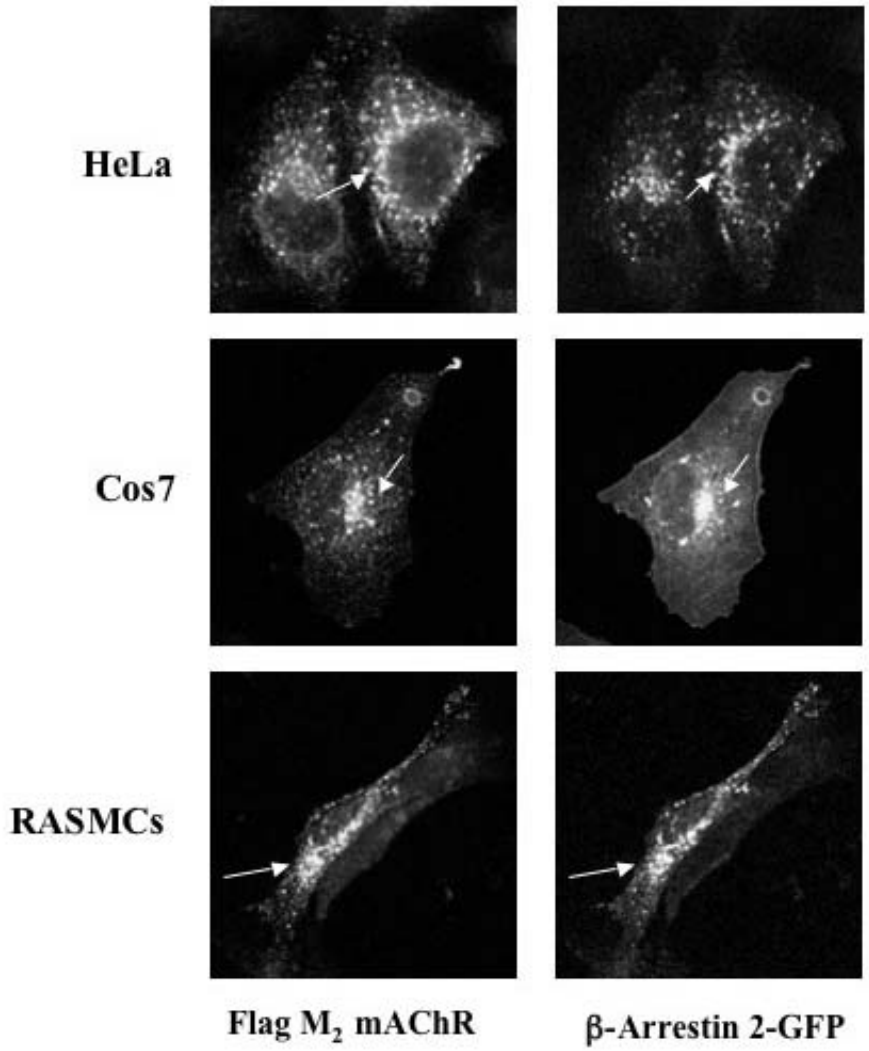

Fig. 16. Agonist-promoted internalized FLAG-tagged M_2 mAChRs exhibit overlap with β-arrestin 2-GFP at intracellular sites in various cell lines. Cells were transiently transfected with FLAG-tagged M_2 mAChR and β-arrestin 2-GFP and treated with 1 mM carbachol for 30 min at 37°C and processed for indirect immunofluorescence. Co-localization of β-arrestin 2-GFP with internalized M_2 mAChRs occurred in HeLa, COS-7 and rat aortic smooth muscle cells (RASMCs). Arrows indicate overlap between β-arrestin 2-GFP and M_2 mAChRs in intracellular compartments. This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

To ensure that our observations are not a result of β-arrestin overexpression, we investigated whether stimulation of M_2 mAChR would lead to recruitment of endogenous β-arrestin to receptor-positive endosomes. Here, we show that HeLa cells transiently expressing M_2 mAChR significantly colocalized with both endogenous and overexpressed β-arrestin 1 following 30 minute carbachol stimulation (Fig. 17). Taken together, these results demonstrate that internalized M_2 mAChRs stably associate with either β–arrestin isoform in multiple cell lines when overexpressed and endogenously.

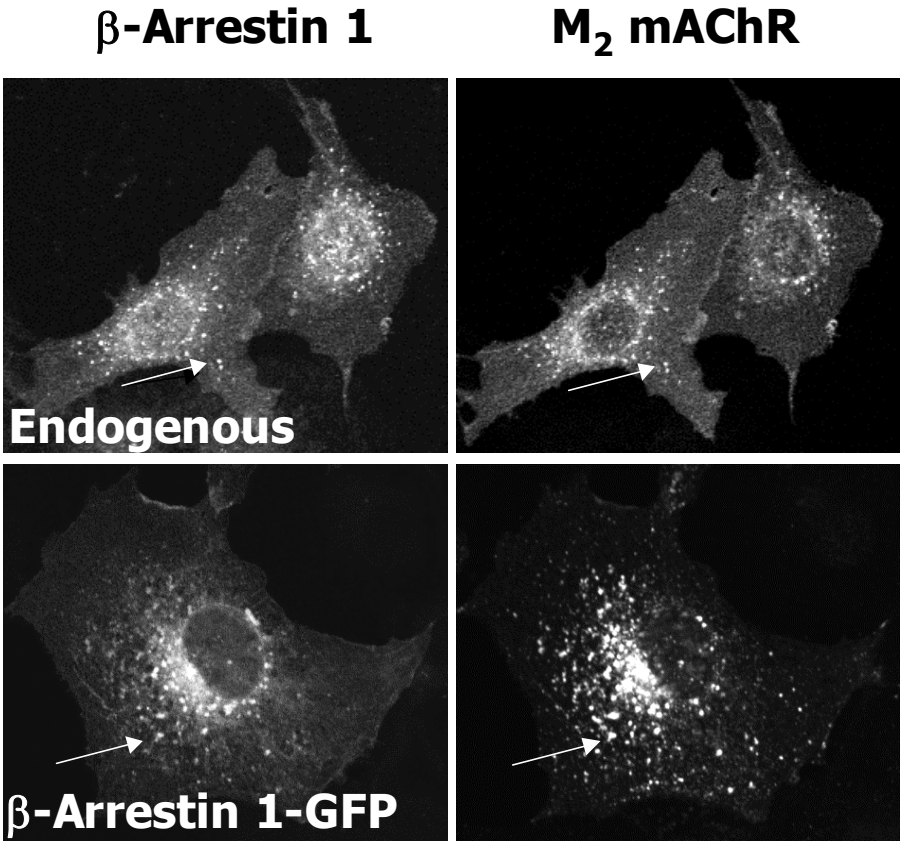

Fig. 17. Addition of agonist leads to the redistribution of both endogenous and transfected β-arrestin 1 to internalized M_2 mAChRs in HeLa cells. HeLa cells were transiently transfected with FLAG-tagged M_2 mAChR alone or with β-arrestin 1-GFP and treated with 1 mM carbachol for 30 min at 37^0C. Singly transfected cells were processed for indirect immunofluorescence microscopy using mouse anti-β-arrestin 1 and rabbit anti FLAG antibodies followed by Alexa488-labeled goat anti-mouse secondary and Alexa594-labeled goat anti-rabbit antibodies. Co-transfected cells were stained for the FLAG epitope. Arrows indicate endosomal structures that contain both M_2 mAChR and β-arrestin 1. This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

Specificity of mAChR Interaction with β-arrestin

To address whether other muscarinic receptor subtypes stably associate with β-arrestin in endosomes, we co-expressed HA-tagged M_1, M_3, M_4, and M_5 mAChRs with β-arrestin 2-GFP in MEF wild type cells and assessed β-arrestin localization using confocal microscopy (Fig. 18 and Fig. 19). Each frame of the figure shows localization of the muscarinic receptor subtype co-expressing β-arrestin 2-GFP. Overlay images indicate co-immunostaining of mAChRs (red) with β-arrestin 2-GFP (green) and their extent of co-localization (yellow). In the absence of carbachol, β-arrestin 2-GFP was primarily diffusely localized in the cytosol of cells expressing M_1 - M_5 mAChR subtypes (Fig. 18). Following 30 minute carbachol stimulation only cells expressing FLAG-tagged M_2 mAChRs exhibited β-arrestin 2-GFP localization in intracellular compartments as shown by arrows indicating overlap and corresponding overlay image (Fig. 19); in cells expressing other receptor subtypes, β-arrestin 2-GFP remained diffusely distributed. Hence, only cells expressing the FLAG-tagged M_2 mAChR subtype exhibited a stable interaction with β-arrestin at intracellular sites compared to the other muscarinic subtypes.

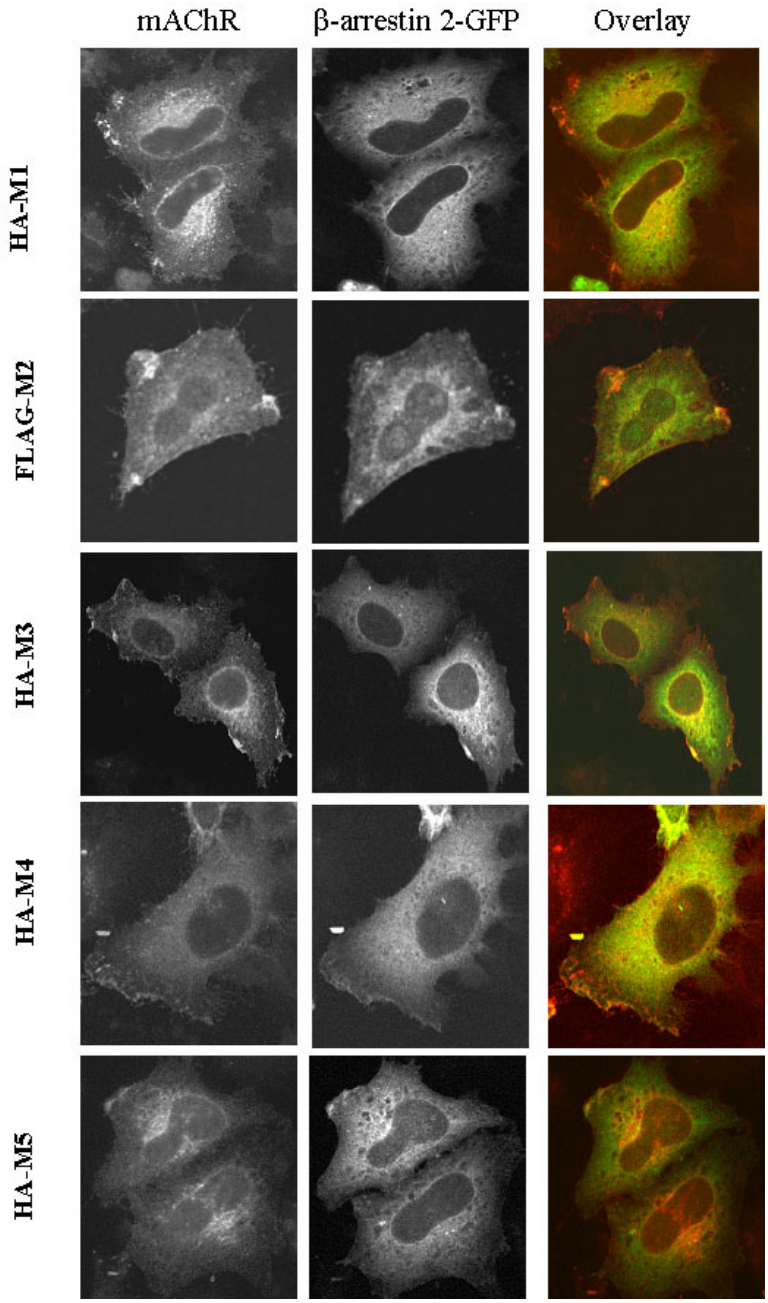

Fig. 18. Unstimulated mAChR subtypes are diffusely distributed at the cell surface while β-arrestin 2-GFP is found in the cytosol. HeLa cells were transiently co-transfected with plasmids encoding β-arrestin 2-GFP and either HA-tagged M_1, M_3, M_4, M_5 mAChR or FLAG-tagged M_2 mAChR. Cells were fixed and processed for confocal microscopy. Grayscale images indicate β-arrestin 2-GFP and mAChR subtype while the overlay represents co-immunostaining of mAChR (red) and β-arrestin 2-GFP (green) expression.

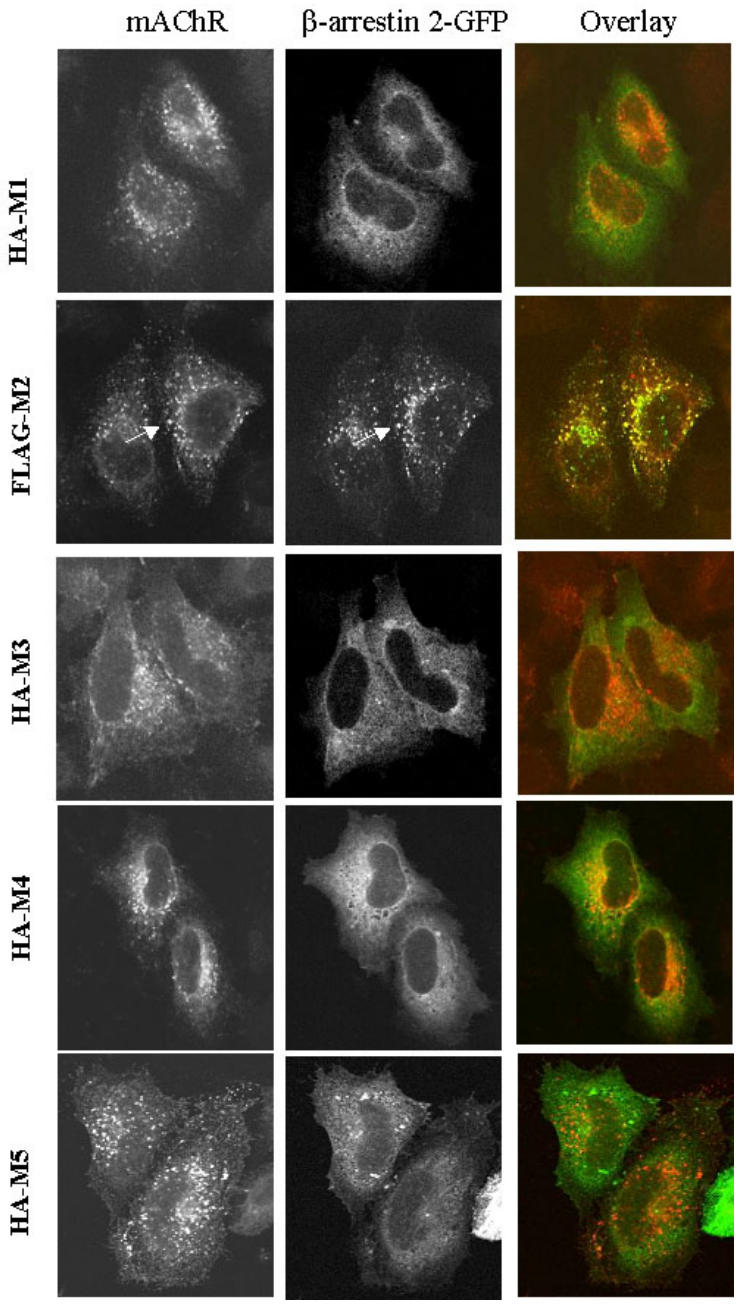

Fig. 19. Internalized M_2 mAChRs exhibit a differential affinity for β-arrestin 2-GFP compared to other muscarinic subtypes. HeLa cells were transiently co-transfected with plasmids encoding β-arrestin 2-GFP and either HA-tagged M_1, M_3, M_4, M_5 mAChR or FLAG-tagged M_2 mAChR. Cells were treated with 1 mM carbachol for 30 minutes at 37^0C. Arrows indicate overlap between internalized M_2 mAChRs and β-arrestin 2-GFP. This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

Intracellular Trafficking of the M_2 mAChR to Early Endosome and Late Endosome Compartments

Based upon the findings described above, we sought the identity of the endosomal structures to which β-arrestin/receptor complexes reside. Delaney and coworkers have shown previously that M_2 mAChRs expressed in HeLa cells internalized in an Arf6 GTPase sensitive fashion that quickly merges with clathrin-derived early endosomes [26]. Therefore, we wanted to determine whether β-arrestin/receptor complexes localized to early endosomal compartments following agonist addition. To determine this we performed co-localization analyses using markers of the early endosome, the early endosomal autoantigen-1 (EEA-1) and the transferrin receptor (TfnR), in combination with β-arrestin 1-GFP. β-arrestin 1-GFP and FLAG-M_2 mAChRs were co-expressed in HeLa cells, and cells were stimulated with 1 mM carbachol for 30 minutes. Our results showed that β-arrestin 1-GFP partially co-localized with EEA-1 and TfnR following carbachol addition (as indicated by arrows in Fig. 20). β-arrestin 1-GFP was not observed to be associated with EEA-1 or TfnR in unstimulated HeLa cells (Fig. 20). These results indicate that internalized M_2 mAChRs remain co-localized with β-arrestin in clathrin-derived early endosomes.

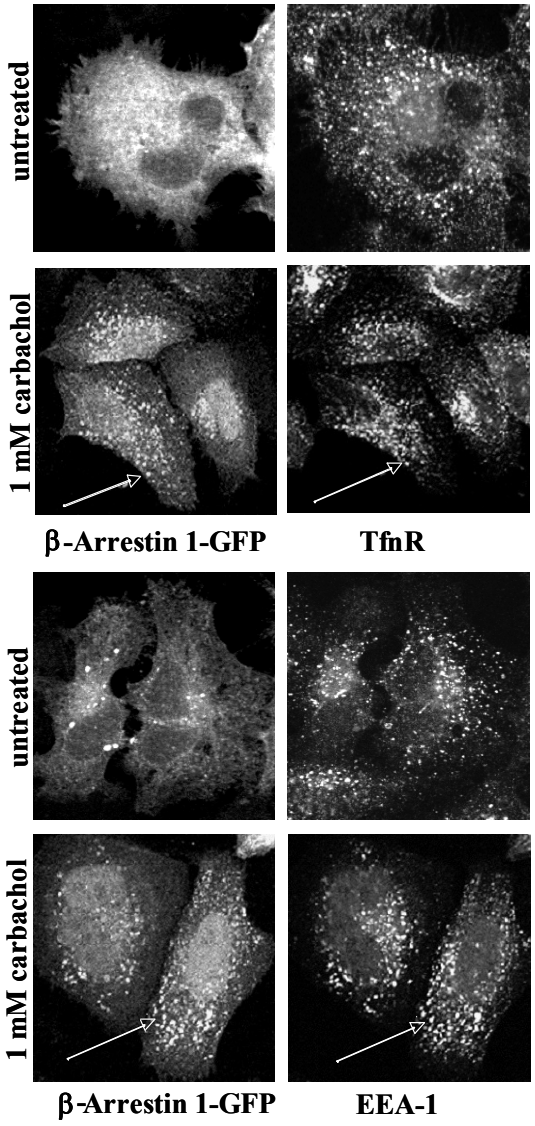

Fig. 20. M_2 mAChR stimulation leads to the redistribution of β-arrestin 1-GFP to early endosomal structures in the cytosol. HeLa cells were transiently transfected with human FLAG-tagged M_2 mAChR and β-arrestin 1-GFP and treated with 1 mM carbachol for 30 minutes. Cells were processed for confocal microscopy. β-arrestin 1-GFP complexes localized to the early endosome as shown by colocalization with markers of that compartment (EEA-1 and TfnR). Arrows indicate signficant overlap between TfnR or EEA-1 with β-arrestin 1-GFP. This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

To better characterize the steps involved in M_2 mAChR intracellular trafficking, we investigated the general role of Rab GTPases, known regulators of GPCR membrane and endosomal trafficking. Given that internalized M_2 mAChRs co-localized with clathrin-dependent, early endosomal markers within 30 minutes of agonist stimulation, we were interested in determining whether Rab5 GTPase regulated the trafficking of internalized M_2 mAChRs. Rab5 GTPase is involved in delivery of primary endocytic cargo to the early endosome and is a known regulator of clathrin-dependent endocytosis [49, 51, 186]. Therefore, we examined whether internalized M_2 mAChRs were delivered to Rab5-positive early endosomes and what effects a dominant-negative Rab5 S34N and constitutively active Rab5 Q79L had on receptor localization. Here we show in HeLa cells that stimulated M_2 mAChR significantly co-localized with wild type GFP-tagged Rab5, further supporting the notion that internalized M_2 mAChRs localize to the early endosome (Fig. 21). Expression of Rab5 S34N led to small, vesicular structures containing M_2 mAChRs while expression of Rab5 Q79L induced hypertrophy of M_2 mAChR positive compartments (Fig. 21). The dominant inhibitory mutant, Rab5 S34N, appeared to prevent fusion of the primary M_2 mAChR vesicles into larger endosomal structures while internalization remained unaltered. These results indicate a role for Rab5 GTPase in regulating the post-endocytic trafficking of the M_2 mAChR and also support the notion that M_2 mAChRs undergo clathrin-independent endocytosis in the HeLa cell line. Overexpression of Rab5 S34N in the MEF wild type cells significantly blocked agonist-induced endocytosis of the M_2 mAChR (*data not shown*).

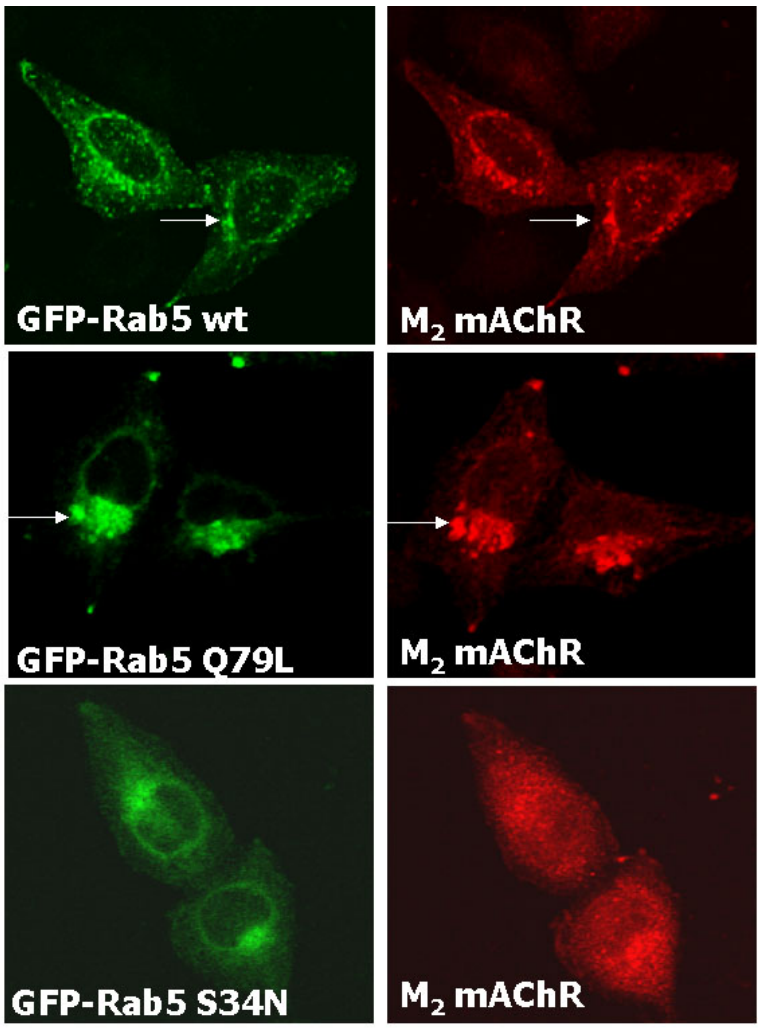

Fig. 21. Rab5 GTPase regulates post-endoctyic trafficking of the M_2 mAChR. HeLa cells were transiently transfected with plasmids encoding M_2 mAChR and either GFP-tagged Rab5 wild type, Rab5 Q79L, or Rab5 S34N. Cells were treated with 1 mM carbachol for 15 minutes, fixed and processed for confocal microscopy. Dominant negative Rab5S34N blocks the formation of distinct vesicular structures although internalization appeared to occur. Arrows indicate extensive co-localization between GFP-Rab5 wt and GFP-Rab5 Q79L with internalized M_2 mAChR. This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

We next sought to determine the role of Rab7 GTPase in regulating the post-endocytic trafficking of the M_2 mAChR. Rab7 GTPase has been shown to mediate the progression of cargo from the early endosome to the late endosome/lysosome compartments [59]. Following 1 hour of agonist treatment, M_2 mAChRs partially co-localized with wild type GFP-tagged Rab7 and overexpression of dominant inhibitory Rab7 T22N caused enlargement of M_2 mAChR-containing compartments (Fig. 22). GFP-tagged Rab7 wt and EEA-1 appear to partially co-localize; however, the observed association is eliminated upon the addition of dominant inhibitory Rab7 T22N. Furthermore, Rab7 T22N produced swollen early endosomal structures that presumably contain M_2 mAChRs since this mutant significantly swells early endosome and receptor positive compartments. These data suggest that Rab7 GTPase may regulate the targeting of internalized M_2 mAChRs to the lysosome for receptor down-regulation. Since both Rab5 and Rab7 GTPase mutants perturb the morphology of M_2 mAChR containing endosomes, we can infer that these Rab GTPases are key elements in post-endocytic trafficking of M_2 mAChRs in the HeLa cell line.

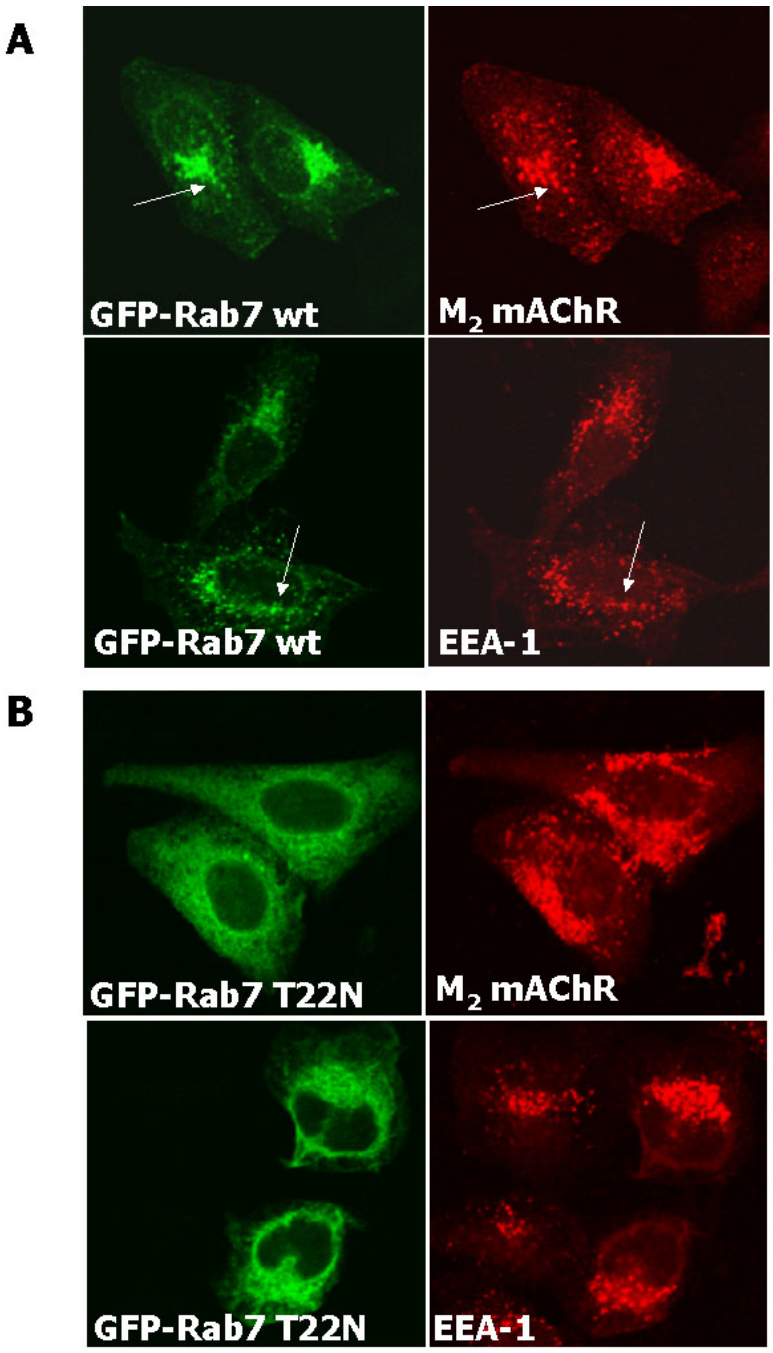

Fig. 22. Rab7 GTPase regulates the post-endocytic trafficking of the M_2 mAChR. HeLa cells were transiently transfected with plasmids encoding FLAG-tagged M_2 mAChR and either GFP-tagged Rab7 wt (A) or the dominant negative mutant, Rab7 T22N (B). Cells were stimulated with 1 mM carbachol for 1 hour, fixed, and immunolabeled using anti-EEA-1 or anti-FLAG antibody. Images were captured by confocal microscopy. Arrows indicate sites of co-localization. This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

CHAPTER 5

DISCUSSION

In the present study, we investigated the role of β-arrestin in agonist-promoted internalization of the M_2 mAChR, which has previously been reported to be β-arrestin independent. In previous studies, heterologous over-expression of wild type and dominant-negative forms of arrestins was used to assess the function of these proteins [156, 175]. Unfortunately, such studies are difficult to interpret because of the complications associated with overexpressed mutants and endogenous proteins. In an attempt to alleviate these complications, we utilized mouse embryonic fibroblasts (MEFs) derived from β-arrestin knockouts in which endogenously expressed β-arrestin 1 and 2 have been genetically eliminated [80]. These cells provide us a unique opportunity to assess whether β-arrestin proteins are involved in the process of agonist-promoted internalization of M_2 mAChRs. Herein, we show that agonist-promoted endocytosis of the M_2 mAChR in MEF cells is β-arrestin- and clathrin-dependent.

Both β-arrestin 1 and 2 isoforms were reported to form high affinity complexes with the agonist-activated M_2 mAChR [152], suggesting that either isoform is capable of mediating agonist-promoted internalization of the receptor. In agreement with these findings, we observed no selectivity between β-arrestin isoforms in mediating agonist-promoted internalization of M_2 mAChRs. Perhaps this lack of selectivity between β-

arrestin 1 and 2 may explain why using over-expression of a single mutant form of β-arrestin fails to completely block the agonist-promoted internalization of M_2 mAChRs.

Interestingly, our studies further revealed that β-arrestin remained stably associated with the M_2 mAChR in juxtanuclear endosomes for prolonged periods of time following agonist exposure. Given that MEF cells do not endogenously express mAChRs, we compared our observations in a physiologically relevant cell line (RASMCs) and two model cell lines (HeLa and COS-7). Similar findings were also observed in these cells. Additionally, we observed recruitment of endogenous β-arrestin to internalized M_2 mAChRs in the HeLa cell line. Since a heterologous overexpression system was used, caution should be taken in intrepretating these results. To confirm these findings, immunohistochemical studies should be employed in an endogenous cell line to examine M_2 mAChR and β-arrestin interaction *in vivo*.

M_2 mAChRs follow the general pattern utilized by most GPCRs in that they are internalized via a β-arrestin-dependent mechanism. Additionally, the stable binding of β-arrestin with activated M_2 mAChRs within microcompartments follows the paradigm of other class B GPCRs. Implications of these findings are that β-arrestin may dictate the intracellular trafficking and/or signaling of the M_2 mAChRs. Since β-arrestin has emerged as a versatile adaptor and scaffolding protein, its role in regulating M_2 mAChR-dependent cellular activity may be significant. It has been shown that β-arrestins interact with trafficking machinery such as Arf6, RhoA, NSF, and a variety of signaling proteins such as ASK1, JNK3, and ERK1/2 [187]. Stable β-arrestin/receptor complexes, as exhibited by class B receptors, appear to redirect signaling complexes to the cytoplasm thereby activating cytoplasmic targets while preventing ERK translocation to the nucleus

[172, 173, 188]. The physiological role of this process may be to participate in actin cytoskeleton reorganization and chemotaxis [71, 189]. With regard to intracellular trafficking, patterns of β-arrestin binding to activated receptors appear to modulate receptor recycling and/or degradation [87]. Class A receptors are typically resensitized and subsequently recycled while class B receptors undergo slow recycling and/or down-regulation. M_2 mAChRs have been shown to undergo slow recycling back to the plasma membrane upon agonist removal [162]. What role or roles β-arrestin plays in M_2 mAChR recycling and/or degradation is currently unknown. The functional consequence of stable β-arrestin/M_2 mAChR complexes remains to be determined.

Previous studies have suggested that M_2 mAChR internalization does not proceed through a clathrin-mediated pathway [24, 153, 156]. Evidence presented here suggests that M_2 mAChR internalization requires β-arrestin association, which would infer a role for clathrin in mediating receptor endocytosis. Therefore, we conducted experiments with arrestin mutants that were selectively deficient in interaction with clathrin, AP-2, or both clathrin and AP-2, to determine whether rescue of agonist-mediated internalization of M_2 mAChR in MEF KO1/2 cells required AP-2 and/or clathrin association. Expression of arrestin mutants defective in interaction with either clathrin (β-arrestin 2-ΔLIELD) or AP-2 (β-arrestin 2-F391A) failed to antagonize M_2 mAChR internalization. Moreover, over-expression of a dominant-negative β-arrestin mutant that was defective in interaction with both clathrin and AP-2 only modestly antagonized M_2 mAChR internalization in MEF KO1/2 cells. Thus, it is reasonable to conclude that these data corroborate previous studies indicating that M_2 mAChR internalization is clathrin-independent. However, Santini and co-workers have reported that arrestin mutants with

impaired binding to clathrin or AP-2 were still capable of displaying recruitment of β_2-AR to clathrin-coated pits, albeit to a reduced degree [178]. Therefore, it may be premature to conclude that M_2 mAChR internalization is β-arrestin-dependent but clathrin/AP-2-independent.

Expression of the truncated carboxy-terminal region of β-arrestin 1, which functions as a dominant negative to sequester available clathrin while lacking receptor binding, has been shown to completely abrogate β_2-AR mediated clustering of clathrin coated pits without altering the integrity of clathrin coats [177]. Introduction of this mutant completely blocked agonist-promoted internalization of M_2 mAChRs in wild type MEFs. In addition, potent inhibitors of clathrin mediated endocytosis, DIII and EΔ95/295 Eps15 mutants, significantly blocked agonist induced endocytosis of the M_2 mAChR in MEFwt cells. Collectively, these results indicate that agonist-promoted internalization of M_2 mAChRs is β-arrestin-dependent and most likely clathrin/AP-2-dependent in MEFwt cells. Interestingly, exogenous expression of Eps15 mutants in the HeLa cell line had no effect on agonist-mediated internalization of the M_2 mAChR. Similarly, previous studies using dominant negative mutants have shown that β-arrestin, dynamin, and clathrin do not play a role in mediating endocytosis of the M_2 mAChR in the HEK 293 cell [145, 153, 155-157, 175]. However, upon further analysis, expression of a N-terminal deletion dynamin-1 mutant N272 that lacks the complete GTP-binding domain strongly inhibited agonist-promoted M_2 mAChR internalization [158]. Based on these observations it is conceivable that M_2 mAChRs are delivered to a membrane environment that is less sensitive to dynamin-, arrestin-, and clathrin-dominant negative mutants but still require these proteins for endocytosis. Regardless, reports have also

shown that M_2 mAChRs utilize a caveolae-dependent pathway in cardiac myocytes while an alternate, ill-defined pathway takes form in HeLa and HEK 293 cells [26, 27, 160]. Therefore this evidence raises the intriguing possibility that the internalization profile of the M_2 mAChR may depend on the cellular system in which it is expressed.

Closer examination of β-arrestin post-endocytic trafficking revealed that M_2 mAChR stimulation led to β-arrestin partial redistribution into Tfn and EEA-1 positive compartments, markers of the early endosome. In accordance with our findings, Delaney and coworkers have reported that stimulated M_2 mAChRs internalize in a manner that quickly merges with clathrin-derived early endosomes [26].

This study further provides evidence for the role of Rab GTPases in regulating the intracellular trafficking of internalized M_2 mAChRs. Rab GTPases regulate trafficking of endocytic cargo between distinct microcompartments and therefore serve as markers for these compartments [190, 191]. Using these markers, we showed that M_2 mAChR is internalized to Rab5 GTPase positive early endosomes in HeLa cells. Subsequently, the receptor appeared to transit to Rab7 late endosome/lysosome compartments. Partial co-localization of M_2 mAChR with Rab7 GTPase endosomes suggests that a portion of receptors may undergo lysosomal targeting and subsequent down-regulation. These results are consistent with the radioligand degradative assay that indicates chronic stimulation of the M_2 mAChRs leads to appreciable down-regulation [163].

Previous studies have shown that expression of a dominant-negative Rab5 S34N mutant could prevent clathrin-mediated endocytosis [48, 192]. Seachrist and coworkers have shown that the GTP-binding defective Rab5 S34N potently inhibits agonist endocytosis of the β_2-AR while a constitutively active form, Rab5 Q79L, causes

accumulation of the receptor in swollen early endosomes [48]. The overexpression of this mutant blocked M_2 mAChR internalization in the MEF cells while internalization remained unaltered in the HeLa cell line. These results suggest that M_2 mAChR may undergo an atypical pathway in the HeLa cell line. Nevertheless, this mutant appeared to block entry into early endosomal compartments in both cell lines thereby preventing proper receptor sorting.

The differential trafficking of β-arrestin with mAChRs to endosomes appears to be subtype specific. There are five muscarinic subtypes termed M_1 mAChR- M_5 mAChR. M_1, M_3, and M_5 mAChRs couple to G_q proteins and activate phospholipase C whereas M_2 mAChR and M_4 mAChR couple to $G_{i/o}$ to inhibit adenylyl cyclase and activate K^+ channels [143, 166]. As shown in Figure 19, stimulated muscarinic subtypes aside from M_2 mAChRs are sequestered into endocytic vesicles that are devoid of β-arrestin. It has been shown that M_1 mAChR, M_3 mAChR, and M_4 mAChR require β-arrestin in mediating agonist-promoted internalization [24] so we do not rule out the possibility that arrestin is recruited to the plasma membrane following stimulation and then rapidly disassociates from the receptor. It is possible that carbachol may induce receptor conformations that may not promote stable β-arrestin associations with the other mAChR subtypes. However, sequence alignment of the M_2 and M_4 mAChR (using the T-coffee program) revealed that the subtypes exhibit high sequence similarities; interestingly, the sequence differences lie in the third intracellular loop, specifically at residues 293-313 within the M_2 mAChR. As described by Pals-Rylaarsdam and others, a cluster of serine and threonine sites at positions 307-311 undergo agonist promoted phosphorylation, which is necessary and sufficient for β-arrestin interaction [149]. This site may be

important for designating stable interactions with β-arrestin. M_2 mAChR sequences downstream from this site at 348-368 also differ significantly from the M_4 mAChR suggesting that an additional motif may be involved. The observed difference between mAChR subtypes and the nature of their interaction with β-arrestin is of remarkable interest and should be considered when elucidating the fundamental differences in the role they play within a target tissue.

PART II

DOWN-REGULATION OF MUSCARINIC ACETYLCHOLINE TYPE II RECEPTOR IS BETA-ARRESTIN DEPENDENT

CHAPTER 6

INTRODUCTION

There are five subtypes of muscarinic acetylcholine receptors (mAChRs) with distinct though overlapping tissue distributions. Like all GPCRs, mAChRs couple to downstream effectors through heterotrimeric G-proteins. The M_1, M_3 and M_5 subtypes couple preferentially to $G\alpha_q$ whereas the M_2 and M_4 subtypes couple to G_i and G_o. The M_2 mAChR regulates a variety of physiological responses ranging from cardiac homeostasis to cholinerigic signaling in the brain [193]. A common feature of mAChRs, and in fact all GPCRs, is that their activity and expression are tightly regulated. Agonist-promoted trafficking of mAChRs and most other GPCRs can be broken down into five distinct phases [193]: agonist-binding promotes G-protein dissociation from the receptor (I) which allows phosphorylation of specific serine and threonine residues on internal loops of the receptor (II) by G-protein receptor kinases (GRKs). This phosphorylation allows the binding of β-arrestins (III), which promotes homologous desensitization, and subsequent internalization of the receptor into clathrin coated pits. Following internalization, the receptor can either be dephosphorylated and recycled to the cell surface as a functional receptor (IV) or targeted for degradation in proteasomes or lysosomes (V).

β-arrestins have emerged as a central control point in the trafficking of nearly all GPCRs [194]. In addition to mediating desensitization of GPCRs, it is recognized that β-arrestin participates in clathrin-dependent endocytosis of activated receptors by directly

interacting with clathrin and the clathrin-associated adaptor AP-2 [16, 68]. The binding of the β_2-AR/β-arrestin complex to AP-2 facilitates receptor internalization from the cell surface through clathrin-coated pits [195]. Once internalized, β-arrestins also function to regulate GPCR post-endocytic trafficking and further serve as a scaffolding protein for the recruitment of signaling components [187, 194].

The extent of interaction between β-arrestins and GPCRs led to the classification of two types of GPCRs: class A and class B receptors. Class A receptors, such as the β_2-AR, dopamine 1A receptor, and endothelin type A receptor, interact with β-arrestin transiently so that disassociation occurs prior to receptor internalization. These receptors typically undergo rapid recycling [79]. Class B receptors, such as the vasopressin 2 receptor (V_2R), angiotensin receptor ($AT_{1a}R$), and neurotensin 1 receptor, recycle and resensitize slowly and internalize in a stable association with β-arrestin [81, 84]. Delineation of class A and B receptors has been shown to be directly correlated with the ubiquitination status of β-arrestin [87]. Class A receptors, which do not internalize with β-arrestin, display a pattern of transient β-arrestin ubiquitination whereas class B receptors, which do internalize with β-arrestin, display sustained β-arrestin ubiquitination [87].

Ubiquitination of proteins is a signal for degradation that leads to delivery of those proteins to, and their subsequent degradation in, the 26 S proteasome [196]. However, recent studies have revealed that ubiquitination of proteins can further serve as a signal in endocytic and post-endocytic sorting of these proteins [90]. There are a number of examples where ubiquitination has been shown to be involved in the regulation, both endocytosis and degradation, of GPCRs, including the opioid receptors

[197], yeast pheromone receptor [98], human immunodeficiency virus co-receptor CXCR4 [102], protease activating receptor PAR_2 [103], β_2-AR and its associated protein β-arrestin [85], and neurokinin receptor NK_1R [47]. In the case of the β_2 -AR, Shenoy and coworkers [85] showed that agonist stimulation led to the ubiquitination of both β-arrestin, an essential step for receptor internalization, and β_2-AR, a necessary event for receptor degradation. Lysine deficient mutants of these receptors abolished agonist-promoted degradation while internalization remained unaltered [47, 85, 102, 103].

In the present study, we sought to determine what role β-arrestin has in regulating the post-endocytic sorting of the M_2 mAChR and whether ubiquitination contributes to this process. Initial reports examining the role of β-arrestin, clathrin, and dynamin in mediating the internalization of the M_2 mAChR indicated that agonist-promoted internalization of the receptor was largely independent of clathrin and dynamin and that the M_2 mAChR internalized via both β-arrestin-dependent and -independent pathways [153, 156]. Later work, however, revealed the essential role of β-arrestin and clathrin in M_2 mAChR internalization, and suggests that M_2 mAChR internalization is, in fact, dynamin-dependent [158, 198]. Moreover, M_2 mAChR internalized into perinuclear compartments remained associated with both isoforms of β-arrestin for prolonged periods of time [198]. This observation, coupled with the fact that M_2 mAChRs display a pattern of slow recycling [162], suggests that M_2 mAChR behaves as a class B receptor. Here we show that β-arrestin mediates agonist-promoted down-regulation of the M_2 mAChR which is dependent upon specific lysine residues and ubiquitin in β-arrestin. In addition, the data indicate that down-regulation occurs in lysosomes and involves proteasomal function.

CHAPTER 7

MATERIALS AND METHODS

Materials

Reagents were purchased as follows: [^{3}H]-*N*-methyl-scopolamine ([^{3}H]-NMS) (81-84 Ci/mmol) and [^{3}H]-L-quinuclidinyl benzilate ([^{3}H]-QNB) (43 Ci/mmole) from Amersham Corp. (Buckinghamshire, England); Dulbecco's Modified Eagle's Medium from Mediatech (Herndon, VA); penicillin/streptomycin, fetal bovine serum, and LipofectAMINE 2000 from Invitrogen (Carlsbad, CA); n-dodecyl-β-D-maltoside was from Calbiochem (San Diego, CA); BCA protein assay kit and Supersignal West Pico from Pierce Biotechnology (Rockford, Il); Atropine, N-ethyl maleimide (NEM), carbamyl choline chloride (carbachol), monoclonal anti-FLAG M2 antibody (F3165), rabbit anti-LAMP1 antibody (L1418), 100X protease inhibitor and all other chemicals from Sigma Aldrich (St. Louis, MO); Anti-HA affinity matrix from Roche Applied Science (Indianapolis, IN); monoclonal anti-Ub antibody P4D1 (sc 8017) from Santa Cruz Biotech (Santa Cruz, CA); The anti-HA.11 monoclonal antibody was purchased from Covance Research Products (Berkley, CA); HRP-conjugated secondary antibodies from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA) and Bio-Rad Laboratories (Hercules, CA); pIRESeGFP from Clontech (Palo Alto, CA). Alexa594-conjugated goat anti-mouse and Alexa488-conjugated goat anti-rabbit antibodies were purchased from Molecular Probes (Eugene, OR).

Expression Constructs and Cell Lines

Expression constructs and cell lines were generously provided by the following: MEF wild-type cells, β-arrestin 1 and 2 single knockout cells, β-arrestin 1 and 2 double knockout cells, FLAG-tagged β-arrestin 1 and 2, YFP-β-arrestin-2-Ub, β-arrestin $2^{K11R,K12R}$, and β-arrestin $2^{K18R,K107R,K108R,K207R,K296R}$ by Dr. Robert Lefkowitz (Duke University Medical Center, Durham, NC); HA tagged human M_2 mAChR by Dr. Audrey Claing (University of Montreal, Montreal, Canada).

Cell Culture and Transient Transfection

Mouse embryonic fibroblast (MEF) cells (wild-type and knockouts) were maintained in Dulbecco's Modified Eagle's Medium (DMEM). Media were supplemented with 10% fetal bovine serum (FBS), 100 IU /mL penicillin, and 100 μg/mL streptomycin. All cells were maintained at 37°C with 5% CO_2. At 24 hr prior to transfection, cells were plated at $7.5x10^4$ cells/well (12-well plate), $1.5x10^5$cells/well (6-well plate), or $1.5x10^6$ cells/dish (10 cm dish) in serum containing DMEM without antibiotics. Cells were transfected using LipofectAMINE 2000 according to the manufacturer's protocol with 1.6, 2.0 or 2.5 μg total DNA per well/plate on a 12-well, 6-well and 10 cm dish, respectively. Transfection efficiencies of 20 – 50 % were routinely obtained [determined by including 10% of total DNA as eGFP construct (pIRESeGFP) or indirect immunofluorescence against proteins and visualizing transfected cells using an Olympus 1X71 fluorescence microscope].

Indirect Immunofluorescence

Cells were treated as described in figure legends, 24 hours after transfection. Cells were then fixed in 2% formaldehyde in phosphate buffered saline (PBS) for 10 minutes and rinsed with 10% adult calf serum and 0.02% azide in PBS (PBS/serum). Fixed cells were incubated with primary antibodies diluted in PBS/serum containing 0.2% saponin for 45 minutes, and then washed with PBS/serum (3 x 5 min.). The cells were then incubated with fluorescently labeled secondary antibodies in PBS-serum and 0.2% saponin for 45 minutes, washed with PBS/serum (3 x 5 min.) and once with PBS, and mounted on glass slides. Images were acquired using a Zeiss LSM 510 scanning confocal microscope. All images were processed with Adobe Photoshop 7.0 software.

Crude Membrane Preparation

Two wells of a 6-well plate were rinsed twice with ice cold PBS and cells were scraped in 50 mM sodium phosphate pH 7.0, pooled, and homogenized with 20 strokes in a Dounce homogenizer. Homogenate was spun at 10,000 rpm for 20 min at 4°C in a Sorvall Mach 1.6R fixed angle rotor. Pellet was resuspended in 50 mM sodium phosphate pH 7.0 and spun again. Pellet was resuspended in 0.55 mL 50 mM sodium phosphate pH 7.0 and used for protein assay and radioligand binding.

Detection of Ubiquitinated β-arrestin 2

At 24 hr post transfection with FLAG-β-arrestin 2 and HA-tagged M_2 mAChR, MEFwt cells were serum-starved for 1 hr prior to 1 mM carbachol treatment. Cells were then rinsed twice with 1X PBS and scraped in 0.5 mLs of 1% Triton X-100 lysis buffer on ice

(50 mM HEPES, pH 7.5, 1% Triton X-100, 10% glycerol, 10 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM NEM, and protease inhibitors). Lysates were solubilized end-over-end for 2 hours at 4°C and subsequently centrifuged at 14,000 rpm for 30 minutes at 4°C. Supernatant was diluted 1:1 with Tris buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT) containing fresh protease inhibitor and 10 mM NEM. Samples (600 μg of protein) were then incubated with 30 μl of anti-FLAG agarose (Sigma) overnight at 4°C. Samples were washed three times in 0.1% Triton X-100 lysis buffer and eluted in 2X SDS sample buffer for 15 minutes at 37^0C. Proteins were resolved by SDS-PAGE (7.0%), transferred to nitrocellulose, and blocked for 1 h at room temperature in 2% milk powder and 0.02% Tween 20 in TBS. Blots were then incubated overnight at 4°C in TBST with mouse anti-FLAG antibody (Sigma) or mouse anti-Ub antibody (Covance). After overnight incubation, blots were washed twice with TBST for 10 minutes each and incubated with HRP-labeled secondary antibodies for 1 h at room temperature. Following three washes with TBST for 10 minutes each, detection was performed by chemiluminescence with Supersignal West Pico reagents (Pierce) according to manufacturer's protocol, then developed on film.

Detection of Ubiquitinated M_2 mAChR

At 24 hr post transfection, treated cells were rinsed with 1X PBS and scraped in 2% n-dodecyl-β-D-maltoside lysis buffer on ice (2% maltoside, 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1.0 mg/mL iodoacetamide, 10 mM N-ethyl maleimide, and protease inhibitor cocktail). Lysates were solubilized end-over-end for 2 hours at 4°C and subsequently centrifuged at 14,000 rpm for 30 minutes at 4°C.

Supernatant was diluted 1:1 with Tris buffer containing fresh protease inhibitor and 10 mM NEM. Samples (600 μg of protein) were then incubated with 30 μL of anti-HA affinity matrix (Roche) overnight at 4°C. Samples were washed three times in 0.2% n-dodecyl-β-D-maltoside lysis buffer and eluted in 2X SDS sample buffer for 15 minutes at 37°C. Proteins were resolved by SDS-PAGE (7.0%), transferred to nitrocellulose, and blocked for 1 h at room temperature in 2% milk powder and 0.02% Tween 20 in TBS. Blots were then incubated overnight at 4°C in TBST with mouse anti-HA antibody (Covance) or mouse anti-Ub antibody (Covance). After overnight incubation, blots were washed twice with TBST for 10 minutes each and incubated with HRP-labeled secondary antibodies for 1 h at room temperature. Following three washes with TBST for 10 minutes each, detection was performed by chemiluminescence with Supersignal West Pico reagents (Pierce) according to manufacturer's protocol, then developed on film.

Radioligand Binding:

Internalization - Internalization was determined by measuring the binding of the membrane impermeable muscarinic antagonist [^{3}H]-NMS to intact cells. Briefly, 24-48 hr after transfection, MEF cells were treated or not treated with 1 mM carbachol for 60 min at 37°C. Cultures were washed 3 x 1 mL with serum-free media and incubated with 100 nM [^{3}H]-NMS in 1 mL PBS for 30 min at 37°C or 4 hr at 4°C. Nonspecific binding was determined as the bound radioactivity in the presence of 1 μM atropine. Labeled cells were washed 3 x 1 mL with ice-cold PBS, solubilized in 0.5 mL 1% Triton X-100, and combined with 3.5 mL scintillation fluid followed by measurement of radioactivity. Cpm were converted to receptors per well which were then corrected to receptors per cell

by dividing by cells/well. Receptor internalization is defined as percent of surface M_2 mAChR not accessible to [^{3}H]-NMS at each time relative to untreated or control cells.

Down-regulation – Down-regulation was determined by measuring the binding of the membrane permeable muscarinic antagonist [^{3}H]-L- quinuclidinyl benzilate (QNB). Crude membranes (100 μL) were incubated with 30 nM [^{3}H]-QNB in a total volume of 1 mL 50 mM sodium phosphate pH 7.0 for 2 hr at 25°C. Nonspecific binding was determined as the bound radioactivity in the presence of 1 μM atropine. Membranes were harvested on glass fiber filters using a Brandell cell harvester (Gaithersburg, MD) and washed 3 x 2 mL with ice-cold 50 mM sodium phosphate pH 7.0. Filter discs were combined with 3.5 mL scintillation fluid followed by measurement of radioactivity.

Cpm per tube were converted to fmoles receptor, which was then corrected to mg of total protein per tube. Percent down-regulation was determined as percent of sites remaining compared to untreated control membranes.

Densitometry:

Band intensity of Western blot signals was quantified using Metamorph imaging software (Universal Imaging, West Chester, PA). To quantify relative ratios of protein ubiquitination, ubiquitinated protein signals were compared with total protein bands immunoprecipitated.

CHAPTER 8

RESULTS

Down-Regulation of M_2 mAChR is β-arrestin Dependent

For a number of years there has been some controversy as to the role of β-arrestin in the internalization and down-regulation of M_2 mAChR. A significant body of work on the role of β-arrestin in the internalization of mAChRs has been performed using dominant-negative or knockdown strategies. In order to avoid any possible complications that could arise from the presence of low levels of endogenous arrestin proteins, we performed our down-regulation experiments in MEF cells derived from β-arrestin 1, β-arrestin 2 or β-arrestin 1 and 2 double knockout mice (MEF KO1, MEF KO2, and MEF KO1/2, respectively). These cells have been characterized to confirm the absence of mAChR expression using both PCR and radioligand binding assays [198].

To investigate whether any selectivity between β-arrestin isoforms existed, we transiently transfected MEF KO1 and KO2 cells with constructs encoding HA-tagged M_2 mAChRs and examined the time course of agonist-promoted down-regulation by measuring receptor abundance using [^{3}H]-QNB. After 24 hr, cells were treated with 1 mM carbachol for 3, 6, 12, or 24 hr. M_2 mAChRs were similarly down-regulated (~ 35%) in both MEF KO1 and KO2 cells following 6 hr of agonist stimulation (Fig. 23A). It did appear that the KO1 cell line (which expresses only endogenous β-arrestin 2) showed a slight recovery at 18 and 24 hours where the KO2 cell line (expressing only endogenous β-arrestin 1) did not. We then performed similar time course experiments

with MEFwt cells expressing HA-M_2 mAChR and MEF KO1/2 co-expressing FLAG-β-arrestin 2 and HA-M_2 mAChR since internalization and subsequent down-regulation was not observed in the MEF KO1/2 cells (*data not shown*). After 24 hr, cells were treated with 1 mM carbachol for 6, 12, 18 or 24 hr. The MEFwt and KO1/2 cells had slightly different time courses of down-regulation, with what appeared to be a maximum of ~ 21 and 31% at ~ 6 and 12 hr, respectively (Fig. 23B). However, stastical analysis revealed that the time courses of receptor down-regulation were similar enough that we could confidently perform all subsequent single point down-regulation experiments at 12 hr in all cell lines (Fig. 23B).

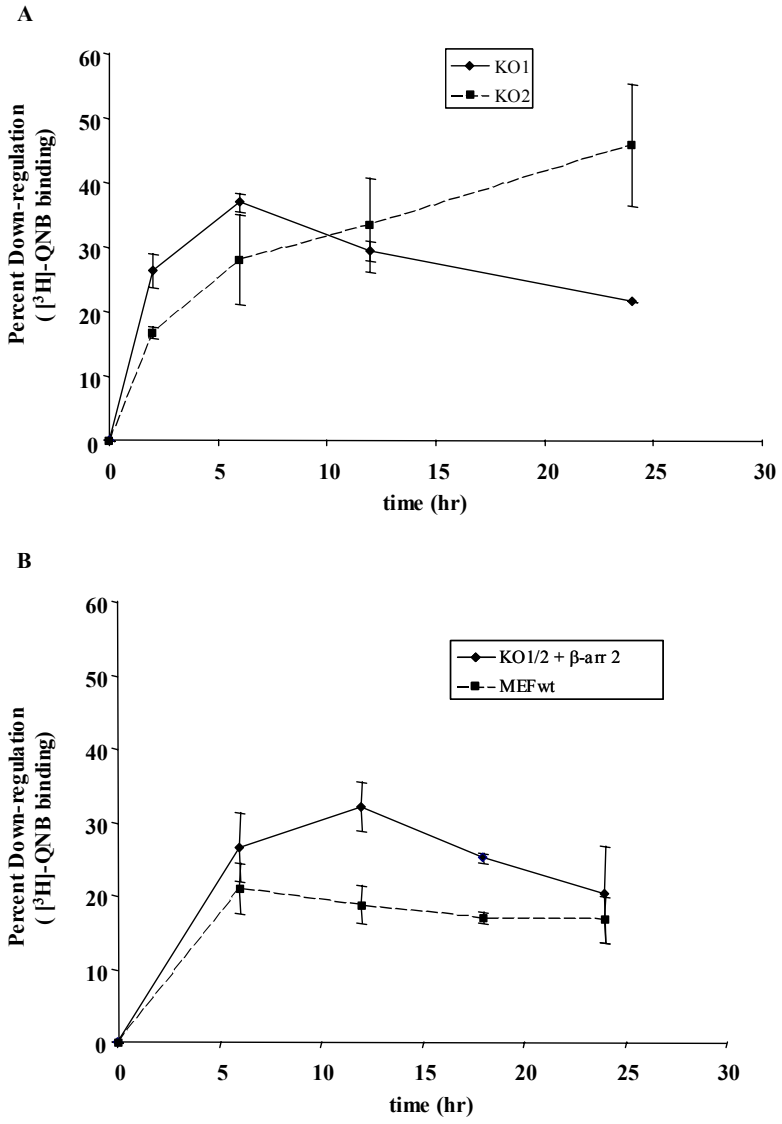

Fig. 23. Time-course of agonist-promoted down-regulation of the M_2 mAChR in MEF cells. All cells were transfected with HA-tagged M_2 mAChR. MEF KO1/2 cells were also co-transfected with FLAG-β-arrestin 2. At 24 hrs following transfection, cells were treated with 1 mM carbachol for the indicated time. Down-regulation was determined using [^{3}H]-QNB binding (fmol/mg protein) in crude membranes as described in Materials and Methods. (A) **MEF KO1 and KO2.** Down-regulation was ~ 35% after 6 hr for both MEF KO1 and KO2. (B) **MEFwt and KO1/2.** Down-regulation was at a maximum at ~ 21 and 31% at ~6 and 12 hr for MEFwt and MEF KO1/2, respectively. Data are expressed as percent down-regulation compared to t = 0 control and are presented as mean ± standard error of the mean for three independent experiments with duplicate data points.

Next, we examined the ability of the different isoforms of β-arrestin to rescue the agonist-promoted down-regulation of the M_2 mAChR in MEF KO1/2 cells. The MEF KO1/2 cells were transfected in 6-well plates with constructs encoding HA-M_2 mAChR and either FLAG-β-arrestin 1 or 2. After 24 hr, cells were treated with 1 mM carbachol for 12 hr. In the absence of exogenous β-arrestin there was no down-regulation in response to agonist in the double knockout cell line (Fig. 24A and B). When, however, cells were co-transfected with either β-arrestin 1 or β-arrestin 2 there was a rescue of the ability to down-regulate in response to agonist (Fig. 24A and B). The rescue of agonist-promoted down-regulation was greater in cells expressing β-arrestin 2 (40%) vs. β-arrestin 1 (25%). In addition, although not stastically significant, there does appear to be some constitutive (agonist-independent) down-regulation of M_2 mAChR levels in non-stimulated MEF KO1/2 expressing β-arrestin 2 (15%) that was not observed in cells expressing β-arrestin 1 (Fig. 24A and B open bars).

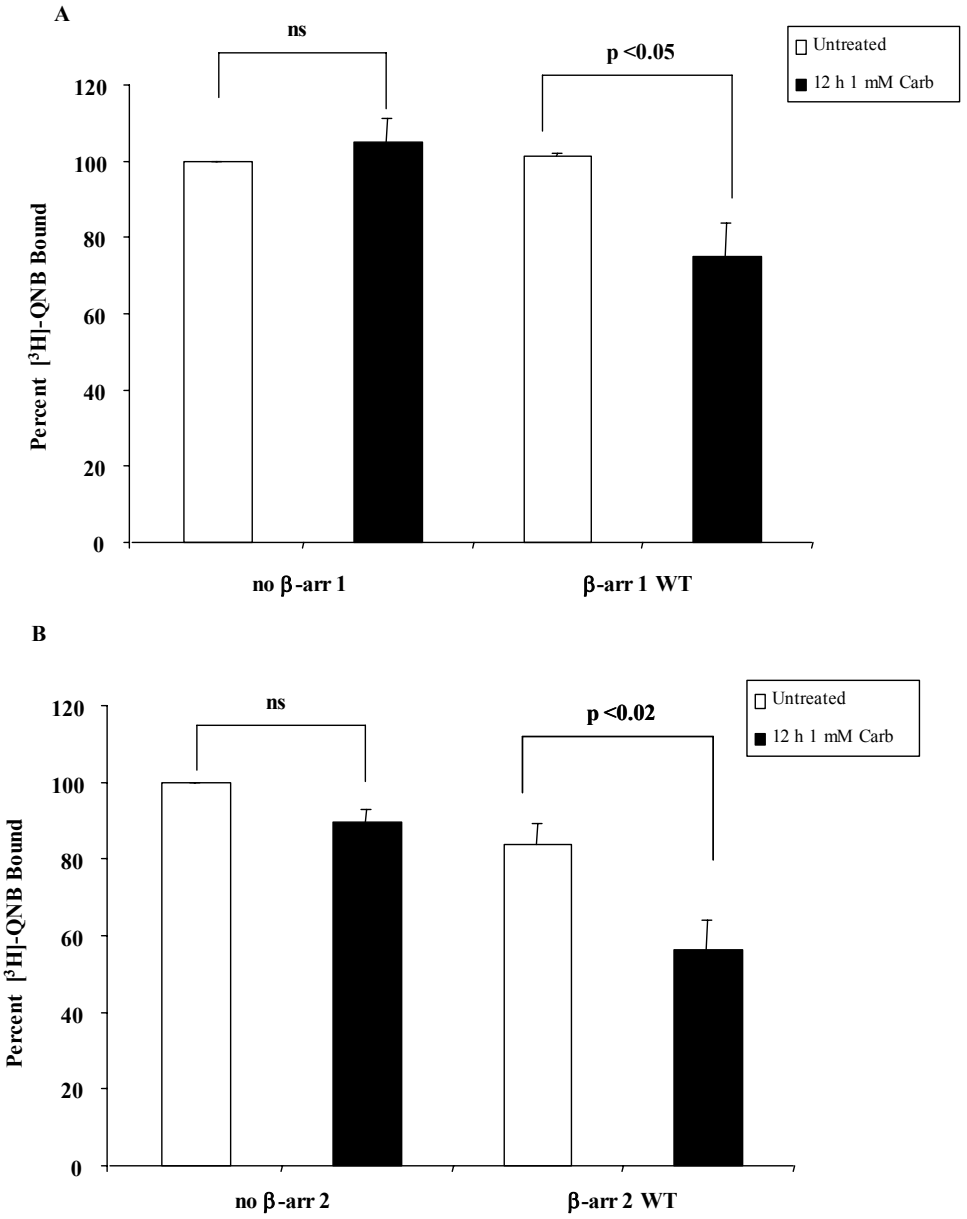

Fig. 24. Rescue of M_2 mAChR down-regulation in MEF KO1/2 cells with β-arrestin 1 or β-arrestin 2. MEF KO1/2 cells were transfected with HA-M_2 mAChR with and without either FLAG-β-arrestin 1 (A) or 2 (B). At 24 hr following transfection, cells were treated with 1 mM carbachol for 12 hr. Down-regulation was determined using [^{3}H]-QNB binding (fmol/mg protein) in crude membranes as described in Materials and Methods. Note the effect of β-arrestin 2 on constitutive down-regulation (B). Data are expressed as percent of [^{3}H]-QNB bound (fmol/mg total protein) compared to untreated control and are presented as mean ± standard error of the mean for three independent experiments with duplicate data points. Statistical analysis was performed using a paired t-test; ns indicates not significant.

β-arrestin Ubiquitination is Important in Mediating M_2 mAChR Down-Regulation

Recent efforts in our laboratory have revealed that stimulation of the M_2 mAChR in MEF cells leads to a stable interaction of the M_2 mAChR and β-arrestin in endosomes, suggesting that M_2 mAChRs are class B receptors [198]. Shenoy and coworkers reported that stable association of class B receptors with β-arrestin involves agonist-promoted ubiquitination of β-arrestin [87]. The data demonstrated a direct relationship between stable association of the receptor with β-arrestin and sustained ubiquitination of β-arrestin. Having established that agonist-promoted down-regulation of the M_2 mAChR in MEF cells is β-arrestin-dependent, we examined whether receptor stimulation led to sustained β-arrestin ubiquitination. To do this, MEFwt cells co-expressing FLAG-tagged β-arrestin 2 and HA-tagged M_2 mAChR were incubated with carbachol for 0-30 minutes. FLAG-β-arrestin 2 was immunoprecipitated via the FLAG tag and Western blots were probed with antibodies against ubiquitin and the FLAG epitope (Fig. 25). In untreated cells, basal levels of ubiquitination were detected as broad smears with apparent molecular masses of ~100-170 kDa. There were no significant changes following 3 min carbachol stimulation. Exposure to carbachol for 15 and 30 min, however, led to an increase in the levels of ubiquitination by ~2 and 3.5 fold, respectively, over basal levels as compared by densitometry (Fig. 25). Metamorph software allowed the generation of relative ratios of ubiquitination by quantifying the intensity of the ubiquitin smear normalized to the amount of FLAG-β-arrestin 2 pulled down. The ubiquitination appeared to be stable rather than transient, since the signal continued to rise after 30 min of agonist exposure indicated by the broader range (~70-170 kDa) and intensity of immunoreactivity. Analysis of immunoprecipitates from mock-transfected cells shows

no detectable signal of β-arrestin or ubiquitin indicating specificity. Stable ubiquitination of β-arrestin in response to agonist adheres to previous findings [87], which further implies that M_2 mAChRs belong to class B.

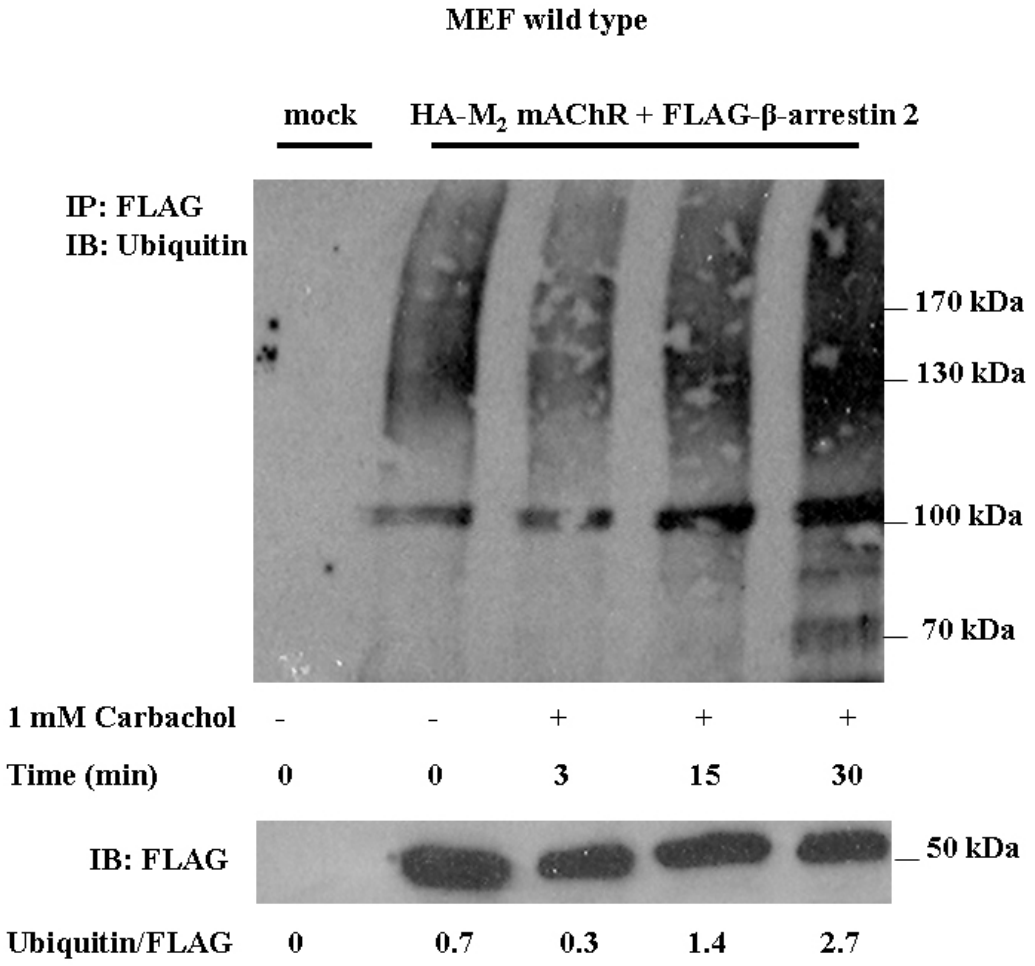

Fig. 25. Agonist treatment promotes ubiquitination of β-arrestin2 in MEFwt cells co-expressing HA-tagged M_2 mAChR. MEFwt cells were co-transfected with HA-tagged M_2 mAChR and FLAG-β-arrestin 2 DNA constructs. At 24 hr following transfection, cells were treated with 1 mM carbachol for the indicated times. **Top panel**: cells were lysed and immunoprecipitated (IP) with anti-FLAG PAb and blotted (IB) with anti-ubiquitin-MAb (P4D1). There was an increase in ubiquitination of β-arrestin 2 with 15 and 30 min exposure to 1 mM carbachol. The blot is representative of two independent experiments. **Bottom panel**: lysates were blotted with anti-FLAG MAb, demonstrating β-arrestin 2 expression levels. Numbers under blot correspond to densitometric quantification of ubiquitin band intensity normalized against the corresponding densitometric value of β-arrestin.

Given the ubiquitination status of β-arrestin following treatment with carbachol, we sought to determine the effects of β-arrestin ubiquitination on regulating down-regulation of stimulated M_2 mAChRs. To do this we employed a constitutively ubiquitinated β-arrestin 2 chimera in which ubiquitin is fused to the C-terminus and yellow fluorescent protein (YFP) is fused to the N-terminus (YFP-β-arrestin 2-Ub). MEF KO1/2 cells were transfected with HA-M_2 mAChR and either FLAG-β-arrestin 2 or YFP-β-arrestin 2-Ub. After 24 hr, cells were treated with and without 1 mM carbachol for 12 hr and the extent of down-regulation was assessed using [^{3}H]-QNB. There was a 26% decrease in total receptor abundance with expression of β-arrestin 2 wild type due to the constitutive (agonist-independent) receptor turnover, which increased to 62% upon treatment with agonist (Fig. 26). The 26% decrease rose to 87% in the presence of constitutively ubiquitinated β-arrestin 2 (YFP-β-arrestin 2-Ub), which was further increased to 95% in the presence of agonist (Fig. 26). Total receptor abundance was approximately the same in all untreated samples (*data not shown*). It is clear from these data that ubiquitination enhances the ability of β-arrestin 2 to mediate both constitutive and agonist-promoted down-regulation of the M_2 mAChR.

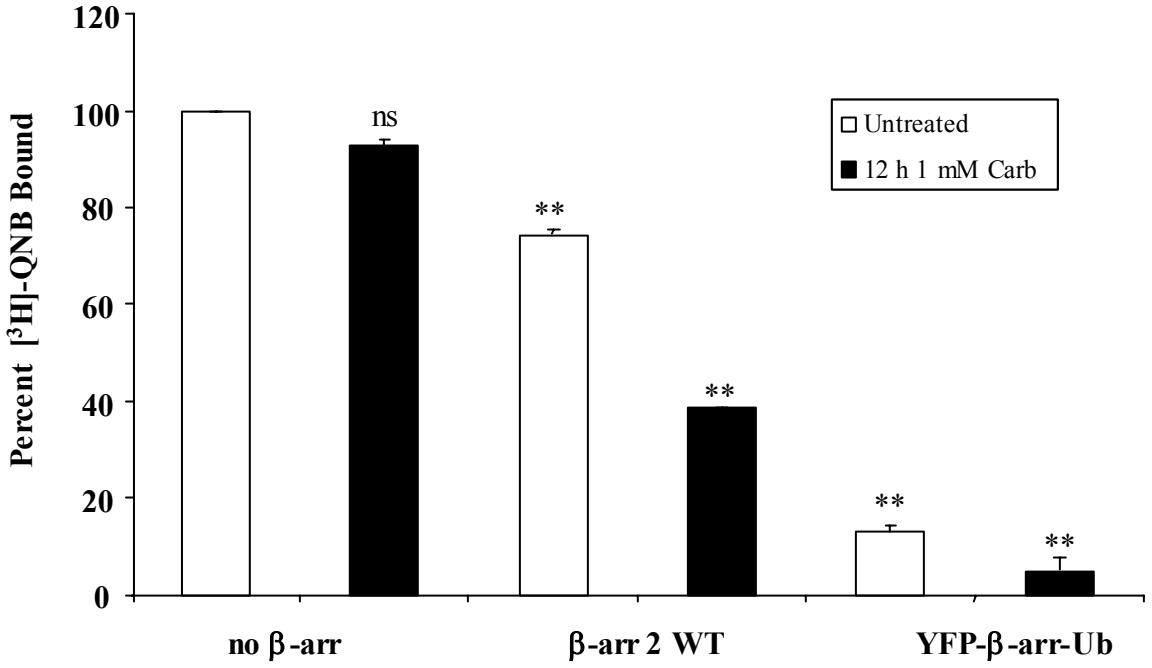

Fig. 26. Expression of a constitutively ubiquitinated form of β-arrestin 2 enhanced the agonist-promoted down-regulation of the M_2 mAChR in MEF KO1/2 cells. MEF KO1/2 cells were transfected with HA-M_2 mAChR and either FLAG-β-arrestin 2 or YFP-β-arrestin 2-Ub. 24 hr following transfection cells were treated with 1 mM carbachol for 12 hr. Down-regulation was determined using [^{3}H]-QNB binding (fmol/mg protein) in crude membranes as described in Materials and Methods. Total receptor abundance was decreased 26% with wildtype β-arrestin 2 alone, 62% in the presence of both β-arrestin 2 and agonist, 87% with β-arrestin 2-Ub alone, and 95% in the presence of both β-arrestin 2-Ub and agonist. Data are expressed as percent of [^{3}H]-QNB bound compared to the untreated control and are presented as mean ± standard deviation for two independent experiments with duplicate data points. Statistical analysis was performed using a repeated measures ANOVA with Bonferroni post test ;** indicates $p<0.001$ (compared to untreated control); ns indicates not significant.

Because ubiquitinated β-arrestin appeared to be involved in agonist-promoted degradation, we were interested in disrupting potential ubiquitination sites in the β-arrestin sequence. Several lysine residues on β-arrestin are known to be putative sites of ubiquitination [199]. In order to determine which residues are most important and to further confirm a role of ubiquitination in agonist-promoted down-regulation, we utilized two β-arrestin 2 mutants in which specific lysine residues are mutated to arginine so as to prevent ubiquitination at these sites. Therefore, MEF KO1/2 cells were transfected with HA-M_2 mAChR and either empty vector (control), FLAG-β-arrestin 2 (wild type), FLAG-β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ or FLAG-β-arrestin $2^{K11R,\ K12R}$ constructs. After 24 hr, cells were treated with and without 1 mM carbachol for 12 hr and extent of degradation was assessed using [^{3}H]-QNB. As shown previously, there was no down-regulation in the control cells lacking β-arrestin (Fig. 24 and 27). Both wild-type β-arrestin 2 (24%) and β-arrestin $2^{K11R,\ K12R}$ (27%) were able to mediate agonist-promoted down-regulation (Fig. 27). β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$, however, was not able to rescue agonist-promoted down-regulation (Fig. 26). Therefore, lysine residues at one or more of these sites in β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ appeared to be important in mediating agonist-promoted degradation of the M_2 mAChR.

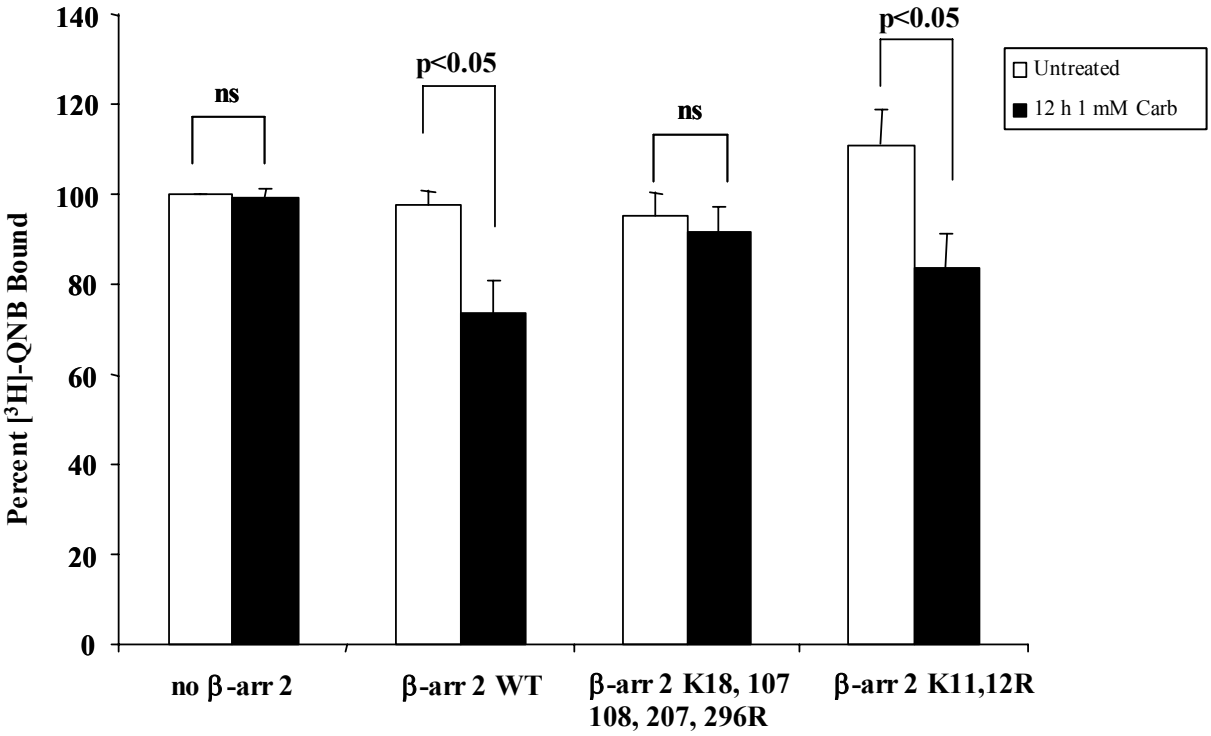

Fig. 27. Effect of expression of β-arrestin 2 lysine mutants on the agonist-promoted down-regulation of the M_2 mAChR in MEF KO1/2 cells. MEF KO1/2 cells were transfected with constructs encoding HA-M_2 mAChR and either empty vector (control), FLAG-β-arrestin 2 (WT), FLAG-β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ or FLAG-β-arrestin $2^{K11R,\ K12R}$. After 24 hr cells were treated with 1 mM carbachol for 12 hr. Down-regulation was determined in crude membranes (fmol/mg protein) as described in Materials and Methods. All β-arrestin 2 constructs were able to mediate agonist-promoted down-regulation except the β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ mutant. Data are expressed as percent of [^{3}H]-QNB bound compared to untreated control and presented as mean ± standard deviation for three independent experiments with duplicate or quadruplicate data points. Statistical analysis was performed using a repeated measures ANOVA with Bonferroni post test; ns indicates not significant.

We used [^{3}H]-NMS to measure internalization to ensure that the effect of β-arrestin 2 lysine mutants on down-regulation was not due to an effect on agonist-promoted internalization. After 24 hr, cells were treated with and without 1 mM carbachol for 1 hr. All β-arrestin 2 constructs (wild type and/or lysine mutants) were able to rescue agonist-promoted internalization in the MEF KO1/2 cells (Fig. 28). These data suggest that lysine residues at either one or more sites that have been mutated in β-arrestin 2$^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ is critical for receptor down-regulation but not for receptor internalization.

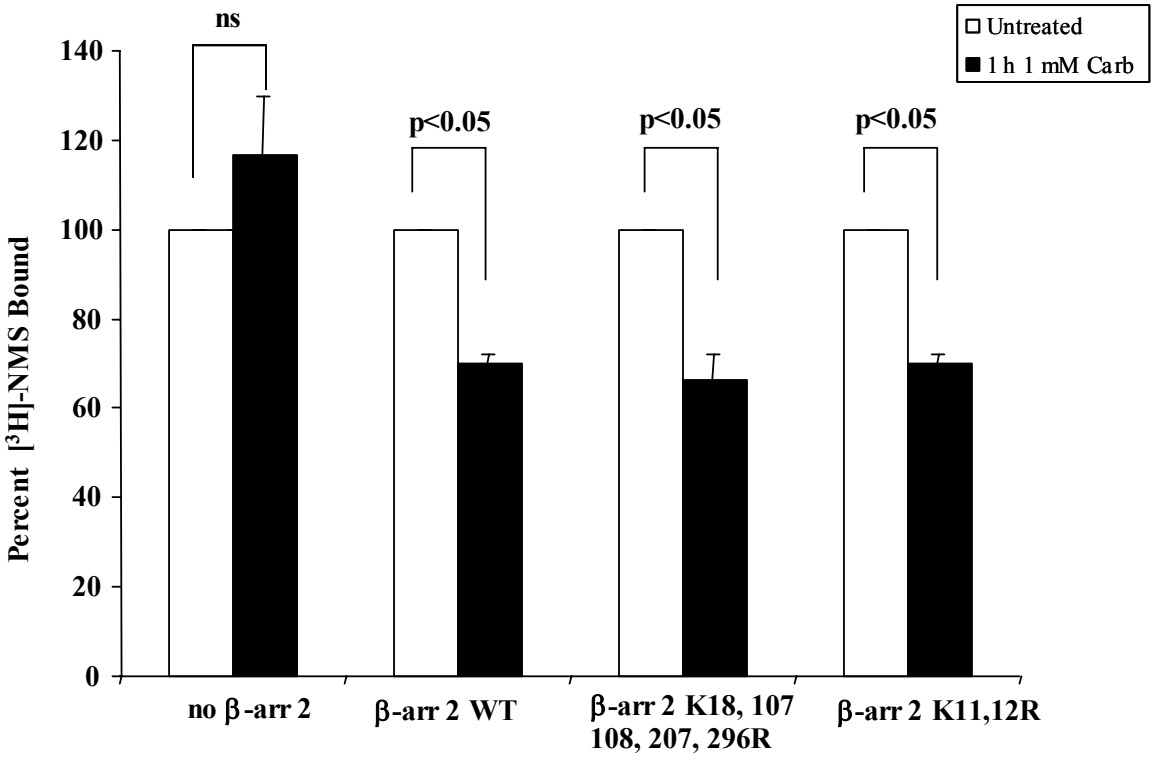

Fig. 28. Effect of expression of β-arrestin 2 lysine mutants on the agonist-promoted internalization of the M_2 mAChR in MEF KO1/2 cells. MEF KO1/2 cells were transfected with constructs encoding HA-M_2 mAChR and either empty vector (control), FLAG-β-arrestin 2 (WT), FLAG-β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ or FLAG-β-arrestin $2^{K11R,\ K12R}$. After 24 hr cells were treated with 1 mM carbachol for 1 hr. Sequestration was determined in whole cells as described in Materials and Methods. All constructs were able to rescue agonist-promoted sequestration. Data are expressed as percent of [^{3}H]-NMS bound compared to untreated control and presented as mean ± standard deviation for three independent experiments with duplicate data points. Statistical analysis was performed using a repeated measures ANOVA with Bonferroni post test; ns indicates not significant.

We have previously shown that agonist-promoted internalization of the M_2 mAChR is β-arrestin dependent and that the receptor exhibits a prolonged interaction with β-arrestin in perinuclear compartments [198]. Therefore, we wanted to determine whether these β-arrestin lysine mutants disrupted the stable β-arrestin/receptor complexes observed previously. To assess whether blockade of M_2 mAChR degradation is due to loss of receptor/β-arrestin complexes, we incubated MEF KO1/2 cells co-expressing FLAG-β-arrestin mutants and HA-M_2 mAChR with 1 mM carbachol for 10 hours and cells were processed for confocal microscopy. Prior to receptor activation, wild type and β-arrestin proteins remained cytosolic and uniformly distributed (*data not shown*). Upon 10 hour agonist treatment, internalized M_2 mAChR remained stably associated with β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ to a similar extent as that observed in the β-arrestin $2^{K11R,\ K12R}$ and wild type β-arrestin 2 cells as indicated by the yellow puncta (Fig. 29). Therefore, these data suggest that β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ maintains a high affinity for the M_2 mAChR thereby ruling out the possibility that this mutant converts the M_2 mAChR from a class B to a class A receptor.

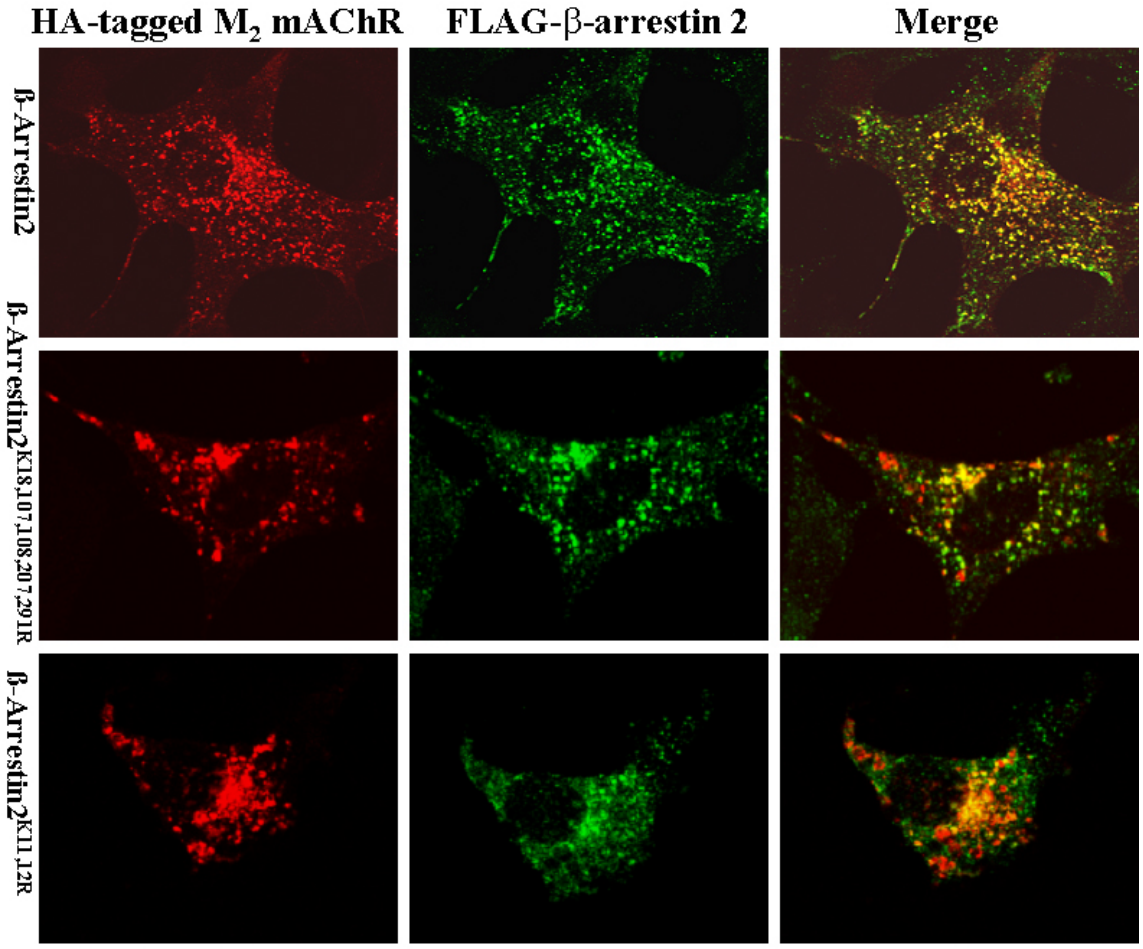

Fig. 29. β-arrestin $2^{K11R, K12R}$ and β-arrestin $2^{K18R, K107R, K108R, K207R, K296R}$ remain stably associated with internalized M_2 mAChRs in MEF KO1/2 cells. MEF KO1/2 cells were transiently transfected with plasmids encoding HA-M_2 mAChR and FLAG-β-arrestin $2^{K18R, K107R, K108R, K207R, K296R}$, β-arrestin $2^{K11R, K12R}$, or wild type β arrestin 2 and treated with 1 mM carbachol for 10 hours. Cells were processed for confocal immunofluorescence microscopy and co-localization (merge/yellow) was assessed using Rbanti FLAG (green) and Manti HA (red) primary antibodies followed by secondary antibodies. This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

Since expression of β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ blocked agonist-promoted degradation; we asked whether receptor entry into late endosomal/lysosomal compartments was altered. We have previously shown that internalized M_2 mAChRs partially co-localized with a marker of the late endosome, Rab7 GTPase (Fig. 21), which suggested that M_2 mAChRs undergo lysosomal-mediated degradation. To determine if the degradation of the M_2 mAChR was indeed lysosomal in nature and that β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ disrupted lysosomal targeting; we performed immunocytochemistry to examine the co-localization of the M_2 mAChR with the lysosomal membrane protein LAMP-1. MEF KO1/2 cells were transfected with constructs encoding HA-M_2 mAChR and either empty vector (control), FLAG-β-arrestin 2 (wild type), FLAG-β-arrestin $2^{K11R,\ K12R}$ or β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$. After 24 hr, cells were treated with and without 1 mM carbachol for 12 hr. There was partial co-localization of the receptor with LAMP-1 in the presence of wild type β-arrestin 2 or β-arrestin $2^{K11R,\ K12R}$ (Fig 30). It is clear, however, that expression of β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ significantly reduced the co-localization of the M_2 mAChR with LAMP-1 (Fig. 30). Since ~25% of receptors are degraded following 12 hr carbachol treatment we can assume that our observations are representative of the sub-population of total receptors localized to lysosomes. Taken together, the data suggest that β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ blocks receptor delivery to lysosomes for degradation while the stable interaction with receptor remains intact.

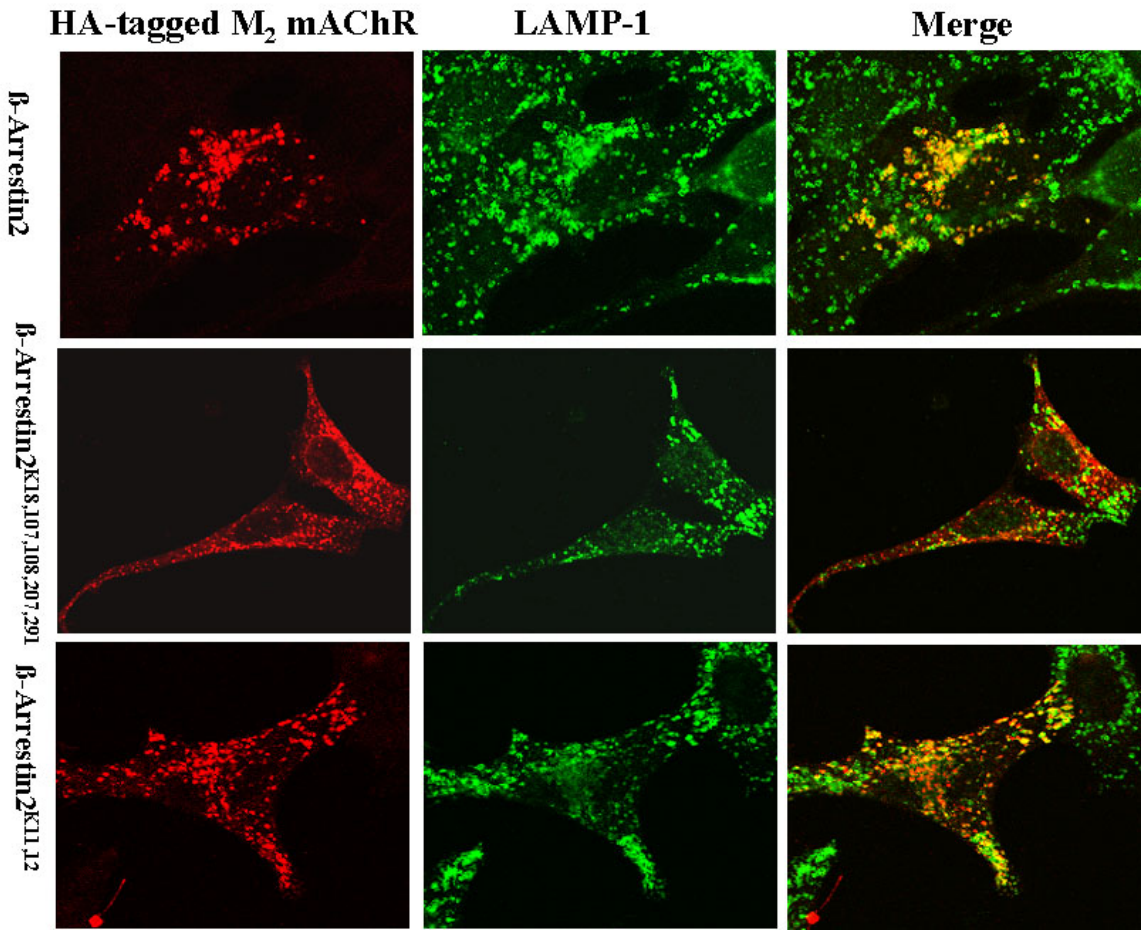

Fig. 30. Exogenous expression of β-arrestin 2$^{K18R, K107R, K108R, K207R, K296R}$ in MEF KO1/2 cells blocked delivery of M_2 mAChR to lysosomal compartments. MEF KO1/2 cells transiently expressing HA-M_2 mAChR and FLAG-β-arrestin constructs were treated for 12 hours with 1 mM carbachol and processed for confocol immunofluorescence microscopy. Cells were stained for HA-tagged M_2 mAChR (red) and the lysosomal marker, LAMP-1 (green). Merged image shows extent of overlap (yellow). This figure is representative of the results obtained in 3 independent experiments where more than 50 cells were examined.

M_2 mAChR Undergoes Agonist-Promoted Ubiquitination in a β-arrestin-Independent Manner

Although agonist induced ubiquitination of β-arrestin appears to be tightly linked to M_2 mAChR degradation, it is conceivable that M_2 mAChR itself may undergo agonist-induced ubiquitination on intracellular lysine residues and that this modification may serve as the signal for delivery and degradation in the lysosome. Indeed, it has been shown that activation of the CXCR4 [102], PAR_2 [103], NK_1R [47], and β_2-AR [85] induces ubiquitination of the receptor which is necessary for receptor sorting and degradation. Therefore, we asked whether M_2 mAChRs are modified with ubiquitin moieties following agonist stimulation. Serum-starved MEFwt cells expressing HA-M_2 mAChR were incubated with 1 mM carbachol at 0, 45, 90, and 180 minutes. Following stimulation, the receptor was immunoprecipitated and immunoblotted for the presence of ubiquitin and receptor via the HA-tag. As shown in Figure 31, in the absence and presence of agonist, significant levels of receptor ubiquitination were detected with antibody (P4D1) that recognizes both mono- and polyubiquitinated proteins. However, densitometric analysis revealed that the relative ratios of ubiquitination increased ~ 3-fold after 3 hrs of receptor stimulation when compared to untreated cells (Fig. 31). Since M_2 mAChR interacts with β-arrestin with high affinity, it is plausible that immunoprecipitation pulls down these complexes and we are observing β-arrestin ubiquitination. To rule out this possibility, we tested the immunoprecipitates from MEFwt cells co-expressing FLAG- β-arrestin 2 and HA-M_2 mAChR and found that modest levels of FLAG-β-arrestin 2 were pulled down with receptor (*data not shown*). Therefore, it is plausible that the ubiquitination pattern observed arises from both

receptor and β-arrestin 2 ubiquitination although we cannot rule out the presence of other protein partners that are ubiquitinated. Irrespective of this finding, the data suggest that although basal levels of receptor ubiquitination are evident, agonist-induced increases in receptor ubiquitination are observed. This modification could potentially play a role in the delivery and degradation of the M_2 mAChR in the lysosome.

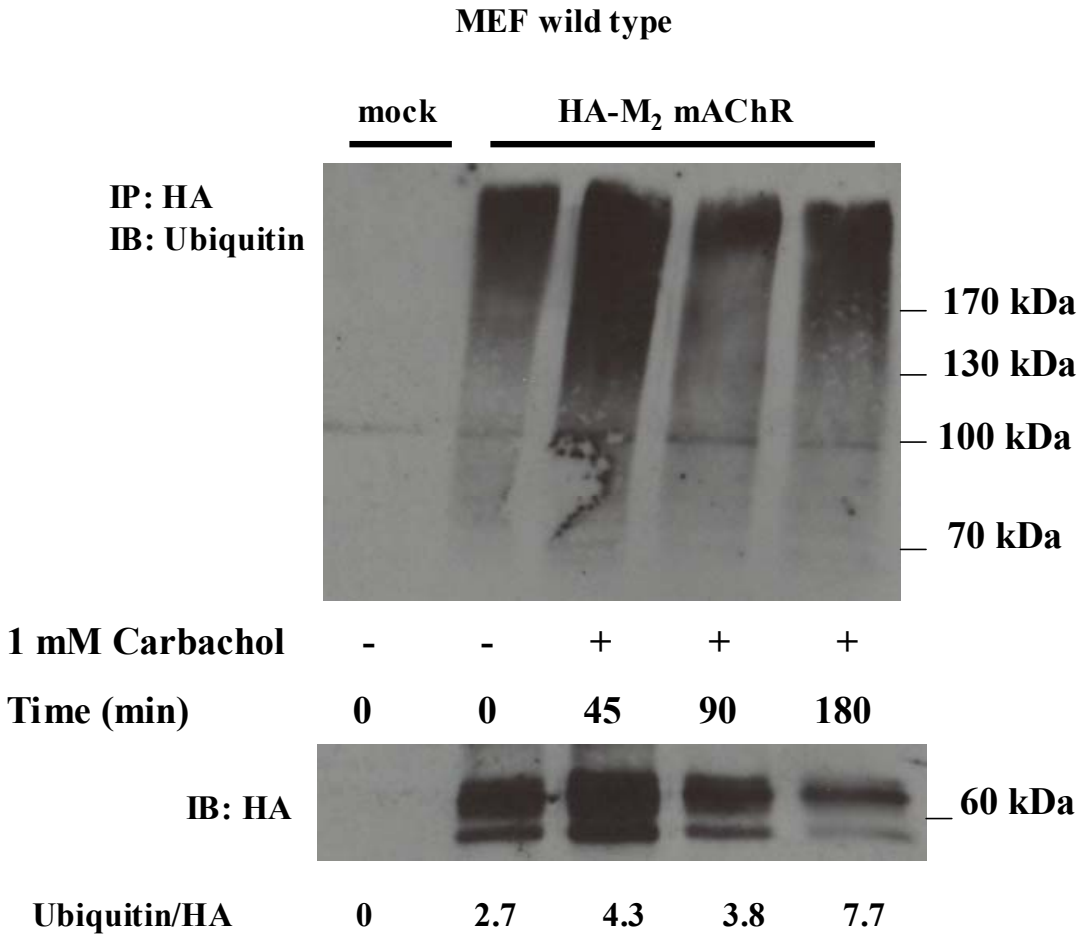

Fig. 31. Agonist addition promotes further ubiquitination of M_2 mAChR. MEF wt cells were transfected with a construct encoding HA-tagged M_2 mAChR and following 24 hrs post-transfection cells were serum starved for 1 hr prior to 1 mM carbachol treatment for various time points. Equal amounts of cell lysates were immunoprecipitated (~600 μg of protein) with anti-HA affinity matrix, resolved on a 7% polyacrylamide gel, and transblotted onto nitrocellulose. **Top panel**: Blots were probed with an anti- ubiquitin antibody (PD41). **Bottom panel**: lysates were blotted with a monoclonal anti-HA antibody, demonstrating receptor levels. Shown is a representative blot from two independent experiments. Numbers under blot correspond to the densitometric quantification of ubiquitin band intensity normalized against the corresponding densitometric value of receptor.

Because receptor ubiquitination can contribute to receptor degradation, we examined whether β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ affected ubiquitination of stimulated receptors. The rationale behind examining β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ effects on receptor ubiquitination resides in the fact that the V_2R and β_2-AR require β-arrestin 2 to mediate their ubiquitination in response to agonist [85, 86]. Moreover, ubiquitination of these receptors is required for efficient degradation. To examine this question, MEF KO1/2 cells were co-transfected with constructs encoding HA-tagged M_2 mAChR and FLAG-tagged β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$, FLAG-tagged β-arrestin 2wt, or receptor alone. Cells were treated with and without 1 mM carbachol for 30 min and the extent of receptor ubiquitination was assessed using immunoprecipitation and subsequent western blotting analysis. Immunoblot analyses revealed that basal ubiquitination is evident in cells co-expressing HA-M_2 mAChR and β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ that increase ~2-fold following receptor stimulation (Fig. 32, far right). This suggests that β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ can mediate agonist-induced increases in ubiquitination of the M_2 mAChR; therefore, β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ does not alter the ubiquitination state of the M_2 mAChR. However, the control experiments conducted alongside β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ samples revealed interesting results. Receptor expressed alone in the MEF KO1/2 cells exhibited basal levels of ubiquitination that did not increase following receptor stimulation suggesting that the M_2 mAChR is basally ubiquitinated in a β-arrestin 2 independent manner (Fig. 32, far left). As mentioned above, this finding is in sharp contrast to previous reports. Introduction of β-arrestin 2wt, however, lowered basal levels of receptor ubiquitination that increased ~3 fold following receptor stimulation (Fig. 32,

middle). Collectively, this result suggests that M_2 mAChR is basally ubiquitinated in a β-arrestin independent manner while increases in ubiquitination require β-arrestin expression. Whether receptor ubiquitination serves as a signal for lysosomal targeting requires further investigation.

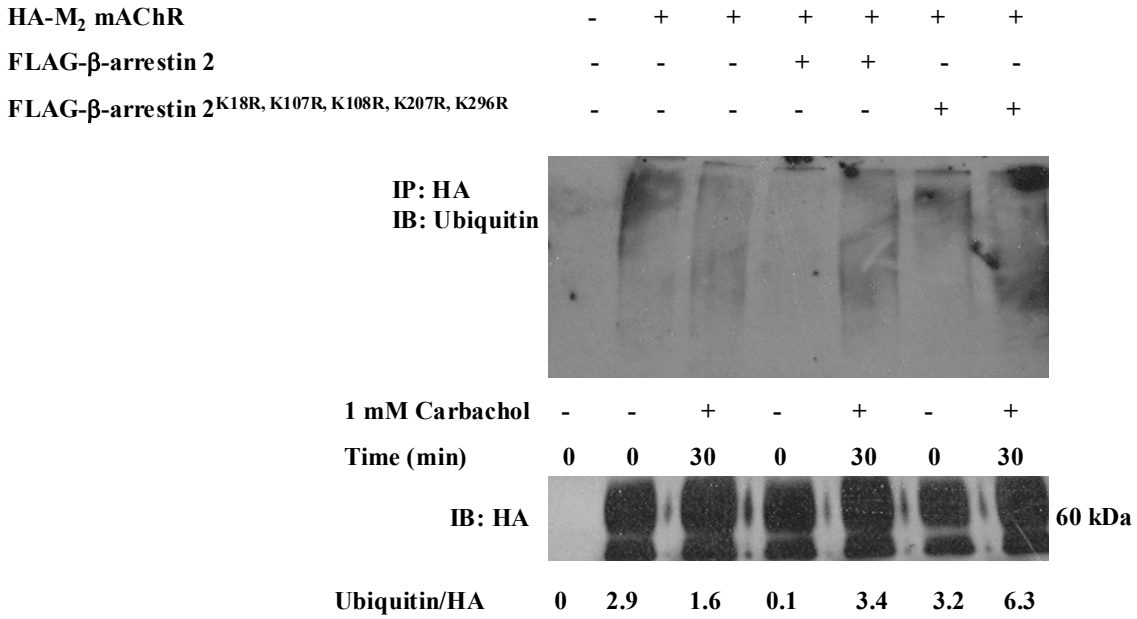

Fig. 32. β-arrestin $2^{K18R, K107R, K108R, K207R, K296R}$ did not prevent agonist promoted ubiquitination of the M_2 mAChR. MEF KO1/2 cells were transfected with HA-tagged M_2 mAChR and FLAG-tagged β-arrestin $2^{K18R, K107R, K108R, K207R, K296R}$ or FLAG-tagged β-arrestin 2 wild type. Cells were treated with or without 1 mM carbachol for 30 minutes. Receptor was immunoprecipitated and immunoblotted for ubiquitin (upper panel) and HA epitope (bottom panel). Relative ubiquitination levels are reported below bottom panel. Shown is a representative blot from four independent experiments.

Inhibition of Proteasome Blocks M_2 mAChR Down-Regulation by Preventing β-arrestin Ubiquitination

Since ubiquitin appears to serve as a signal for protein degradation [196], we asked whether disruption of the ubiquitin/proteasome pathway would affect agonist-promoted degradation of the M_2 mAChR. We examined agonist-promoted down-regulation of the M_2 mAChR in the presence of lactacystin with the intent to block proteasome dependent recycling of ubiquitin thereby indirectly depleting available ubiquitin pools in the cell [200, 201]. Therefore, a reduction in the levels of free ubiquitin would affect the ubiquitination state of target proteins. To address this question, MEFwt cells expressing HA-tagged M_2 mAChR were pretreated with and without 10 μM lactacystin for 20 minutes prior to agonist exposure. In control cells, following 4 hrs of carbachol treatment, M_2 mAChR underwent agonist-promoted degradation by ~30% (Fig. 33). In cell pretreated with lactacystin, agonist-promoted degradation of the receptor was abolished (Fig. 33) suggesting that ubiquitin and/or a proteasome-dependent pathway is involved in agonist-promoted degradation of the M_2 mAChR.

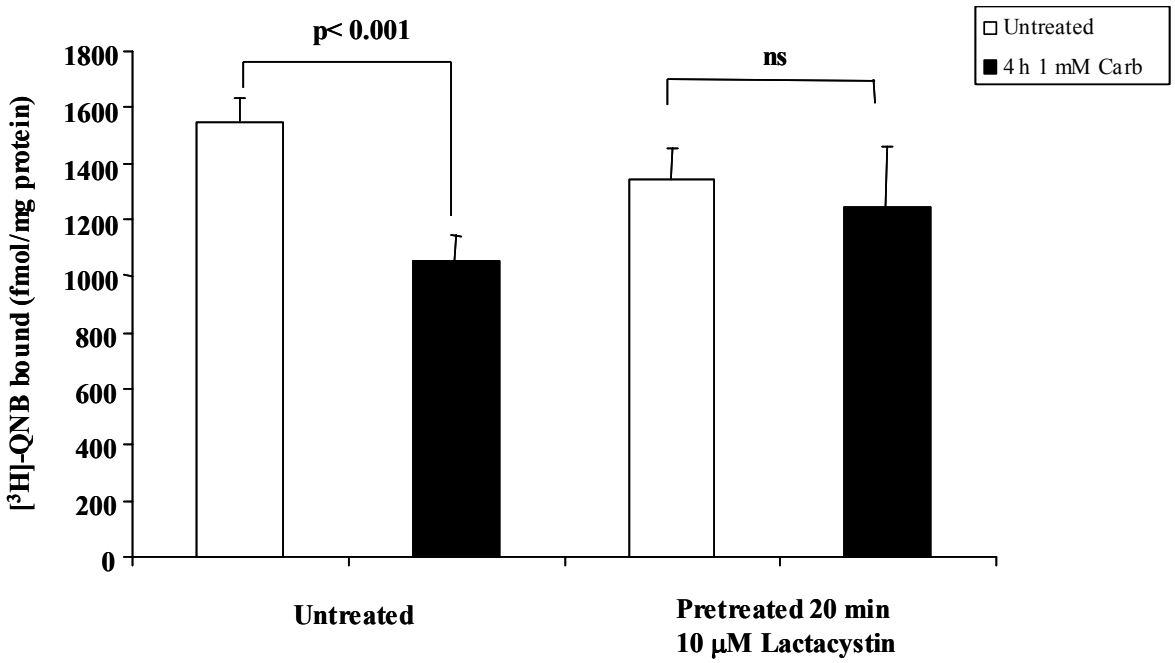

Fig. 33. The proteasome inhibitor lactacystin interfered with agonist-promoted down-regulation of the M_2 mAChR in MEFwt cells. MEFwt cells were transfected with a construct encoding HA-M_2 mAChR, and after 24 hr cells were incubated for 20 min with or without 10 μM lactacystin, then treated with 1 mM carbachol for 4 hr. Lactacystin inhibited the β-arrestin 2 mediated down-regulation of the M_2 mAChR receptor in response to agonist. Down-regulation was determined using [^{3}H]-QNB binding in crude membranes as described in Materials and Methods. Data are expressed as [^{3}H]-QNB bound (fmol/mg total protein) and are presented as mean ± standard deviation from three independent experiments with duplicate data points. Statistical analysis was performed using a paired t-test; ns indicates not significant.

Since MG 132, another inhibitor of proteasome activity, strongly blocked agonist-induced endocytosis of the β_2-AR [85], we also determined whether or not the effects of lactacystin were occurring at the level of receptor internalization. We examined the effect of lactacystin on the agonist-promoted internalization of M_2 mAChR using the membrane impermeable ligand [^{3}H]-NMS. MEFwt cells were transfected with plasmid encoding HA-M_2 mAChR, and after 24 hr cells were incubated for 20 min in the absence or presence of 10 μM lactacystin, then treated with 1 mM carbachol for 30 min. Pretreatment with lactacystin had no effect on agonist-promoted internalization (Fig. 34) suggesting that the ability of lactacystin to block receptor degradation was not occurring at the level of internalization.

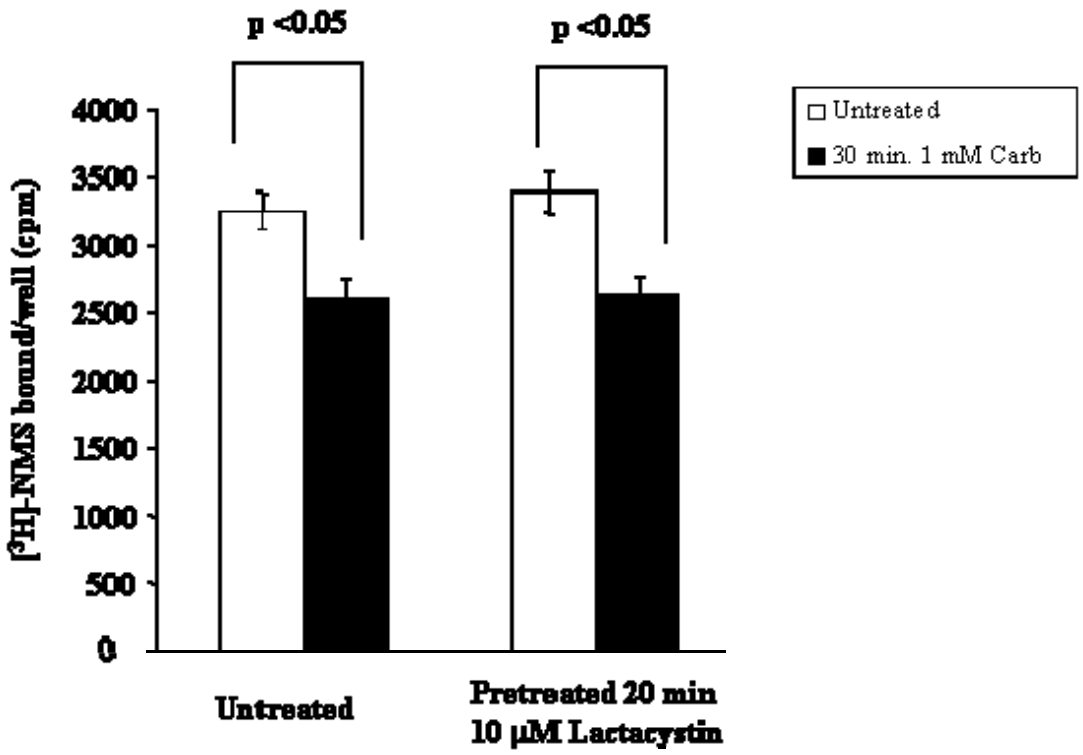

Fig. 34. Agonist-mediated internalization of M_2 mAChR is not impaired following pretreatment with proteasomal inhibitors. MEFwt cells were transfected with a construct encoding HA-M_2 mAChR and after 24 hr cells were incubated for 20 min with or without 10 μM lactacystin then treated with 1 mM carbachol for 30 min. The presence of lactacystin had no effect on the agonist-promoted internalization of the M_2 mAChR. Internalization was determined using [^{3}H]-NMS binding in whole cells as described in Materials and Methods. Data are expressed as cpm [^{3}H]-NMS bound per well (plated at $1x10^5$ cells) and presented as mean ± standard deviation from three separate experiments with duplicate data points. Statistical analysis was performed using a paired t-test.

Our data suggested that ubiquitination of β-arrestin or M_2 mAChR promotes degradation of the M_2 mAChR. Disruption of the proteasome/ubiquitin system involved in maintaining ubiquitin levels blocked receptor degradation. Therefore, we next determined the ubiquitination status of HA-tagged M_2 mAChR as well as FLAG-β-arrestin in the presence of proteasome inhibitors. To address this question, we pretreated MEFwt cells expressing HA-M_2 mAChR and FLAG-β-arrestin 2 with 50 μM MG 132 or 20 μM lactacystin before carbachol stimulation. Receptor was immmunoprecipitated and assayed for agonist-stimulated ubiquitination. Although preliminary, both treatments appeared to enhance ubiquitination of the receptor in both stimulated and unstimulated conditions as compared to Figure 31. We see that proteasome inhibitor treatment increased the relative ratio of ubiquitination (~10.4) to the same extent as receptor stimulation for 3 hr with carbachol (~7.7) (Fig. 31 and 35). Interestingly, lactacystin treatment promoted ubiquitination in a ligand-independent manner while agonist-promoted ubiquitination of the M_2 mAChR was retained upon exposure to MG 132 (Fig. 35). In contrast, preliminary data reveal that pretreatment of lactacystin strongly diminished basal and stimulated levels of β-arrestin 2 ubiquitination (Fig. 36). Comparison to Figure 25 reveals that the relative ratio of ubiquitination of FLAG-β-arrestin 2 decreased from ~0.3 to ~0.04 upon pretreatment with lactacystin. The relative ratio remained low even after agonist-stimulation (Fig. 36). This experiment should be repeated in conjunction with untreated cells to confirm that this observation is valid. To ensure that β-arrestin and M_2 mAChR association remained intact, we visualized the extent of co-localization in lactacystin-treated cells using indirect immunofluoroscence. We observed extensive overlap of the M_2 mAChR and β-arrestin 2 in discrete punctate

vesicles following 30 min stimulation (*date not shown*). These findings, although preliminary, suggest that β-arrestin ubiquitination may potentially play an important role in mediating down-regulation of the M_2 mAChR.

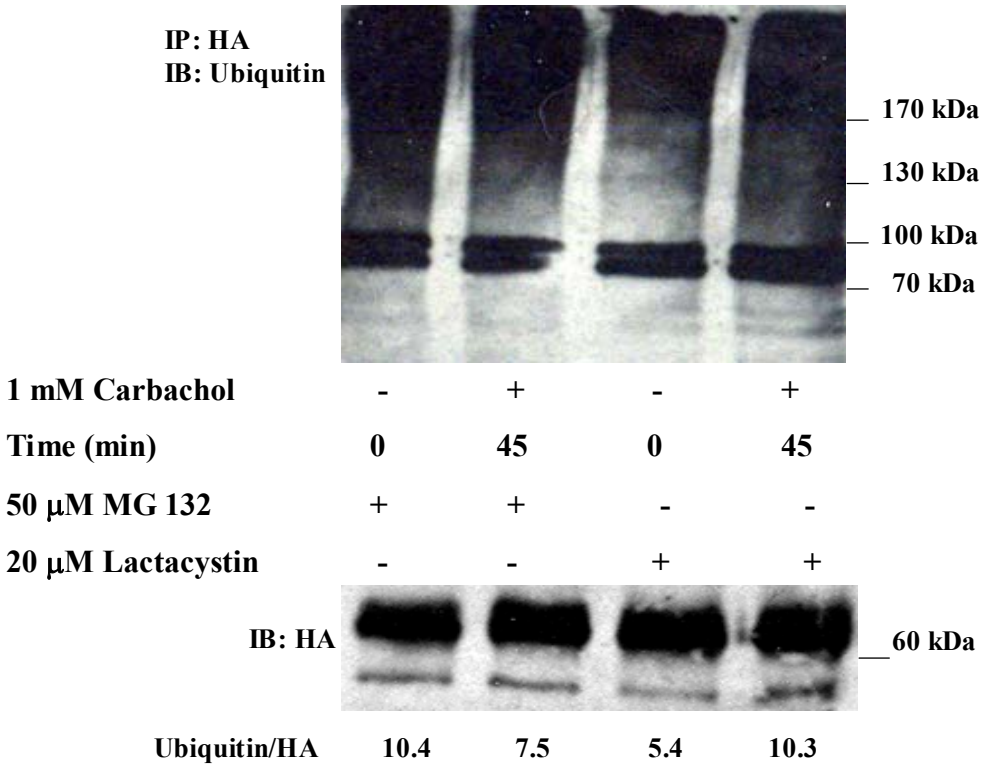

Fig. 35. Inhibition of the proteasome/ubiquitin pathway enhances agonist-promoted ubiquitination of the M_2 mAChR. Serum starved MEFwt cells expressing HA-tagged M_2 mAChR were incubated with or without 50 μM MG 132 or 20 μM lactacystin for 2 hrs before treatment with 1 mM carbachol for 0 and 45 minutes. Receptor was immunoprecipitated and immunoblotted for ubiquitin (upper panel) and HA epitope (bottom panel). Western blot is representative of two independent experiments.

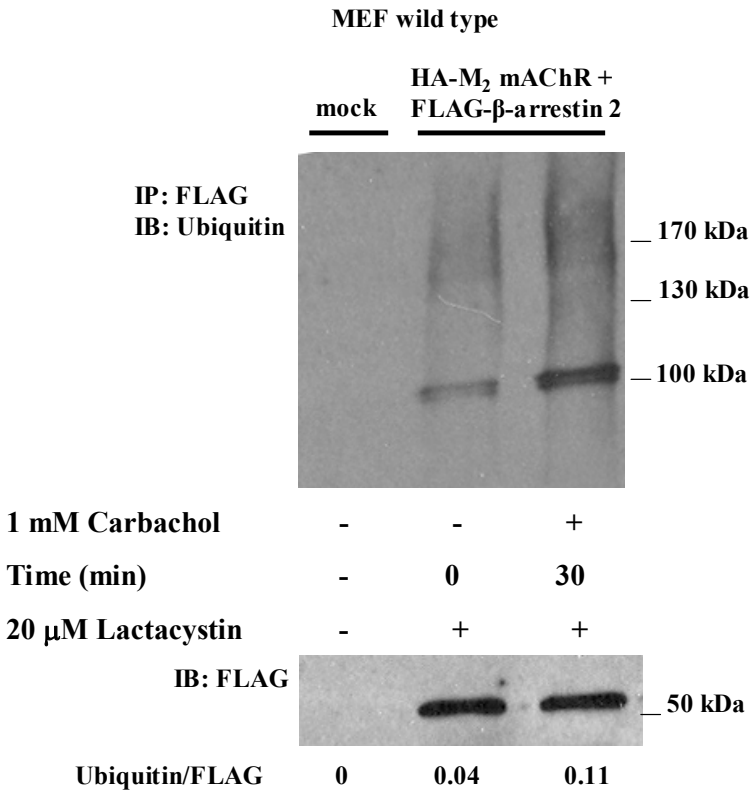

Fig. 36. Inhibition of the proteasome/ubiquitin pathway by addition of lactacystin significantly reduces FLAG-β-arrestin 2 ubiquitination. Serum starved MEF wt cells co-expressing HA-M_2 mAChR and FLAG-β-arrestin 2 were incubated with or without 20 μM lactacystin for 2 hrs prior to 1 mM carbachol treatment for 0 and 30 minutes. FLAG-β-arrestin 2 was immunoprecipitated using anti-FLAG agarose and immunoblotted for ubiquitin (upper panel) and FLAG epitope (bottom panel). Western blot is representative of one experiment.

CHAPTER 9

DISCUSSION

The role of β-arrestin in mediating agonist-promoted internalization and post-endocytic trafficking of the M_2 mAChR has been unclear and inconclusive. Initial reports suggested that M_2 mAChR utilized both β-arrestin-dependent and -independent pathways upon exposure to agonist [153, 156]. Other work suggested that Arf6 GTPase was important in regulating agonist-promoted endocytosis of the receptor [26, 27]. However, our recent work performed in MEF cells lacking both isoforms of β-arrestin has demonstrated that agonist-promoted endocytosis of the M_2 mAChR is β-arrestin-dependent and that the receptor remains bound to β-arrestin in endosomes [198]. Accordingly, the present study extends the observations of previous work and examines the role of ubiquitination of β-arrestin in the agonist-promoted down-regulation of the M_2 mAChR.

Our first set of experiments demonstrated that M_2 mAChRs undergo time- and β-arrestin-dependent down-regulation in response to agonist. All four cell lines displayed a similar down-regulation between 6 and 12 hr. The MEF KO1 cell line, which expresses only endogenous β-arrestin 2, reproducibly showed a recovery period late in the time course, which can easily be ascribed to new receptor synthesis whereas MEF KO2 cells, which express only endogenous β-arrestin 1, did not display any type of recovery in total receptor abundance at the same time points. This difference suggests an ability of the M_2

mAChR to differentiate between the two subtypes of β-arrestin, particularly under chronic stimulation.

The M_2 mAChR displayed a similar β-arrestin 2 preference in the down-regulation rescue experiments in MEF KO1/2 cells. Both isoforms of β-arrestin were able to rescue down-regulation but there was selectivity, demonstrated by more down-regulation via both the agonist and constitutive (agonist-independent) pathways seen with β-arrestin 2 vs. 1. Although not statistically significant these differences may reflect a preferential interaction of the M_2 mAChR with the β-arrestin 2 isoform under chronic conditions. However, *in vitro* binding experiments [152] and *in vivo* β-arrestin translocation experiments [82] were not able to detect a difference in binding affinity between β-arrestin 1 and 2 for the M_2 mAChR. Additionally, class B receptors have been shown to form high affinity and prolonged interactions with both β-arrestin 1 and 2 [82].

A hallmark of class B receptors is that β-arrestin proteins stably associated with the receptor exhibit a sustained ubiquitination pattern [87]. This stable ubiquitination correlates with the affinity of receptor/β-arrestin complexes since deubiquitination of β-arrestin leads to rapid disassociation of this complex. In agreement with this study, we previously showed that M_2 mAChRs form stable complexes with arrestin within discrete intracellular microcompartments for prolonged periods of time [198]. Herein, we demonstrated carbachol-induced ubiquitination of β-arrestin 2 that displayed a slower onset (15 min) that further increased over time. The time course of ubiquitination of β-arrestin 2 in response to muscarinic stimulation was quite different from that observed following stimulation of the β_2-AR, where β-arrestin 2 is ubiquitinated rapidly (within 1 min) and transiently, showing prompt deubiquitination within 15 minutes [85]. These

data combined with the observations of Jones *et al.* [198] suggest that the M_2 mAChR behaves as a class B receptor.

Once we had established that β-arrestin is gradually ubiquitinated via activation of M_2 mAChR, we examined whether constitutive ubiquitination of β-arrestin enhances its ability to mediate agonist-promoted down-regulation. Shenoy and Lefkowitz have previously demonstrated that co-expression of a constitutively ubiquitinated β-arrestin 2 chimera with β_2-AR converted the β_2-AR from a class A to a class B receptor and significantly enhanced agonist-promoted down-regulation under chronic conditions [87]. In accordance with this finding, M_2 mAChR levels were nearly ablated in the presence of β-arrestin 2-Ub compared to wild type β-arrestin 2. It was clear from these data that constitutive ubiquitination of β-arrestin greatly increased its ability to mediate down-regulation of the M_2 mAChR. These data support the role of ubiquitinated β-arrestin in promoting agonist-mediated M_2 mAChR down-regulation, but the question remained as to which lysine residue(s) of β-arrestin are ubiquitinated. Shenoy and coworkers have generated mutants of β-arrestin 2 at two lysines near the amino terminus (β-arrestin $2^{K11R, K12R}$) as well as other sites (β-arrestin $2^{K18R, K107R, K108R, K207R, K296R}$) [199]. Wild type β-arrestin 2 is stably ubiquitinated upon stimulation of the AT_{1a} receptor, a prototypic class B receptor. However, when co-expressed with β-arrestin $2^{K11R, K12R}$, stimulation of the $AT1_a$ receptor resulted in transient β-arrestin 2 ubiquitination and transient association with the receptor, thereby converting the receptor into a class A type. This same mutant had no effect on endosomal recruitment of β-arrestin to other class B receptors, which included the V_2R and NK_1R [199]. Since these β-arrestin mutants affected trafficking patterns of β-arrestin in complex with various GPCRs, we were interested in determining

the functional consequences of these mutations on trafficking of the M_2 mAChR itself. We found that exogenous expression of β-arrestin $2^{K11R,\ K12R}$ had no effect on agonist-promoted endocytosis, down-regulation, or complex formation with the M_2 mAChR. In contrast, the β-arrestin $2^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ mutant, shown to impair association of V_2R with β-arrestin on endosomes [199], significantly reduced M_2 mAChR degradation while internalization and stable association remained unaltered. Our data are consistent with Shenoy's conclusion [199] that the trafficking of different GPCRs is dictated by the ubiquitination state of β-arrestin on specific lysines, which is determined by distinct receptor bound conformation states of β-arrestin. The resulting ubiquitination state of β-arrestin appears to have different consequences for different GPCRs as in the case of the M_2 mAChR.

However, what is unclear is whether receptor sorting to the degradative pathway arises from ubiquitination of β-arrestin and/or the receptor, itself. Indeed, recent studies of CXCR4, β_2-AR, PAR_2, PAF, NK_1R, and V_2R receptors provided evidence that agonist-induced ubiquitination of the receptor is essential for lysosomal or proteasomal targeting and degradation [85, 86, 102-104]. Our results showed that ubiquitination of M_2 mAChR was detected under unstimulated conditions and increased over time. This is in partial agreement with previous findings, which showed an agonist-independent ubiquitination of the PAF receptor [104]. Because ubiquitination of the receptor increased with time, the prospect of additional lysine residues modified with ubiquitin is considerable. This ubiquitin modification may participate in the pathway that leads to degradation as reported with other GPCRs. To establish a role for lysine and ubiquitin in receptor degradation, attempts to engineer mutant receptors lacking lysine residues in the

third intracellular loop were made. The M_2 mAChR mutants, HA-M_2 mAChR$^{K375R,\ K376R,\ K377R,\ K383R}$ and HA-M_2 mAChR$^{K383R,\ K384R}$, were found to localize in intracellular compartments that did not traffick to the cell surface (*data not shown*). These mutants were not studied further. Thus, we cannot rule out the possibility that ubiquitination of the M_2 mAChR facilitates receptor sorting to the lysosome.

Since ubiquitination of receptor may be important for degradation, we were interested to see whether β-arrestin 2$^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ had any effect on the ubiquitination state of the receptor. It has been shown that agonist-promoted ubiquitination of the V_2R and β_2-AR requires β-arrestin 2 but not β-arrestin 1 [85, 86]. Our results indicated that ubiquitination of the receptor is not affected when expressed in MEF KO1/2 cells suggesting that the receptor is constitutively ubiquitinated in a β-arrestin-independent manner. This is in sharp contrast to previous reports [85, 86]. Moreover, the ubiquitination state of the receptor increases following receptor stimulation in the presence of both β-arrestin 2$^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$ and β-arrestin 2wt suggesting that arrestin is required for agonist-induced increases in receptor ubiquitination and that receptor ubiquitination is not affected by the expression of β-arrestin 2$^{K18R,\ K107R,\ K108R,\ K207R,\ K296R}$. Since ubiquitination of endogenous β_2-AR was analyzed in MEF cells, it is feasible that we are observing an artifact of heterologous overexpression system. However, Martin and coworkers showed that ubiquitination of exogenously expressed V_2R does not occur in MEF KO1/2 cells but is restored when expressed in MEF KO1 cells [86]. Therefore, we may have a case where basal ubiquitination exists. Interestingly, we still observe a dose-dependent increase in

ubiquitination of the M_2 mAChR in MEFwt cells as well as MEF KO1/2 cells co-expressing β-arrestin 2.

To further characterize the mechanism of agonist-promoted down-regulation of M_2 mAChR, we employed the use of a proteasomal inhibitor lactacystin, a specific inhibitor of the 26 S proteasome that functions by covalently modifying the active site and inhibiting the enzymatic activity of the proteasome [196]. Because inhibition of the proteasome inhibits recycling of cellular ubiquitin, this results in depletion of available ubiquitin pools that could indirectly affect the ability of other substrates to be modified with ubiquitin. Lactacystin was able to completely block agonist-promoted down-regulation with no effect on agonist-promoted sequestration. Upon further analysis, we found that while ubiquitination levels of M_2 mAChR were slightly enhanced, β-arrestin 2 ubiquitination was drastically reduced. Accumulation of ubiquitinated M_2 mAChR in cells treated with lactacystin and MG 132 is consistent with previous findings suggesting that preventing receptor down-regulation leads to the stabilization of ubiquitinated receptor species [86, 197, 202]. It is possible that inhibition of receptor degradation by proteasome inhibitors arises from either an associated regulatory protein, normally degraded by the proteasome, which becomes stabilized so as to prevent receptor sorting and degradation, or that depletion of available ubiquitin prevents the conjugation of regulatory proteins with ubiquitin that is known to be a factor in proper sorting. The latter is consistent with our preliminary finding in that β-arrestin 2 does not undergo agonist-promoted ubiquitination in the presence of lactacystin. However, it is plausible that protein normally degraded by the proteasome may obscure the ubiquitination signal of β-arrestin 2 in the presence of lactacysin. One approach would be to analyze

immunoprecipitation eluents for changes in protein interaction using 2D-gel analysis. Further investigation is also required to establish that ubiquitin pools are in fact depleted by testing the stability of a known proteasome target. Moreover, caution must be employed when intrepretating these results since a dose dependent effect of lactacystin should be included to analyze agonist-promoted M_2 mAChR degradation, internalization, and ubiquitination. Thus, these preliminary results suggest that the mechanism behind lactacystin blockade of M_2 mAChR degradation may involve the prevention of β-arrestin ubiquitination. Collectively, our data may support the notion that ubiquitin modification of β-arrestin provides the signal for receptor sorting to the lysosome.

Many studies indicate that post-endocytic sorting of cargo to the lysosome involves interactions of ubiquitin with sorting machinery such as hepatocyte growth factor-regulated tyrosine kinase (HRS) [56, 62]. To successfully enter into the degradative pathway (multivesicular bodies/late endosomes), cargo is required to be ubiquitinated, which serves to interact with sorting proteins. It has been shown that M_2 mAChR activation in neurons leads to their redistribution into multivesicular bodies [164]. Therefore, it is conceivable that β-arrestin/receptor complexes interact with lysosomal sorting machinery at the site of mulitivesicular bodies via ubiquitinated β-arrestin. Other mechanisms may also be involved in the lysosomal targeting of the M_2 mAChR which include association between receptor and proteins such as G-protein-coupled receptor-asscociated sorting protein (GASP) and sorting nexin-1 (SNX-1) [41-43]. These proteins are shown to bind to the C-terminal tails of receptors to facilitate lysosomal sorting. Whether or not arrestin or M_2 mAChR interacts with sorting machinery remains to be determined.

We conclude that agonist-promoted down-regulation of M_2 mAChR is β-arrestin-dependent and is modulated by the ubiquitination state of β-arrestin, which specifically targets the receptor for degradation in lysosomes. Muscarinic stimulation leads to ubiquitination of β-arrestin 2 and this ubiquitination enhances the agonist-promoted down-regulation of the receptor. Finally, where it has been suggested that class A receptor down-regulation proceeds primarily via receptor ubiquitination, these data imply that ubiquitination at specific lysine residues on β-arrestin 2 are critical in mediating the down-regulation of the class B M_2 mAChR.

CHAPTER 10

PERSPECTIVE AND FUTURE DIRECTIONS

Regulation of the M_2 mAChR

The first study of this thesis provides evidence to support the notion that agonist-induced internalization of the M_2 mAChR is β-arrestin- and clathrin-dependent in MEF cells. However, the role of clathrin in mediating receptor sequestration may depend on the cell line expressed. Moreover, expression of M_2 mAChR in multiple cell lines led to a stable interaction with β-arrestin in early endosomal compartments. This suggests that β-arrestin may be facilitating receptor recycling or degradation kinetics given its role in these processes for other GPCRs. Additionally, β-arrestin may serve as an intracellular signaling scaffold to intiate a second wave of signaling which may give rise to a M_2 mAChR-dependent cellular response. Following internalization, the fate of M_2 mAChR appears to involve Rab GTPases, known regulators of cargo trafficking.

In the second study of this thesis, M_2 mAChR regulation is further extended to include a role for β-arrestin in agonist-induced down-regulation. Lysosomal degradation of the M_2 mAChR appears to be dependent on the ubiquitination state of β-arrestin since expression of a mutant form of β-arrestin 2 lacking five lysine residues abolished agonist-promoted down-regulation of the M_2 mAChR. Similarly, blockade of receptor degradation in the presence of a proteasome inhibitor, lactacystin, may perhaps be explained by its effect on diminishing β-arrestin 2 ubiquitination. In contrast, constitutive ubiquitination of β-arrestin 2 strongly induced basal and agonist-promoted

down-regulation. The hypothesis is that M_2 mAChR/β-arrestin 2 complexes undergo lysosomal sorting via interactions between ubiquitin on β-arrestin 2 and ubiquitin-dependent sorting machinery at the site of multivesicular bodies/late endosomes. Disruption of β-arrestin 2 ubiquitination traps the receptor in unknown microcompartments so that lysosome-mediated degradation does not occur. The functional consequence of this process remains to be determined. Given the findings from this study, a proposed mechanism of M_2 mAChR regulation is provided (Fig. 37).

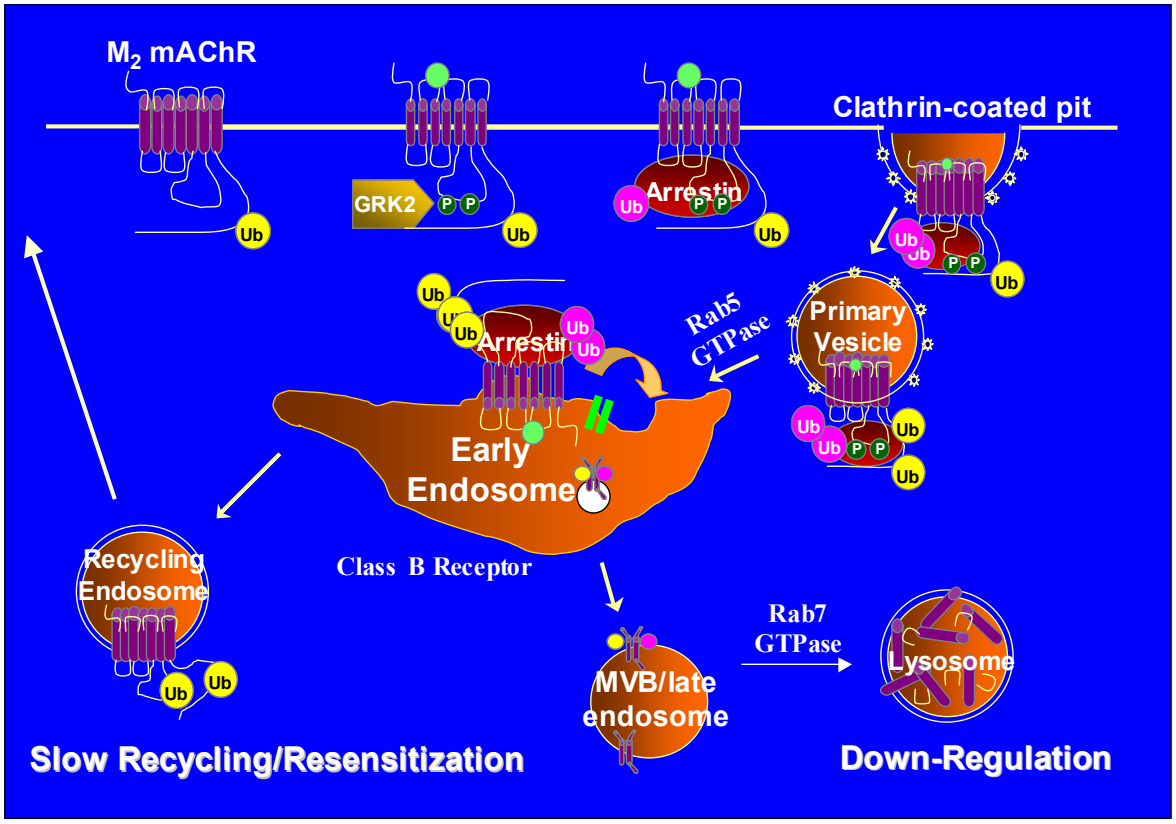

Fig. 37. Proposed model summarizing internalization and endocytic sorting of the M_2 mAChR. Stimulation of the M_2 mAChR leads to phosphorylation at specific serine/threonine residues in the third intracellular loop by GRK2 resulting in β-arrestin translocation and binding to the receptor. Concurrently, β-arrestin and M_2 mAChR undergo an enhanced modification with ubiquitin moieties (yellow and pink). Following internalization in a β-arrestin- and clathrin-dependent manner, M_2 mAChR/β-arrestin complexes are delivered to the early endosome via activation of Rab5 GTPase. Ubiquitin proteins conjugated to β-arrestin or M_2 mAChR may then interact with sorting machinery such as HRS (green) for delivery to multivesicular bodies/late endosomes. The cargo is then delivered to the lysosome via activation of Rab7 GTPase. Presumably, receptors that do not enter the degradative pathway are delivered to recycling compartments where recovery to the cell surface occurs very slowly.

The two studies presented in this thesis have broadened our knowledge regarding the regulation of M_2 mAChR intracellular trafficking and the role for β-arrestin in regulating this process, which has been contentious in the literature. Moreover, although β-arrestin has been implicated in promoting down-regulation of class B GPCRs, a mechanism for this process has not been fully established. Here, we provide evidence to support a mechanism that includes β-arrestin-mediated GPCR sorting to the lysosome for degradation. Given the multifaceted function of β-arrestin proteins as well as other key regulators, many questions have arisen, some of which are addressed below.

Future Directions

The basic mechanism regarding the entry route and subsequent post-endocytic sorting of the M_2 mAChR has been characterized to a certain degree in these studies. We found that β-arrestin regulates the levels of receptor at the cell surface in a ligand-dependent manner and further can participate in the sorting of the M_2 mAChR to the degradative pathway. However, approximately 30% of receptors undergo agonist-induced down-regulation suggesting that a significant fraction of total receptors are unaccounted for. While this study focused on the mechanism and kinetics of receptor down-regulation, Roseberry and coworkers demonstrated that a significant portion of internalized M_2 mAChRs exhibit slow recycling [162]. It would be of interest to examine the effects of β-arrestin mutants and proteasome inhibitors in the recycling kinetics of the M_2 mAChR.

Earlier studies have proposed that whether a receptor enters the recycling or degradative pathway depends upon the strength and duration of agonist-mediated

stimulation [203]. Addition of high concentrations or longer exposure to agonist is thought to promote robust phosphorylation of the receptor that distinguishes whether the receptor interacts transiently or stably with β-arrestin [203, 204]. With respect to these findings, it would be interesting to ask how acute or chronic stimulation with varying concentrations of ligand would affect the phosphorylation state of the M_2 mAChR, affinity with β-arrestin proteins, as well as degradation kinetics. This may have enormous implications as to how surface receptor levels are modulated when exposed to acetylcholine in a native tissue.

This leads us to another important question. These studies were performed in a model cell line that does not endogenously express the receptor; therefore, it is feasible that the fate of the receptor depends on the cell in which it is expressed. Indeed, many studies including one presented in this thesis have shown that the endocytic mechanism of the M_2 mAChR differs in different cell lines [26, 27, 160, 161]. So far, it is not known if receptor entry through these alternate pathways leads to a different sorting fate of the M_2 mAChR. Future work in dissecting the intracellular trafficking pathway should be considered in a neuronal cell line such as PC12 that endogenously express mAChRs and would thus provide a better model to study M_2 mAChR trafficking [205]. Furthermore, since our work was performed in MEF cells derived from knockout mice it may be relevant to examine endogenous M_2 mAChR trafficking *in vivo* using immunohistochemical studies in tissues such as the brain and heart harvested from the β-arrestin single knockout or wild type mice.

Since an overexpression system was used to study the role of β-arrestin in regulating the M_2 mAChR, we initially performed a DNA titration experiment to achieve

optimal transfection efficiency. No major differences were observed from 0.3-3.0 μg although higher concentrations resulted in cell death. Massive overexpression was observed so that in some cases intracellular staining was evident as a result of *de novo* synthesis. To eliminate this intracellular staining, cells could be treated with cyclohexamide, a protein synthesis inhibitor, to chase receptors retained in the Golgi or Endoplasmic Reticulum to the plasma membrane. Therefore, following treatment only surface receptors would be studied. Since overexpressed receptors and β-arrestin proteins are used, the stable interaction we observed with M_2 mAChR and both β-arrestin isoforms may be a result of differences in expression levels. Caution should be taken in interpretating these results. A way to circumenvent this problem is to generate a stable cell line so that a constant level of receptor expression is achieved.

The observation that both β-arrestin and M_2 mAChR exhibits a high basal steady-state of ubiquitination suggests that perhaps we may be observing an artifact of heterologous overexpression system or that we are detecting ubiquitination of associated proteins that have been pulled down during the immunoprecipitation process. Although we do observe agonist-promoted changes in ubiquitination suggesting our key findings are preserved, the use of heterologous expression systems has many limitations. One potential caveat is that exogenous overexpression of proteins can lead to a partial accumulation of misfolded proteins or protein aggregates in the endoplasmic reticulum. These unfolded proteins are removed by proteasome-dependent ER-associated degradation (ERAD) that involves polyubiquitination of substrate proteins [206]. Therefore, we may be detecting polyubiquitination of unassembled membrane receptors that do not reach the cell surface. This may explain why we observe augmented levels of

ubiquitinated receptor in the presence of proteasome inhibitors. Future studies will need to be performed to rule out this possibility. One likely approach to circumvent this limitation is to examine endogenous M_2 mAChR ubiquitination using biochemical studies in native cells or tissues. This may lead to a better understanding of M_2 mAChR regulation in a physiologically relevant cell line. Alternatively, one could tag surface-resident receptors to ensure that only those receptors that reached the cell surface and were functional were included in biochemical analyses. This approach could also be used to assess the time course of agonist-promoted degradation of surface M_2 mAChRs. For example, a surface biotinylation assay could be used prior to immunoprecipitation and subsequent immunoblotting with HRP-conjugated streptavidin to assess the effects of β-arrestin and lactacystin on M_2 mAChR down-regulation. Moreover, the aforementioned findings and proposed studies should be conducted in animal models of human diseases such as dementia associated with Alzheimer's type. Comparisons between the studies could elucidate potential mechanisms of M_2 mAChR-associated pathologies.

Because β-arrestin was found to stably associate with the receptor at early endosomal compartments, questions arise as to the purpose or function of high affinity interactions with the M_2 mAChR aside from its role in receptor sorting. Once again, a fraction of these receptors are degraded while presumbably the other fraction is slowly recycled; however, the extent of co-localization between β-arrestin and M_2 mAChR is significant. Do cellular events depend on this tight interaction? Do receptor/arrestin complexes remain active in signaling at these intracellular sites? It has been shown that β-arrestin can function to assemble multi-protein signaling complexes within intracellular sites [187, 194]. These signaling complexes were shown to function in either

reorganization of the cytoskeleton or in inducing proliferative signaling pathways, to promote cell survival and migration, respectively [76-78]. Speculation would suggest that a similar signaling pathway would be initiated from intracellular M_2 mAChR/β-arrestin complexes, perhaps depending on the cellular system. Future directions would be to identify what signaling events occur and what cytoplasmic targets are activated. Another obvious question would be to address whether these β-arrestin mutants or proteasome inhibitors alter these intracellular signaling pathways.

The physiological consequence of the differences in trafficking between mAChR subtypes, particularly stable β-arrestin association, may be attributed to their differential localization in various cells [207]. M_2 mAChR modulates neurotransmitter release (autoreceptors) and therefore predominate presynaptically in the brain and the lung while other mAChR subtypes are found postynaptically to mediate acetylcholine signals within the target cell. It has been shown that activation of the M_2 mAChR blocks voltage-operated calcium channels via $G_{\beta\gamma}$ while M_2 mAChR interacts directly with proteins of the exocytic apparatus, specifically syntaxin and SNAP-25 to prevent neurotransmitter release [208, 209]. Therefore, it is likely that a portion of these autoreceptors are desensitized and internalized into intracellular compartments with β-arrestin intact. Given that β-arrestin can promote receptor-mediated signaling inside the cell to induce cytoskeletal rearrangements, we can hypothesize that this further prevents neurotransmitter release since Moralles and coworkers have shown that actin depolymerization is required for neurotransmitter release [210]. Therefore, this could serve as a compensatory mechanism to ensure proper neurotransmitter levels within the synapse. Moreover, Ilouz and coworkers have suggested that when an action potential

reaches the nerve terminal, M_2 mAChRs undergo a conformational change which converts it to a lower affinity state for acetylcholine resulting in the release of its interaction with syntaxin and SNAP-25 inside the cell [208]. Depolarization further serves to open voltage-operated calcium channels for Ca^{2+} influx to further allow for neurotransmitter release. Therefore, one can speculate that the purpose of M_2 mAChR retention inside the cell with β-arrestin serves as a major mechanism to control acetylcholine release, a primary function of the M_2 mAChR.

Since the majority of the mAChR subtypes are found postsynaptically, the receptor must be able to constantly sample the environment to relay acetylcholine-mediated signaling events. Therefore, these subtypes that include M1, M3, M4, and M5 mAChR tend to rapidly recycle [157, 211]. Therefore, the differences observed in post-endoctyic trafficking and differential affinities with β-arrestin may be due to their fundamental differences in the role they play inside the cell. Since M_2 mAChRs are also expressed postsynaptically, the ability of M_2 mAChR to sequester β-arrestin inside the cell could potentially prevent desensitization and internalization of other GPCRs at the plasma membrane. This could allow acetylcholine-mediated signaling events to persist through other mAChR subtypes.

The characterization of M_2 mAChR sorting along the degradative pathway represents one of the major questions for future research. While we provide evidence that ubiquitination of β-arrestin targets the M_2 mAChR to the lysosome, the complete molecular mechanism and machinery involved remains to be determined. Does sorting of M_2 mAChR to the lysosome involve HRS binding via interactions with ubiquitin conjugated to receptor or β-arrestin? Do other sorting mechanisms play a role? It would

be of interest to investigate whether interaction of the M_2 mAChR occurs in a stoichometric manner with proteins that control lysosomal sorting of other GPCRs such as GASP and SNX-1 [41-43]. In the case of the cannabinoid 1 receptor, endogenous ligands induce internalization and subsequent functional receptor recycling whereas chronic stimulation leads to down-regulation. They propose that GASP forms a higher affinity complex with the C-terminal tail of the receptor that has been chronically stimulated. Moreover, expression of GASP appears to show regional differences in tissues which may explain why some of the cannabioid receptors are more prone to enter the degradative pathway [43].

Additionally, since we have not ruled out the possibility that ubiquitination of M_2 mAChR serves as a signal for sorting to lysosomes, it may be worth investigating other mutant forms of M_2 mAChR, particularly phosphorylation defective mutants. It has been shown that receptor ubiquitination requires receptor phosphorylation [85, 102]. Lee and coworkers have generated many phosphorylation deficient mutants of the M_2 mAChR that could potentially elucidate whether receptor phosphorylation preceeds receptor ubiquitination [212]. To further define whether receptor or β-arrestin contains the sorting signal presumbably via ubiquitin modification, a M_2 mAChR mutant in which mutation of a serine/threonine cluster to a series of alanines in residues 307-311 within the third intracellular loop should be examined. Mutation of this sequence within the M_2 mAChR has been shown to impair its ability to associate with β-arrestin 1 and 2 while signaling, densensitization and subsequent internalization remained unaltered [149, 153, 212]; however, our data strongly support a role β-arrestin in receptor internalization. Testing the above mutant may clarify whether β-arrestin, M_2 mAChR, or some other accessory

protein such as GRK2 participates in ubiquitin-dependent or -independent sorting to the lysosome.

Broad Implications of Research

The M_2 mAChR is abundantly expressed in the central nervous system where its activation is critical to the regulation of several functions, including neurotransmission, cardiac heart rate, and smooth muscle contraction/bronchoconstriction. Receptor availability at the cell surface directly controls the signaling events involved in mediating these physiological events. The molecular mechanisms that regulate the cell surface density of M_2 mAChR are an important component of maintaining proper signaling events. Thus, the studies provided here have broadened our understanding of the cellular mechanisms that regulate M_2 mAChR surface expression.

Further characterization of the regulation of M_2 mAChR including post-endocytic trafficking could offer additional targets for the development of neuronal and cardiovascular therapeutics. There is growing evidence that alterations in M_2 mAChR cell surface density contribute to the pathology of aging and disease in both the heart and brain [118, 138, 141, 142, 213]. Therefore, manipulation of the mechanisms that regulate M_2 mAChR activity could provide potential therapeutic hope for those afflicted with M_2 mAChR related disorders.

APPENDIX A

PROTOCOLS

Splitting Mammalian Cells

1. Aspirate media and add 2 mL trypsin and incubate for 2-3 minutes at 37^0C.
2. Add 4 mL complete media to cells and pipet cells 20 times to get uniform suspension.
3. Add 0.4-1.0 mL suspended cells to 10 cm dish containing 10 mL of complete media.
4. Label dish with date, split number, cell line, dilution, and initials.
5. If using cells for experimental purposes, count number of cells using hemacytometer.

Plating Mammalian Cells

1. Flame ethanol soaked coverslips and let air dry for a few seconds before adding to 10 cm dish or 6-well plate.
2. After counting number of cells, place appropriate amount of cells into a conical tube containing media and mix well.
3. Dispense 2 mL or 6 mL of cells into 6-well plate or 10 cm dish, respectively.

Lipofectamine 2000 Transfection

1. For a 10 cm dish, plate 0.6 x 10^6 MEF KO1/2 cells or 1.3 x 10^6 MEF wt cells in complete media without antibiotics for ~ 24 hrs. (density depends on cell type)
2. Following day, aspirate media and rinse cells with 1 mL prewarmed OPTI-MEM.
3. Add 4 mL of OPTI-MEM media to cells prior to transfection.
4. For each transfection, dilute 3.0 µg of DNA total into 200 µl of OPTI-MEM.
5. Dilute 6 µl of lipofectamine 2000 into a separate tube containing 200 µl of OPTI-MEM.
6. Incubate for 5 minutes at room temperature.
7. Combine diluted DNA and diluted reagent together and mix gently.
8. Incubate at room temperature for 20 minutes.
9. Add to cells and incubate for 5 hrs.
10. Change media to complete and assay following day.

ExGen 500 Transfection

1. For a 10 cm dish, plate 1.0 x 10^6 HeLa cells in complete media without antibiotics for ~ 24 hrs.
2. Following day, dilute 2.0-3.0 µg of DNA into 200 µl of 150 mM NaCl.
3. Add 8 ul of ExGen 500 to DNA solution and vortex for 10 seconds.
4. Incubate at room temperature for 10 minutes.
5. Add DNA solution to cells.
6. Incubate overnight and assay following day.

Indirect Immunofluorescence

1. Day 1: Plate 0.10 x 10^6 HeLa, 0.13 x 10^6 MEF wt, 0.08 x 10^6 MEF KO 1/2 cells on flamed 12 mm circle glass coverslips in a 6-well dish.
2. Day 2: Begin transfection protocol according to manufacturer's protocol.
3. Day 3: Treat as required for experimental protocol.
4. Transfer coverslips to a 12-well dish containing 1 mL of chilled 2% formaldehyde in PBS pH 7.4
5. Incubate for 10 minutes at room temperature.
6. Remove fixative and add 1 mL of 10% adult calf serm and 0.02% sodium azide in PBS (PBS/serum).
7. Incubate for 5 minutes at room temperature.
8. Dilute primary antibodies into PBS/serum containing 0.2% saponin and spin for 5 minutes at 14,000 rpm.
9. Add parafilm to the bottom of a 150 mm Petri dish and label for each corresponding coverslip in the 12-well plate.
10. Add 25 µl of the diluted antibody solution to appropriate spot on the parafilm.
11. Using forceps pick up individual coverslips, wick off excess fluid on paper towel, and add cell side down directly onto 25 µl of diluted antibody.
12. Place cover on 150 mm Petri dish and incubate for 45 minutes.
13. Carefully transfer coverslip, cell-side up, back into 12-well dish containing PBS/serum.
14. Wash cells with 1 mL PBS/serum (3 x for 5 minutes)
15. Dilute fluorescently-labeled secondary antibodies in PBS/serum + 0.2% saponin and spin for 5 minutes at 14,000 rpm.
16. Invert coverslips onto 25 µl of diluted secondary on parafilm as described above.
17. Incubate for 45 minutes.
18. Wash coverslips 3 x 5 minutes with PBS/serum.
19. Rinse coverslips with 1X PBS and mount onto glass slides with fluoromount G.
20. Seal coverslips with nail polish.

Cell Lysis and Immunoprecipiation of M_2 mAChR

1. Rinse cells with twice with ice-cold 1X PBS.
2. Add 600 µl of Tris Buffer + 2% DβM to each sample
    a. 50 mM Tris pH 7.4 0.24g in 30 mls
    b. 150 mM NaCl 0.35g

c. 5 mM $MgCl_2$ 0.04g
d. 1 mM EDTA 400 µl of 100 mM EDTA pH 7.4
e. 1mM DTT 0.006g
    i. Fill to 40 mLs with ddH_2O
    ii. DβM: n-dodecyl-β-D-maltoside, Calbiochem (#324355)
        1. 0.4g in 20mls of Tris buffer
f. Add fresh 1X protease inhibitor cocktail (Roche or Sigma)
g. Iodoacetamide Final = 1.0 mg/ml
h. 10 mM N-ethylmaleimide
    i. Dilute to 0.2M in ethanol.

3. Incubate on ice 15 minutes, then scrape cells into centrifuge tube.
4. Solubilize end-over-end at 4^0C for 2 hrs.
5. Add lysate to ultracentrifuge tubes, and spin at 100,000 x g for 30 minutes at 4^0C to pellet insoluble material.
6. Remove soluble lysate to new tube. Save ~ 100 µl of sample to run on SDS-PAGE and to perform BCA assay (store at -20^0C).
7. Dilute lysate 1:1 in Tris buffer containing fresh protease inhibitor, iodoacetamide, NEM so that the final concentration of DβM is 1%.
8. Add 50 µl of HA-affinity matrix (Roche).
9. Incubate overnight with end-over-end rotation at 4^0C.
10. Following day, wash beads 3Xs with Tris buffer + 0.2% DβM.
    a. For 6 samples, wash 500 µl each.
        i. 1 ml of 2% DβM + 9 mls of Tris + PI cocktail, NEM, iodoacetamide.
11. Add ~50 ul of 2X Laemmli Sample buffer with β-mercaptoethanol to beads.
    a. 950 µl of 2X Laemmli Sample buffer + 50 µl of β-mercaptoethanol.
12. Incubate at 37^0C for 15 minutes to elute receptor.
13. Spin down beads and add supernatant (~20 ul) to SDS-PAGE.

SDS-PAGE Gel recipe:

MINIGEL: SEPARATING GEL (10 mL)

Reagent:	**7%**	**10%**
40% Acrylamide	1.75 mL	2.5 mL
1.5M Tris, 0.4% SDS pH 8.8	2.5 mL	2.5 mL
ddH_2O	5.25 mL	5.0 mL
10% APS *fresh	50 µl	50 µl
TEMED	10 µl	10 µl

MINIGEL: STACKING GEL (5 mL)

Reagent:	
40% Acrylamide	0.375 mL
0.5M Tris, 0.4% SDS pH 6.8	1.25 mL
ddH_2O	3.375 mL
10% APS *fresh	50 µl
TEMED	7.5 µl

SDS-PAGE Set Up

1. Assemble two glass plates using green casting stand from Bio-Rad.
2. Place cast upright in the Bio-Rad assembly stand to seal bottom of gel.
3. Pour mixed separating gel using a Pasteur pipet ensuring no air bubbles form.
4. Overlay gel with isopropanol to ensure a flat surface and to exclude air.
5. Allow ~45 minutes for gel to polymerize.
6. Pour stacking gel onto top of set separating gel and insert comb.
7. Allow gel to set. May store gel overnight at 4^0C.
8. Place one or two gels into the slots of the Mini Tran Blot Cell, ensuring that the short plate faces the interior of the cell.
9. Remove comb and remove bubbles from wells using a syringe.
10. Load 5 µl of protein standard and ~20 µl of protein sample to corresponding wells.
11. Fill the middle buffer chamber with 1X SDS running buffer (196 mM glycine, 50 mM Tris-Cl pH 8.3, 0.1% SDS). Once running buffer reaches lanes slowly add rest of running buffer and ensure that samples do not run over into adjacent lanes. Fill to top.
12. Fill the lower buffer chamber with 1X SDS running buffer until it covers the wire found on the inside of the gel apparatus.
13. Connect the electrode cables to power supply.
14. Run gel at 150V for ~1 hour or until dye runs off gel.

Western Blotting (ECL Detection)

1. Following SDS-PAGE, carefully separate glass plates and float the gel off the glass plate under chilled transfer buffer.
2. Assemble sandwich in this order on top of black side of sandwich.
    a. Scotch-brite pad (presoaked in transfer buffer)
    b. Whatman filter paper (presoaked in transfer buffer)
    c. SDS-PAGE Gel
    d. Nitrocellulose paper (presoaked in transfer buffer)
        i. Roll out air bubbles
    e. Whatman filter paper (presoaked in transfer buffer)
    f. Scotch-brite pad
3. Seal cassette, place in Mini Protean II Cell, fill with chilled transfer buffer, and add ice pack.
4. Connect the electrode cables to power supply.
5. Run gel at 50V for 1.5 hrs.
6. Transfer nitrocellulose to blocking buffer (Tris buffered saline with 0.1% Tween 20 (0.1% TBST) and 2% milk)
    a. For better resolution of ubiquitinated species, add 20 mL denaturation solution and place at 60^0C for 30 minutes.
    b. Wash 3Xs with 0.1% TBST.

7. Block nitrocellulose for 30 minutes at room temperature.
8. Transfer nitrocellulose to ziplock bag containing diluted primary antibody in 10 mL of blocking buffer.
9. Incubate on rocker overnight at 4^0C.
10. Wash nitrocellulose 2Xs with 0.1% TBST.
11. Incubate with secondary antibody diluted in blocking buffer for 1 hour on rocker at room temperature. (HRP conjugated donkey 1:5000)
12. Wash nitrocellulose 3Xs with 0.1% TBST.
13. Add 1.5 mL of ECL solution (1:1 mixture of Soln A and Soln B) for 5 minutes.
14. Transfer nitrocellulose to syran wrap and place in film cassette.
15. Expose film for 1 minute.
16. Develop film in dark room.

BCA Assay

1. Add 0, 5, 10, 15, 20 µl of 1 mg/mL of BSA standard to corresponding wells in a 96-well plate.
2. Add 5 µl of protein sample (lysate) to 96-well plate.
3. Add 200 µl of BCA mixture to each well containing sample.
    a. 1 part solution B to 50 parts solution A.
4. Incubate plate for 30 minutes at 37^0C.
5. Read plate at 562 nm using a microplate reader.

[^{3}H]-NMS Assay

1. Day 1: Plate 1.2 x 10^6 cells in a 10 cm dish.
2. Day 2: Transfect cells using Lipofectamine 2000 protocol.
3. Day 3: Trysinize cells and replate in a 24-well plate at a density of 0.1 x 10^6 cells/well.
4. Day 4: Treat cells as required for experimental protocol.
5. Rinse cells 3Xs with chilled 1X PBS over a ice-water bath.
6. To indicated wells add 1.0 µM of the atropine (muscarinic antagonist) diluted in 1X PBS. This step accounts for nonspecific [^{3}H] NMS binding.
7. Dilute 10 µl of [^{3}H]-NMS into 990 µl of 1X PBS.
8. Calculations of cpm needed for each well (sample):
    a. Determine efficiency of scintillation counter. (0.65 cpm/dpm)
    b. The specific activity of [^{3}H]-NMS = 81 Ci/mmol (found on container)
       Conversion factor: 2.2 xs 10^{-6} dpm/uCi
       The K_D of NMS = 120pM
    c. Determine the specific activity of [^{3}H]-NMS in terms of cpm/fmol:
       $\frac{81\text{Ci}}{\text{mmol}} \times \frac{2.2 \times 10^{-6}\ \text{dpm}}{1 \times 10^{-6}\ \text{Ci}} \times \frac{0.65\ \text{cpm}}{\text{dpm}} \times \frac{\text{mmol}}{10^{12}\ \text{fmol}} = \frac{115.83\ \text{cpm}}{\text{fmol}}$
    d. Determine the number of cpm to use per well:
        i. For saturation binding experiment use 6 x K_D.

       6 x 120pM = $\frac{720\ \text{pmol}}{} = \frac{720{,}000\ \text{fmol}}{} = \frac{720\ \text{fmol}}{}$

liter 1000 mL mL

For 0.5ml reaction use: $\frac{360\ \text{fmol}}{\text{well}} \times \frac{115.83\ \text{cpm}}{\text{fmol}} = \frac{41{,}698\text{cpm}}{\text{well}}$

9. Each well should contain *41,698 cpm* of [^{3}H]-NMS for complete saturation. To test activity of diluted [^{3}H]-NMS, add 10 µl of [^{3}H]-NMS into 4 mL of scintillation fluid and test radioactivity in scintillation counter.
    a. Example: The scintillation counter gave a reading of 54, 477 cpm/ 10 µl. Therefore, the diluted [^{3}H]-NMS contains 5,447 cpm/µl.
    b. 41,698 cpm/5,477 cpm/µl = 7.3 µl per sample
    c. 7.3 µl x 66 samples = 481.8 µl in 34 mLs of 1X PBS.
10. Dispense 0.5 mL of [^{3}H]-NMS in 1X PBS to each well.
11. Incubate on ice-water bath for >5 hrs.
12. Remove [^{3}H]-NMS solution using glass pipette and discard in liquid waste container.
13. Rinse wells 3 x 1ml with ice cold PBS, discarding all liquids in radiation waste container. Use new pipette each wash.
14. Solubilize cells with 0.5 mL of 1% Triton X in 1X PBS. Once triton is added, the plates can be removed from ice.
15. After 5 minutes, pipette up and down and add 400 µl of solution into 4 mL of scintillation fluid.
16. Vortex each sample immediately. The sample should go from cloudy to clear.
17. Read counts in a scintillation counter.
18. Amount of [^{3}H]-NMS detected in the presence of 1 µM atropine (nonspecifically bound, cpm) is subtracted from total bound (cpm) to get specifically bound [^{3}H]-NMS.

[^{3}H]-QNB Assay

1. Day 1: Plate 0.5 x 10^6 cells in a 6-well plate.
2. Day 2: Transfect cells using Lipofectamine 2000 protocol.
3. Day 3: Treat cells as required for experimental protocol.
4. Rinse cells 2Xs with chilled 1X PBS over a ice-water bath.
5. Scrape cells in 50 mM $NaPO_4$, pH 7.0, pool two wells, and homogenize with 20 strokes in a Dounce homogenizer.
6. Homegenate was spun at 10,000 rpm for 20 min at 4^0C in a Sorvall Mach 1.6R fixed angle rotor.
7. Resuspend pellet in 50 mM $NaPO_4$, pH 7.4 and spin again.
8. Resuspend pellet in 500 µl of 50 mM $NaPO_4$, pH 7.4
9. Add 100 µl of membrane homogenate to 0.67 nM [^{3}H]-QNB in a final volume of 1 mL with 50 mM $NaPO_4$, pH 7.0.
10. Incubate at room temperature for 90 minutes.
11. Add 5 ml of ice-cold 50 mM $NaPO_4$ to quench binding.
12. Pass samples through a Whatman glass fiber filter (2.5 cm G/C, presoaked in a 0.1% solution of BSA in 50 mM $NaPO_4$ buffer.

13. Rinse three times with $NaPO_4$ buffer.
14. Place filter in vials containing 4 mL of scintillation fluid.
15. Read counts in a scintillation counter.
16. Amount of [^{3}H]-QNB detected in the presence of 1 μM atropine (nonspecifically bound, cpm) is subtracted from total bound (cpm) to get specifically bound [^{3}H]-QNB.

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