The Structural Basis of Gβ5-RGS7 Interaction with the Muscarinic M3 Receptor and Implications for its Role in Functional Pathway Selectivity

Darla M. Karpinsky-Semper

University of Miami, dkarpinsky@med.miami.edu

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THE STRUCTURAL BASIS OF Gβ5-RGS7 INTERACTION WITH THE
MUSCARINIC M3 RECEPTOR AND IMPLICATIONS FOR ITS ROLE
IN FUNCTIONAL PATHWAY SELECTIVITY

By

Darla Karpinsky-Semper

A DISSERTATION

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Darla Karpinsky-Semper

Approved:

Vladlen Z. Slepak, Ph.D.
Professor of Molecular and Cellular Pharmacology

R. Grace Zhai, Ph.D.
Associate Professor of Molecular and Cellular Pharmacology

Charles W. Luetje, Ph.D.
Professor of Molecular and Cellular Pharmacology

Christian Faul, Ph.D.
Assistant Professor of Medicine

Nirupa Chaudhari, Ph.D.
Professor of Physiology and Biophysics

M. Brian Blake, Ph.D.
Dean of the Graduate School
The neurotransmitter acetylcholine (Ach) is arguably the most important in the CNS. Two classes of receptors are activated by Ach: nicotinic (ionotropic) and muscarinic (metabotropic). Muscarinic receptor pharmacology is well-defined, but still lacks receptor sub-type specificity seen in other GPCR families like adrenergic receptors. We have found that Gβ5-RGS7, a unique G-protein obligate heterodimer complex, selectively attenuates Ca^{2+} signaling through the muscarinic M3 receptor (M3R).

The G protein beta subunit Gβ5 uniquely forms heterodimers with R7 family regulators of G protein signaling (RGS) proteins (RGS6, RGS7, RGS9, and RGS11) instead of Gγ. While the Gβ5-RGS7 complex attenuates Ca^{2+} signaling mediated by M3R, the route of Ca^{2+} entry (i.e., release from intracellular stores and/or influx across the plasma membrane) is unknown. Here I show that in addition to suppressing carbachol-stimulated Ca^{2+} release, Gβ5-RGS7 enhanced Ca^{2+} influx. This novel effect of Gβ5-RGS7 was blocked by nifedipine and 2-APB. Experiments with pertussis toxin, RGS domain-deficient mutant of RGS7 and UBO-QIC, a novel inhibitor of Gq, showed that Gβ5-RGS7 modulated a Gq-mediated pathway. These studies indicate that Gβ5-RGS7, independent of RGS7 GAP activity, couples M3R to a nifedipine-sensitive Ca^{2+} channel.
In neurons and glands, muscarinic signaling plays a major role in secretion. The novel finding that Gβ5-RGS7 enhances M3R-stimulated insulin secretion can explain why loss of Gβ5 results in impaired insulin secretion in mice. In insulin secreting cells, I found that the mechanism of Gβ5-RGS7-enhanced Ca\(^{2+}\) signaling is similar to the one identified in CHO-K1 cells as it is sensitive to nifedipine.

I also compared the action of Gβ5-RGS7 on M3R-induced Ca\(^{2+}\) influx and release elicited by different muscarinic agonists. Responses to oxotremorine-m were insensitive to Gβ5-RGS7. Pilocarpine responses consisted of a large release and modest influx components, of which the former was strongly inhibited whereas the latter was insensitive to Gβ5-RGS7. McN-A-343 was the only compound whose total Ca\(^{2+}\) response was enhanced by Gβ5-RGS7, attributed to, in part, by the relatively small Ca\(^{2+}\) release this partial agonist stimulated. Together these results show that distinct agonists not only have differential M3R functional selectivity, but also confer specific sensitivity to the Gβ5-RGS7 complex.

A deeper understanding of the structural basis of this ligand bias towards sensitivity to Gβ5-RGS7 may lead to new strategies for selective therapeutics. My biophysical studies provide additional insights into the structural basis of Gβ5-RGS7 regulation of M3R signaling.

Altogether, this dissertation work led to the novel finding that Gβ5-RGS7 has a dual effect on M3R-stimulated Ca\(^{2+}\) signaling, and the newly discovered positive effect on Ca\(^{2+}\) influx plays an important role in hormone and/or neurotransmitter secretion stimulated by the M3R pathway.
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CHAPTER 1

INTRODUCTION

1.1 Signal transduction through G protein-coupled receptors

History

The last century has seen remarkable advances in all areas of science and technology. Understanding the mechanisms of action of biologically active substances was not an exception. Pharmacology as a discipline came to existence as the result of fusion of knowledge in chemistry, biology, medicine and physics. A particularly significant concept that came about from this science is that of a receptor, a molecule in the body that binds to a drug. The term ‘receptive substance’ was first coined by Jonathan Langley in 1905 for his work with nicotine and curare on skeletal muscle (Langley, 1905). In 1909, Langley’s student, Archibald Hill, was the first to mathematically describe drug receptor interactions with the law of mass action (Hill, 1909). The relationship between drug concentration and biological response was described in 1926 by A.J. Clark and Gaddum independently, which combined with Langley and Hill’s work constructed the receptor occupancy model (Clark, 1926a, b; Gaddum, 1926; Hill, 1909; Langley, 1905). In 1948 Raymond Ahlquist hypothesized that there are two distinct subtypes of adrenergic receptors, α and β, based on differences in biological responses in several tissues, although biochemical evidence of distinct pools of adrenergic receptors did not exist until the 1960s (Ahlquist, 1948). This work eventually lead to the development of the first subtype selective class of GPCR drugs, β-blockers, by James
Black in the early 1960s for which Black was awarded the Nobel Prize for Medicine in 1988 and confirmed Ahlquist’s theory (Black et al., 1964; Black and Stephenson, 1962).

A major breakthrough in receptor biology came from physics, particularly the application of radioactive isotopes. Chemists used isotopes to synthesize radiolabeled analogs of pharmaceutical drugs which were applied to tissues to identify receptors. Radioligand analysis provided the first crucial line of biochemical evidence that distinct receptor populations exist. Because ligand binding provided the means to trace the activity of receptors in vitro, ligands also facilitated biochemical purification which was accomplished in the late 1970s (Lefkowitz and Michel, 1983).

In the 1980s, the advent of molecular cloning led to determination of amino acid sequences of GPCRs and rapidly expanded our knowledge of their structure and molecular mechanisms of as illustrated in Fig. 1.1. The first GPCR to be cloned, and one of the most extensively studied, was the β2-adrenergic receptor (β2AR) (Dixon et al., 1986). Dozens of receptors were cloned thereafter, including receptors of acetylcholine (Lechleiter et al., 1989), one of which I focus on in my dissertation. All GPCRs have a very characteristic 7-pass transmembrane structure and many are monoexonic, allowing them to be easily recognized in the genome.

When sequencing of human and mouse genomes was accomplished, scientists identified hundreds of GPCRs. Many of them are orphan, that is, the ligand is not known. It has been reported that the human genome encodes 367 G protein-coupled receptors (GPCRs) which respond to endogenous ligands (i.e. peptides, lipids, neurotransmitters, or nucleotides), approximately 350 olfactory receptors and about 30 other chemosensory
GPCRs (Vassilatis et al., 2003). Together, GPCRs comprise the largest receptor gene family.

While the work on structure and classification progressed rather quickly, our understanding of signaling pathways downstream of GPCRs is still evolving. Over the past three decades the efforts of Alfred Gilman, Martin Rodbell, Elliott Ross, Jeffrey Benovic, Robert Lefkowitz, Brian Kobilka, Jurgen Wess, Michael Bouvier, Arthur Christopolous, Terry Kenakin and several others shaped our current understanding of GPCR structure and the intricacies of GPCR-mediated signal transduction signaling. I will elaborate on some seminal findings in the subsequent paragraphs.

**Figure 1.1 Progress in the field of G protein signaling.** Graphical representation of a PubMed search for literature on “G protein” showing the number of publications per year from 1980 to 2013 with some seminal findings listed below the year.
The G protein cycle and coupling specificity

In the early 1970s, Martin Rodbell and colleagues showed that application of hormones/drugs lead to activation of adenylyl cyclase that was dependent on guanine nucleotides (Birnbaumer et al., 1969, 1971; Birnbaumer et al., 1972; Birnbaumer and Rodbell, 1969; Rendell et al., 1975; Rodbell, 1980; Rodbell et al., 1971; Rodbell et al., 1975; Schlegel et al., 1979). However, it was not until the early 1980s that Alfred Gilman and colleagues identified the regulatory factor of adenylyl cyclase, guanine nucleotide-binding proteins (G proteins) (Gilman, 1984; Northup et al., 1982; Northup et al., 1983a; Northup et al., 1983b; Northup et al., 1980; Sternweis et al., 1981).

Figure 1.2 – The G-protein cycle. The canonical G protein cycle proceeds from receptor activation which leads to GDP exchange for GTP on the Gα subunit causing dissociation heterotrimeric G protein, allowing each subunit (Gα and Gβγ) to activate effector molecules (ion channels, enzymes, etc.). Termination of the signal is facilitated by intrinsic GTPase activity of the Gα subunit and this activity is accelerated by GTPase-accelerating proteins (GAPs), which belong to the regulator of G protein signaling (RGS) homology (RH) superfamily of proteins.
The canonical G protein cycle proceeds from receptor activation and leads to GDP exchange for GTP on the Ga subunit causing heterotrimeric G protein dissociation, allowing Ga and Gβγ subunits to activate effector ion channels and enzymes (Fig. 1.2). Approximately 25 Ga subunits have been identified and fall into the following broad classes based on sequence identity and function: Gs, stimulates cAMP production; Gi/o, inhibits accumulation of cAMP and hyperpolarizes cells having an overall inhibitory effect; Gq, activates the PLC-β IP3 pathway ultimately leading to a rise in intracellular Ca^{2+} concentration [Ca^{2+}]_i (Neves et al., 2002). The G_{12/13} family is unusual as they do not activate enzymes or ion channels, but rather small G proteins via activation of their GEFs (Worzfeld et al., 2008). The Gβγ dimer also plays a major role in signal transduction as it is capable of activating effector molecules like PLC-β and G protein-coupled inwardly-receifying K⁺ (GIRK) channels (Smrcka, 2008). The active state of the cycle is terminated when the G protein hydrolyzes its GTP. The GDP-bound state of Ga has a higher affinity for Gβγ thereby causing the heterotrimer to re-associate.

How receptors selectively couple to a particular pathway is an area of great interest in pharmacology. Coupling-specificity of receptors to their cognate G proteins is determined by affinity of the G protein for the active state of the receptor. Studies using chimeras of two receptors known to couple to different G proteins identified specific receptor regions involved in selectivity of G protein recognition (Dohlman et al., 1991; Hedin et al., 1993; Savarese and Fraser, 1992; Strader et al., 1994; Wess et al., 1997). Although these studies identified that G protein coupling is primarily determined by amino acids located in the i2 loop and the juxtamembrane i3 loop, all of them were done *in vitro* using overexpressed receptors where coupling can be
pleiotropic (Dohlman et al., 1991; Hedin et al., 1993; Savarese and Fraser, 1992; Strader et al., 1994; Wess et al., 1997). It quickly became apparent that the paradigm of GPCR-G protein-effector was too simplistic and the reality of GPCR signal transduction was more complex, involving parallel and intersecting pathways that rely on the dynamic interaction of multiple second messengers, effectors, regulators and even other GPCRs.

Many factors that contribute to coupling specificity include cell type, receptor oligomerization, stimulus, receptor density and experimental conditions. Cell lines are commonly used to overexpress GPCRs to study signal transduction pathways since many don’t endogenously express the GPCR of interest. However, it has been reported that in these systems coupling is pleiotropic, presumably due to high receptor density (Burford et al., 1995a, b; Hermans, 2003; Kenakin, 1997). This is because the relative stoichiometry of receptors to G proteins can affect the quantity and quality of the response in some systems (Kenakin, 1997).

Several tools have been developed to investigate coupling specificity. ADP ribosylation of Gi by pertussis toxin (PTX) locks Gi in a GDP bound state thereby inhibiting its activity. For many years GPCR pathways were described as PTX-sensitive or -insensitive. In the last decade identification of a novel Gq inhibitor, YM-254890, has allowed researchers to study specific activation of Gq. This depsipeptide is purified from bacteria and has been shown to prevent the exchange of GDP for GTP on Gq (Takasaki et al., 2004). Since inhibition of Gq by does not involve an enzymatic process, incubation times for YM-254890 are relatively short compared to pertussis or cholera toxins. In the course of my dissertation I used UBO-QIC, a depsipeptide nearly identical to YM-254892, to identify the pathway regulated Gβ5-RGS7.
Significance of GPCRs

GPCRs are arguably the most important pharmacologically targeted gene family as they regulate many biological processes including heart rate, arterial pressure, blood volume, neurotransmission, vision, olfaction, metabolism and cell migration. Receptors that respond to the same endogenous ligand are categorized as a subfamily and can be further differentiated as subtypes based on sequence identity and G protein coupling. For example, cholinergic receptors contain members of the ionotropic nicotinic and metabotropic muscarinic subfamilies, and muscarinic receptors can be categorized as subtypes M1-M5 because there are five genes encoding muscarinic receptors. One of the core themes of my dissertation is regulation of the muscarinic M3 receptor (M3R) by a unique G protein β subunit complex, Gβ5-RGS7.

Subtype selective drugs are of great clinical importance as they produce fewer side effects. One major goal in GPCR pharmacology is to develop subtype selective drugs, which has proven successful for some subfamilies, but is more difficult for others. Some lines of evidence suggest that targeting members of GPCR signal transduction pathways, like RGS proteins, may provide some subtype selectivity. My studies on the structural and functional relationship between M3R and Gβ5-RGS7 provide basic knowledge of this molecular mechanism and could be used to selectively target M3R.

GPCRs are integral membrane proteins making them accessible from outside the cell, therefore permeability is not as critical of a requirement for some GPCRS as it is for other targets such as enzymes. Most GPCR therapeutics bind in the same pocket as the endogenous ligand, called the orthosteric site, but many allosteric sites have been
identified (Christopoulos and Kenakin, 2002b). An allosteric modulator binds to the receptor at a site different from the endogenous ligand and can either enhance or inhibit its activity. Identification of allosteric sites expands the chemical space of potential GPCR drugs, possibly offering a higher degree of subtype specificity (Jeffrey Conn et al., 2009). Another key feature of GPCRs is their ability to couple to a particular transduction pathway based on ligand structure and/or concentration.

**GPCRs are platforms for multi-protein complex formation**

A recent concept in GPCR pharmacology is the phenomenon of ligand bias (Stallaert et al., 2011). It is generally accepted that receptors can be stabilized in different active conformations by structurally distinct agonists. Different receptor conformations can selectively activate a particular pathway by changes in affinity to G proteins (Christopoulos and Kenakin, 2002a). The observation that a particular chemical structure of a drug produces a certain biological effect dates back to the 1940s (Pfeiffer, 1948). The effects that Pfeiffer and colleagues observed after muscarinic activation were likely a combination a biased agonism and activation of different muscarinic subtypes, i.e. M2 and M3, as some ligands display higher affinity towards a specific subtype. One part of my thesis attempts to correlate biological action, regulation of M3R by Gβ5-RGS7, to a particular chemical structure of muscarinic agonists (Table 3.1; Fig. 3.16).

The terms biased agonism, functional selectivity, agonist trafficking or ligand bias are interchangeable. Functional selectivity is not only a consequence of receptor G protein interactions, but also receptor interactions with a diverse set proteins called GPCR
interacting proteins (GIPs) (Bockaert et al., 2004). GIPs control GPCR subcellular localization, as well as the nature, kinetics, strength, and fine-tuning of GPCR signaling (Bockaert et al., 2010). There are more than 30 GIP families which include arrestins, GRKs, PDZ domain-containing proteins and RGS proteins (Bockaert et al., 2004). Some of these accessory proteins not only regulate the activity of the canonical GPCR-G protein-effector pathway, but can transduce a signal independent of the G protein. One example that became "canonical" itself is β-arrestin-mediated signaling. Binding of β-arrestin to active receptor and phosphorylation results in assembly of GPCR-arrestin-Src complex, which leads to activation of the MAP kinase cascade (Luttrell et al., 1999). Some agonists are bias towards arrestin signaling, demonstrating that some compounds are capable of enhancing affinity of a GPCR for its other protein binding partners, not just G proteins (Allen et al., 2011; Violin and Lefkowitz, 2007; Wacker et al., 2013). One aspect of my dissertation work is the discovery of agonist directed regulation of a muscarinic receptor signaling by a RGS protein complex (Karpinsky-Semper et al., 2014).

1.2 Regulators of G protein signaling (RGS)

Gatekeepers of GPCR signaling

In the early 1990s before RGS proteins were identified, it was discovered that termination the G protein cycle was facilitated by GAP activity of two effector enzymes (Arshavsky and Bownds, 1992; Berstein et al., 1992). This finding reconciled the discrepancy between the rate of GTP hydrolysis in cells where it occurs on a sub-second
time scale, and purified G proteins where the rate of GTP hydrolysis can be upwards of tens of seconds. However, this also raised other questions like whether all effectors were GAPs or was there another yet undiscovered family of G protein GAPs (Ross, 1992). The first RGS protein was identified in 1995 by a yeast two-hybrid screen for interaction with Gαi and thus was named G Alpha Interacting Protein (GAIP) by Marilyn Farquhar and colleagues, but its function was unknown (De Vries et al., 1995). Shortly thereafter, two groups elucidated the function of GAIP as a GAP for Gi and it was designated as an RGS protein along with RGS4 (Berman et al., 1996; Watson et al., 1996).

**RGS protein structure and function**

RGS family members are related by a conserved RGS domain that is approximately 130 amino acids (Ross and Wilkie, 2000) (Fig. 1.3). To date more than 30 mammalian RGS proteins have been identified and are categorized based on sequence identity and domain architecture (Hollinger and Hepler, 2002). The RGS domain alone is capable of binding Gα subunits and accelerating GTP hydrolysis whereas the other regions confer variety of regulatory functions (Faurobert and Hurley, 1997; Popov et al., 1997). About half of all identified RGS proteins contain multiple domains which facilitate diverse functions such as kinase activity, membrane targeting and scaffolding (Neubig and Siderovski, 2002).

Some RGS proteins are selective for a particular class of G protein (Xie and Palmer, 2007). For example, RGS4 accelerates the hydrolysis of both Gq and Gi/o (Huang et al., 1997), whereas the R7 family is selective for Gi/o (Hooks et al., 2003).
Another mechanism that contributes to selectivity of RGS proteins for GPCR signaling is direct interactions with GPCRs (Hepler, 2003; Neitzel and Hepler, 2006). One of the first studies to demonstrate such selectivity used knockouts of Gq class genes in mice to evaluate the potency and selectivity of RGS4 in modulating Ca\textsuperscript{2+} signaling transduced by different Gq-coupled receptors (Xu et al., 1999). Another example of selectivity for receptors was demonstrated by our lab with the selective attenuation muscarinic M3 receptor (M3R) Ca\textsuperscript{2+} signaling by RGS7 (Sandiford and Slepak, 2009). This finding was puzzling because, as mentioned above RGS7 only accelerates GTPase activity of the Gi/o class of G proteins and M3R is known to signal through Gq (Caulfield, 1993; Hooks et al., 2003). This result demonstrates that the dual functions of some multi-domain RGS proteins may be mediated by their other domains. Furthermore, the R7 family of RGS proteins has been shown to modulate K\textsuperscript{+} channels, adenylyl cyclase 5 and muscarinic M3 receptor-stimulated Ca\textsuperscript{2+} signaling through direct protein-protein interactions, which I will explain in detail below.

### 1.3 The R7 family of RGS proteins

In 1998 our lab was the first to identify that the G protein β subunit, Gβ5, forms an obligate heterodimer with members of the R7 family of RGS proteins (Cabrera et al., 1998). Gβ5 is significantly divergent from the G protein β subunit subfamily and has not been found in vivo to interact with any other G protein subunits. All R7 RGS proteins contain an N-terminal DEP (Desheveled, Egl10, Plekstrin homology), followed by DHEX (DEP Helical EXTension), a GGL (G-Gamma-Like) and a C-terminal RGS
domain. Association of Gβ5 with the R7-RGS GGL domain stabilizes the heterodimer protecting each protein from degradation (Chen et al., 2003; Witherow et al., 2000b).

**Figure 1.3 Mammalian RGS proteins.** Adapted from Figure 3 in (Neubig and Siderovski 2002) showing representative members from each of the RGS protein sub-families. This illustrates the diversity amongst the sub-families showing their sequence identity of the RGS domains compared to the R4 sub-family and the diverse domain architecture across the RGS family. Highlighted is the RGS9 which belongs to the R7 family along with RGS6, 7 and 11. The R7 sub-family forms an obligate heterodimer with the G protein β subunit Gβ5 which stabilizes each member from degradation. Another unique feature of the R7 RGS proteins is the N-terminal DEP domain, which has been implicated in facilitating molecular functions other than GAP activity and membrane targeting (Kovoor, Seyffarth et al. 2005, Ballon, Flanary et al. 2006, Chen and Hamm 2006, Sandiford and Slepak 2009).
Another unique feature of the R7 family is the DEP domain, which is common in about ten other protein families that have diverse functions in signal transduction (Consonni et al., 2014). The main function of DEP domains is facilitating membrane targeting, but they also carry out other actions by using different binding interfaces (Consonni et al., 2014). The core of all DEP domains is highly conserved consisting of three α-helices connected by a β-hairpin arm that is less conserved and presumably confers variety of function (Consonni et al., 2014). For R7 RGS proteins, an additional motif, DHEX, is required for membrane targeting and interaction with membrane anchoring proteins (Anderson et al., 2007; Cheever et al., 2008; Jayaraman et al., 2009; Masuho et al., 2011; Slepak, 2009). Recently, our lab also found that the DHEX domain is required for localization of RGS7 to cytosolic vesicles (Liapis et al., 2012). The DEP/DHEX domain of the R7 family of RGS proteins is also implicated in regulating M3R-stimulated Ca^{2+} signaling, the orphan GPCR GPR158/179 and dopamine D2
receptor (D2R) signaling through direct interaction with the receptors (Celver et al., 2010; Kovoor et al., 2005; Orlandi et al., 2013; Orlandi et al., 2012; Sandiford and Slepak, 2009; Zheng et al., 2011). Another noteworthy role of the R7 RGS DEP domain is that it interacts with Gβ5, leading to our hypothesis that the Gβ5-RGS7 complex may exist in two conformations: “open” and “closed” (Figure 1.3) (Narayanan et al., 2007; Sandiford et al., 2010).

Gβ5 is expressed in all divisions of the nervous system, heart and some glands, but specific R7 RGS isoform expression is more discrete. For example, RGS9-2 is highly expressed in striatum where regulates dopaminergic signaling (Kovoor et al., 2005) and RGS6 is a key modulator of parasympathetic regulation of the heart (Wydeven et al., 2014). RGS7 and RGS11 are key GAPs in the mGluR6 pathway of retinal rod ON bipolar cells that set the sensitivity and time course of light-evoked responses (Cao et al., 2012). Recently, our lab identified that Gβ5-R7 complexes are expressed in pancreatic β-cells and I will discuss my experiments on insulin secreting cells and the role of Gβ5-R7 complexes there in Subchapter 3.2.

1.4 The muscarinic M3 receptor

Similar to adrenergic receptors, studies on muscarinic receptors dates back to the middle of the twentieth century (Heymans and van den Heuvel, 1949; Pfeiffer, 1948; Schueler, 1952). Pharmacological studies and molecular cloning identified five muscarinic receptors (MRs) (Caulfield and Birdsall, 1998). In physiological settings the paradigm is that M1, M3 and M5 are coupled to Gaq whereas M2 and M4 are coupled to
Muscarinic receptors respond to the neurotransmitter acetylcholine and play major roles in synaptic plasticity, muscle contraction, neuronal transmission and neurotransmitter/hormone release (Fig. 1.5; Eglen, 2006; Wess, 1993). Understanding the physiological roles of individual muscarinic receptor poses a challenge since subtype specific ligands have yet to be developed and some cells express multiple subtypes (i.e. M1 and M3 are both found in salivary glands). Using a gene knockout approach, Wess and colleagues showed that in pancreatic islets only M3R regulates insulin secretion whereas both M1 and M3 receptors synergistically stimulate secretion in salivary glands (Gautam et al., 2006; Wess, 2012). These experiments demonstrated that the combination and magnitude of muscarinic subtype activation is required for fine-tuned signaling in some cells (Wess, 2012).

**Figure 1.5 M3R signaling in excitatory cells.** ACh released from the presynaptic cell into the synaptic cleft can bind to metabotropic muscarinic (M1-M5) cholinergic receptors on the postsynaptic/target cell. M2 and M4 are coupled to Gi/o, which inhibits cAMP production and through a βγ-mediated mechanism hyperpolarizes the cell reducing excitability. M1, M3 and M5 are coupled to Gq, stimulating rises in [Ca^{2+}]i, which is a second messenger required for activation of many cellular processes including neurotransmitter release.
Earlier work in our lab identified a novel mechanism of M3R regulation by Gβ5-RGS7: it can selectively attenuate M3R-stimulated Ca\(^{2+}\) signaling in CHO-K1 cells (Sandiford and Slepak, 2009). RGS proteins have been proposed as potential drug targets due to their discrete tissue expression and receptor selectively (Roman and Traynor, 2011). Therefore, fundamental knowledge of the nature of RGS protein interactions with GPCRs is important. My work continues to unravel the mechanism by which Gβ5-RGS7 modulates M3R-stimulated signaling and its physiological significance.

Like many GPCRs, agonist binding to M3R stabilizes an active receptor conformation. These structural changes are predicted to involve multiple receptor regions, primarily distinct segments of TMs III, VI and VII and helix 8 (Wess et al., 2008). G protein binding sites have been mapped to the third intracellular (i3) loop of several GPCRs and it’s widely accepted that G protein specificity arises from this region (Abadji et al., 1999; Avet et al., 2013; Buck et al., 2000; Burstein et al., 1996; Claus et al., 2006; Punn et al., 2012). It is not surprising that this region also serves as an important regulatory site and participates in conformational changes which alter receptor activity (Bellot et al., 2009; Bernstein et al., 2004; Chee et al., 2008; Jewell-Motz et al., 2000; Simon et al., 2012; Wu et al., 2000).

The i3 loop of M3R is unusually long, 239 amino acids, as compared to the other MRs and has several phosphorylation sites which can act as a code for pathway selectivity (Budd et al., 2003; Budd et al., 2001; Butcher et al., 2011; Shockley et al., 1999). The highly conserved NPxxY motif in the distal most part of TMVII is crucial for ligand affinity and pronounced conformational dynamics required for M3R activation.
Helix 8 resides in the proximal cytoplasmic tail (C-tail) right after the NPxxY motif where it partakes in the transition to an active receptor state and dimerization (Hu et al., 2013; Jian et al., 2008; Jian et al., 2007; Wess et al., 2008). Another noteworthy characteristic of the M3R C-tail is a polybasic stretch that possesses anti-apoptotic properties (Budd et al., 2003).

One goal of my study was to explore the structural basis of the interaction between M3R and Gβ5-RGS7 and elucidate the mechanism by which Gβ5-RGS7 regulates M3R-stimulated Ca\(^{2+}\) signaling. My findings regarding the interaction between helix 8 and the i3 loop of M3R with the Gβ5-RGS7 are discussed in Subchapter 3.3.

### 1.5 Modulation of M3R-stimulated signal transduction by Gβ5-RGS7

Rises in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\textsubscript{i}) initiated by M3R stimulation involves the Gq-PLC-IP3 pathway which leads to Ca\(^{2+}\) release from intracellular stores. Plasma membrane ion channels are activated down stream of Gq and mediate movement of Ca\(^{2+}\) ions from outside the cell to the cytoplasm, i.e. Ca\(^{2+}\) influx. Some channels which mediate Ca\(^{2+}\) influx respond to Ca\(^{2+}\) release from the ER and thus are referred to as store-operated or Ca\(^{2+}\)-dependent channels. The other Ca\(^{2+}\) influx pathway is membrane-delimited where the lipid second messenger diacylglycerol (DAG) directly activates some channels independent of Ca\(^{2+}\) release. Prior to the beginning of my project, it was unknown whether the Gβ5-RGS7 complex inhibits M3R-stimulated Ca\(^{2+}\) release and/or influx (Fig. 1.5).
M3R-stimulated Ca\(^{2+}\) release and influx can be differentially regulated (Carroll and Peralta, 1998). At low agonist concentrations, M3R is bias towards Ca\(^{2+}\) release and as concentration increases it becomes more selective for Ca\(^{2+}\) influx (Carroll and Peralta, 1998). Moreover, several lines of evidence suggest that different muscarinic agonists can produce distinct Ca\(^{2+}\) release and influx patterns (Hishinuma et al., 1997; Karpinsky-Semper et al., 2014; Schaalma et al., 2006; Wang et al., 1992). I reasoned that dissecting M3R-stimulated Ca\(^{2+}\) influx from release may provide insight as to where the G\(\beta\)5-RGS7 complex was exerting its action.
CHAPTER 2

MATERIALS AND METHODS

2.1 Production of anti-R7 DEP antibody. As described in (Karpinsky-Semper et al., 2014), antibodies against the DEP domain of RGS7 were raised by immunizing rabbits (Cocalico Biologicals, Inc) with recombinant DEP domain, amino acids 37-112. The DEP domain construct was generated by PCR amplification of nucleotides 443-668 with the addition of a 6xHis on the 5’ end. Then, the PCR product was inserted at BamHI and EcoRI sites of the pGEX-2T vector (GE Healthcare), which has GST tag and a thrombin cleavage site upstream of the ORF. The GST-DEP-6xHis protein was expressed E. coli and purified by glutathione chromatography as previously described (Narayanan et al., 2007). Next, fusion protein bound to glutathione beads was incubated with thrombin at room temperature for 4 h with a thrombin to fusion protein ratio of 1:500 in thrombin cleavage buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂ and 0.1% 2-mercaptoethanol, pH 8.6). The supernatant was collected and DEP-6xHis was purified by Ni-NTA resin chromatography (GE Healthcare) as described in (Crowe et al., 1994), and the resulting eluate used for immunization. The final bleed was used at a 1:5,000 dilution to detect recombinant RGS7 and RGS7\textsuperscript{ARGS} using SuperSignal® West Femto substrate (Thermo Scientific) with a 1:75,000 dilution of secondary HRP-conjugated anti-rabbit antibody.
2.2 Antibodies, compounds and reagents. Polyclonal antibodies were raised against the N-terminus, SGS and CT215, or the C-terminus, ATDG, of Gβ5 in rabbit as previously described (Watson et al., 1994; Zhang and Simonds, 2000; Zhang et al., 1996). Mouse monoclonal antibodies for GFP and HA-tag were purchased from Genscript and Santa Cruz Biotechnology, respectively. A monoclonal guinea pig antibody directed against insulin was purchased from Dako. Secondary antibodies conjugated to FITC (anti-rabbit) or Cy3 (anti-mouse) were acquired from Jackson Laboratories. Carbamylcholine chloride, McN-A-343, oxotremorine-m, pilocarpine hydrochloride, nifedipine, 2-APB and pertussis toxin were purchased from Sigma-Aldrich. The selective Gq/11 inhibitor UBO-QIC was procured from the Institute of Pharmaceutical Biology University of Bonn (www.pharmbio.uni-bonn.de/signaltransduktion). HBSS with or without Ca\(^{2+}\), Lipofectamine 2000\(^\circledR\) transfection reagent, and fura-2AM were acquired from Life Technologies. Glutathione and Ni-NTA resins were purchased from GE Healthcare. SuperSignal\(^\circledR\) West Fempto substrate and FLIPR\(^\circledR\) membrane potential dye were acquired from Thermo Scientific and Molecular Devices, respectively.

2.3 Cell culture, transfection and lysate preparation. Chinese hamster ovary (CHO-K1) cells were cultured in F-12K nutrient mixture with 10% fetal bovine serum and penicillin/streptomycin. Twenty-four hours prior to transfection, cells were seeded on 10 cm plates for pull-down and FLPR Ca\(^{2+}\) experiments, 12 mm glass coverslips for Ca\(^{2+}\) imaging or T-75 flasks for membrane potential assay to achieve 50-75% confluency at the time of transfection. Lipofectamine 2000 transfection reagent was used according to manufacturer guidelines. The Lipofectamine:DNA ratio was 2:1 for all experiments. The
total amount of DNA for 12 mm coverslip transfections was 0.5 μg and was scaled-up according to relative surface for transfection in larger vessels. The ratio of Gβ5 to RGS7 plasmid DNA was always 1:3. For pull-down experiments, constructs encoding YFP-DEP or Gβ5 and RGS7$^{R249}$ were used. At 48 h post-transfection, cells were washed in ice-cold PBS and scraped in a hypotonic lysis buffer containing 20 mM Tris-HCl, 5 mM NaCl, 2 mM MgCl$_2$, 1 mM DTT, 100 mM EDTA, 100 mM EGTA and protease inhibitors, followed by two freeze-thaw cycles at -80°C and centrifugation at 20,000×g for 45 m at 4°C. The resulting supernatant was collected and used in the GST-pulldown assay. Fresh lysate was prepared for each experiment. For Ca$^{2+}$ imaging, to identify transfected cells I co-transfected a vector encoding only EYFP. The DNA ratio of YFP to M3R to Gβ5 to RGS7 was 0.1:1:1:3 with a total of 0.51 μg of plasmid DNA per well. Empty pcDNA3.1 or LacZ vector DNA was used to ensure constant DNA loading in co-transfections. Forty-eight hours after transfection cells were used for Ca$^{2+}$ imaging or immunofluorescence studies. For membrane potential assay, cells were 80-90% confluent at the time of transfection. The ratio of DNA to Lipofectamine2000 was 1:2 with a total of 18.75 μg DNA per T-75 flask. The plasmid ratio of M3R to Gβ5 to RGS7 was 1:1:3.

2.4 Constructs for mammalian expression. The cDNA for Gβ5 and RGS7 was cloned into the pcDNA3 vector at BamHI and NotI sites as described previously (Narayanan et al., 2007). The RGS7$_{ΔRGS}$ construct lacking the RGS domain was generated by PCR amplification of nucleotides 1-1310 and cloned into pcDNA3 vector linearized with BamHI and NotI. Constructs encoding human M3R and HA-M3R in pcDNA3.1 were
purchased from cDNA.org. These constructs were used for subsequent cloning of M3R mutants, M3R\text{Δ}^{304-390} and M3R\text{TP/LP}, by PCR utilizing flanking primers along with mutant primer sets. The resulting PCR products were inserted back into pcDNA3 vector at BamHI and XbaI restriction sites.

2.5 Cloning of GST-fusion proteins. The M3R cytoplasmic tail (M3CT) spanning amino acids 548-590 was cloned using PCR into the pGEX-2T vector (GE Healthcare) at BamHI and EcoRI sties. The resulting GST-M3CT construct was used to create the following mutants:

a) GST-M3CT-K→A - Three Lys residues, K565-567, were substituted with Ala
b) GST-M3CT-NT – Contains the first half of the c-tail, residues 548-567, including the polybasic region.
c) GST-M3CT-CT – Starts after the polybasic stretch and spans the second half of the c-tail, amino acids 568-690.
d) GST-M3CT-TP/LP – Amino acids at positions T553/L558 were substituted with Pro.

2.6 Purification of GST-fusion proteins from bacterial cells. I purified GST fusion proteins that were expressed in \textit{Escherichia coli} on glutathione beads using a protocol I adapted from (Narayanan et al., 2007). Briefly, 1 L bacterial cultures were grown to an OD\textsubscript{600} of 1.0 at 37 °C. Protein expression was induced with the addition of 0.4 mM IPTG for 1.5–2 h at 30 °C. Cells were pelleted and stored at −80 °C until further use. Pellets were resuspended in BugBuster® Master Mix lysis buffer (Novagen) supplemented with
protease inhibitors (Complete, Roche). Protein solubilization was achieved by adding the ionic detergent N-Lauroylsarcosine to 1.5% for 10 min at room temperature. N-Lauroylsarcosine was sequestered by Triton X-100 (final concentration of 2%) and the lysate was centrifuged at 19000×g for 30 min at 4 °C. Glutathione–Sepharose4B beads (GE) were incubated with lysate for 1 h and washed with PBS. Bound GST fusion proteins were eluted with 20 mM glutathione and desalted on Sephadex G-25 pre-equilibrated with buffer containing 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 15% glycerol, and stored frozen in aliquots at −80 °C. The concentration was determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard. The concentration of the frozen GST-M3R protein stock was 2.5 mg/mL (~65 μM). The purity of GST fusions was above 90% as verified by SDS–PAGE.

2.7 GST pull-down assay. As previously described (Narayanan et al., 2007), Glutathione–Sepharose 4B beads were prewashed with PBS plus 0.1% CHAPS, incubated at 4 °C with purified recombinant GST or the GST fusion proteins for 1 h, and washed three times with PBS plus 0.1% CHAPS to remove excess protein. The slurry was incubated for 1–2 h at 4 °C on a rotary shaker with the various pre-cleared lysates as determined by the experiment. At the end of the incubation, the beads were settled by gravity, and the supernatant was collected as the unbound fraction. The resin was extensively washed and subsequently eluted with the addition of SDS-containing sample loading buffer. In a typical assay, the packed volume of the GST resin was 30 μL, the amount of loaded GST fusion protein was 10 μg, and the volume of the protein lysate was 300 μL. The total protein concentration in transfected cell lysates was 2.5–5.0
mg/mL. The beads were washed three times with 600 μL of PBS plus 0.1% CHAPS buffer and eluted with 30 μL of 2× SDS sample loading buffer. The unbound and eluted fractions were resolved by gel electrophoresis and analyzed by western blotting with chemiluminescent detection. Films were scanned and densitometric measurements of the bands were calculated using ImageJ software.

2.8 Western blot analysis and Immunofluorescence. Protein samples were denatured in 5× Laemli buffer containing DTT and placed in a 65°C water bath for 5 min. As required by experiment, denatured samples were loaded on 8% or 12% acrylamide gels and exposed to a 25 mA/gel current in a Tris-Glycine gel running buffer. When the dye-front had reached the bottom of the gels, the acrylamide gels were laid over nitrocellulose paper and placed in a transfer chamber containing 2.6 mM NaHCO3 at 300 mA 1.5 h or 30 mA for overnight transfers. Blocking solution contained 5% milk or 1% BSA in Tris buffered saline plus tween (TBS-T) and was applied for 1 hr at room temperature or overnight at 4°C. Typical antibody incubations were the same as the blocking step with four 5 min washes with TBS-T in between each incubation. Secondary antibodies were conjugated to horseradish peroxidase (HRP) to enable chemiluminescent detection by exposure to light-sensitive film and developing. For immunofluorescence, transfected CHO-K1 cells grown on glass coverslips or tissue sections were fixed with 4% paraformaldehyde and incubated for 30 min in a blocking buffer containing 1% BSA in PBS with or without 0.1% Triton-X100, as determined by the experiment. Primary and secondary antibodies were diluted in blocking buffer and incubated with fixed cells for 30 m each with three washes with PBS in between and after antibody incubations.
Coverslips were affixed to glass slides using ProLong Gold Anti-fade with DAPI. After overnight drying, slides were ready for imaging.

2.9 Preparation of mouse adrenal gland and pancreatic tissue slices. All mice were humanely treated in accordance with guidelines set forth by IACUC and in our approved animal protocol. Age and sex matched mice were anesthetized with isoflurane prior to cervical dislocation. Adrenal glands and pancreas were dissected and placed in ice cold PBS. Visceral fat was trimmed away from the organs and then the tissue was fixed in 4% paraformaldehyde for 30 min at 4°C. The fixed tissues were then prepared for cryo-embedding by consecutive incubations in increasing concentrations (10%, 20% and 30%) of sucrose PBS. Sucrose saturated tissue was placed in a mold containing embedding medium and immediately placed in a -80°C freezer. Sections ranging from 10 – 18 µm in thickness were obtained using a cryostat in the DRI Core Imaging facility at UMMSM. Tissue sections were placed on Fisher Scientific Colofrost Plus glass slides with three tissue sections per slide. Slides were placed on a slide warmer set to 42°C for 20 min followed by a wash in PBS to remove residual embedding medium. Staining procedures were identical to that which was carried out on transfected cells as described above.

2.10 Ca²⁺ fluorescence microscopy. Transiently transfected CHO-K1 cells grown on 12 mm glass coverslips were washed with culture media then incubated at 37°C in culture media containing 2 µM fura-2AM for 25 min. After fura-2AM loading, the cells were kept at ambient temperature for no longer than 1.5 hrs before imaging. Coverslips were
secured in a flow chamber and mounted on the stage of a Nikon TE2000 inverted fluorescence microscope. The cells were continuously superfused by gravity flow with HBSS either with or without CaCl$_2$/MgCl$_2$. As required by the experiment, flow was switched to agonist containing HBSS for a specified time then changed back to agonist free buffer. Images were collected in real time every three seconds using a 20X UV objective lens and recorded using Metafluor software. The excitation wavelengths were 340 (Ca$^{2+}$ bound) and 380 (Ca$^{2+}$ free) nm, and the emission was set to 510 nm. The 340/380 ratio is representative of intracellular free [Ca$^{2+}$]. The entire field of view was selected as a region of interest (ROI). A typical ROI contained 50-70 cells, of which, 30-50 were YFP-positive. The number of cells responding to muscarinic agents varied with agonist concentration, but was typically 30 cells in a particular ROI. Traces shown here are averages of 2-4 independent experiments with three replicate coverslips per experiment.

2.11 FLIPR membrane potential assay. Twenty-four h post transfection, cells were detached with TrypLE Express (Life Technologies) and seeded in a 384-well, black-walled, clear-bottomed plate (Corning) at a density of 10,000 cells per well and allowed to grow for twenty-four hours at 37°C, 5% CO2 and 95% relative humidity (RH). As described previously (Baxter et al., 2002), cells were washed 3 times with assay buffer HBSS containing 20 mM HEPES, pH 7.4 (HHBSS). Then, 25 µl of assay buffer supplemented with 1.26 mM CaCl$_2$ was added to each well, and cells were allowed to equilibrate at room temperature for 10 min. Finally, 25 µl of blue FLIPR® Membrane Potential Reagent (Molecular Devices), reconstituted per the manufacturer’s instructions
(final concentration of 20 µM) was added to the appropriate wells. The plate was
incubated at 37°C for 30 min, and then placed into the Fluorometric Imaging Plate
Reader (FLIPR®) Tetra (Molecular Devices). A baseline read of plate fluorescence (510-
545 nm excitation and 565-625 nm emission) was performed for 10 s. Next, HHBSS
alone or CCh dissolved in HHBSS was dispensed using an integrated 384-well dispenser
to the appropriate wells, during fluorescence measurements. Fluorescence was recorded
every second for the remaining 590 seconds of the assay.

2.12 FLIPR Ca²⁺ imaging

Cells were transfected in 10 cm dishes. At 24 h post-transfection, cells were seeded in a
black-walled 384-well plate at a density of 10,000 cells per well. The Fluo-8 No Wash
Calcium Assay kit (Abcam, ab112129) was used according to manufacturer’s
recommendations. With this system there is no washing step after incubation with the
Ca²⁺ indicator as there is a quenching dye that is not membrane permeant thus reducing
the signal of extracellular dye. Briefly, growth media was removed cells and were
washed with Ca²⁺-free HHBSS. A 2X solution of the Fluo-8 dye was prepared in Ca²⁺-
free HHBSS and 1% plurionic acid. Equal volumes of HHBSS and Fluo-8 were added to
the cells and placed in the 37°C incubator for 30 min. The plate was then allowed to
equilibrate to atmospheric conditions for 20 min before analysis with the FLIPR tetra
system. A baseline read of plate fluorescence of 10 s was performed prior to addition of
agonist. After agonist application, fluorescence intensity was recorded every s for at least
3 m.
2.13 Pharmacological chaperoning

A pharmacological chaperone is a membrane permeant GPCR ligand that binds to and stabilizes misfolded GPCRs allowing them to be targeted to the membrane. For experiments with the M3R\textsuperscript{TP/LP} mutant, which was not expressed on the plasma membrane, atropine was used to increase membrane expression of the receptor. Atropine is a membrane permeant muscarinic antagonist, so the receptor will not be activated under these conditions. Cells were transfected 32 h later, growth media was replaced with one containing 100 nM atropine. Cells were returned to the 37°C incubator for 16 h before imaging or flow cytometry studies.

2.14 Flow cytometry

For the analysis of M3R surface expression, CHO-K1 cells were transfected with HA-tagged WT or mutant M3R. Forty-eight hours after transfection, cells were rinsed with PBS, detached by incubation with 1mM EGTA and 5 mM EDTA in PBS. After cells were washed once in 1 ml of staining buffer (PBS pH7.2, 0.5% BSA, 2 mM EDTA) they were resuspended in 100 ul of staining buffer with 10 ul of mouse anti-HA phycoerythrin-conjugated antibody (130-092-257, Miltenyi Biotec GmbH) and incubated for 10 min in the dark at 8°C. Cells were washed in 2 ml of staining buffer by centrifugation at 300xg for 5 min and resuspended in 1 ml of 0.5% formaldehyde in PBS. Flow cytometry was performed using Becton Dickinson LSR II instrument with 10,000 events acquired per sample.
2.15 Pertussis toxin (PTX) and UBO-QIC (Gq inhibitor) treatment

PTX was applied to cells transfected with pcDNA or M3R or M3R and Gβ5-RGS7 4 h prior Ca²⁺ imaging at the final concentration of 200 ng/ml in culture media and incubated at 37°C. This concentration was maintained during fura2-AM loading in the last 30 m of the 4 h incubation. To validate the activity of PTX, cells were stimulated with 5-hydroxytryptamine (5-HT) to activate endogenous Gi-coupled 5-HT1B receptors that stimulate Ca²⁺ release through a βγ-mediated mechanism. UBO-QIC was dissolved in DMSO to a stock concentration of 2 mM and stored at -20°C in 20 µl aliquots. Incubation with UBO-QIC was carried out in the presence of fura2-AM for 30 m at 37°C at a final concentration of 0.3 µM.

2.16 – 2.17 Biophysical analysis of M3R helix 8 peptides (CD, molecular modelling and dynamics) was done in collaboration with the Farooq Lab (UMMSM)

2.16A Peptide synthesis

20-mer wildtype (WT) and mutant (TP/LP) peptides derived from the C-terminal tail (residues 548-567) of CHRM3 were commercially obtained from GenScript Corporation. The amino acid sequence of these peptides is shown below:

WT  548-NKTFRTTFTKMLLCQCDKKK-567
TP/LP  548-NKTFRPTFKMPMLLCQCDKKK-567

Note that the T553/L558 residues and their Pro counterparts are shown in red.

2.16B Circular dichroism

Far-UV circular dichroism (CD) measurements were conducted on a Jasco J-815 spectropolarimeter thermostatically controlled at 25°C. Briefly, WT and TP/LP peptides
were dialyzed in 10mM sodium phosphate at pH 7.0 and experiments were conducted on 50μM sample of each peptide alone in solution or in the presence of 5mM n-dodecylphosphocholine (DPC) bicelles. Data were collected using a quartz cuvette and normalized against reference spectra to remove the contribution of buffer. Each data set represents an average of four scans acquired at 0.1 nm intervals.

2.17A Molecular modeling

Structural models of WT and TP/LP peptides (residues 548-567) were built using the MODELLER software based on homology modeling (Marti-Renom et al., 2000). In each case, the crystal structure of M3R (PDBID 4DAJ) harboring helix 8 (residues 548-556) was used as a template (Kruse et al., 2012). For each peptide, a total of 100 atomic models were calculated and the structure with the lowest energy, as judged by the MODELLER Objective Function, was selected for further analysis. The structural models were rendered using RIBBONS (Carson, 1991).

2.17B Molecular dynamics

Molecular dynamics (MD) simulations were performed with the GROMACS software (Van Der Spoel et al., 2005) using the integrated AMBER99SB-ILDN force field (Hornak et al., 2006; Lindorff-Larsen et al., 2010). Briefly, the structural models of WT and TP/LP peptides (residues 548-567) were each centered in a cubic box and explicitly hydrated with a water layer that extended 10Å (box size) from the protein surface along each orthogonal direction using the extended simple point charge (SPC/E) water model (Berendsen et al., 1987; Toukan and Rahman, 1985). The ionic strength of solution was
set to 100mM with NaCl and the hydrated structures were energy-minimized with the steepest descent algorithm prior to equilibration under the NPT ensemble conditions, wherein the number of atoms (N), pressure (P) and temperature (T) within the system were kept constant. The Particle-Mesh Ewald (PME) method (Darden et al., 1993) was employed to compute long-range electrostatic interactions with a spherical cut-off of 10Å and a grid space of 1.6Å with a fourth order interpolation. The Linear Constraint Solver (LINCS) algorithm was used to restrain bond lengths (Hess et al., 1997). For the final MD production runs, data were collected every ns over a time scale of 1μs. All MD simulations were performed on a Linux workstation using parallel processors at the High Performance Computing (HPC) facility within the Center for Computational Science (CCS) of the University of Miami.

2.18 TR-FRET based assay for cAMP accumulation

In order to assess Gs activation, I used the Perkin Elmer LANCE Ultra cAMP competitive immunoassay kit. In this assay, the interaction between anti-cAMP monoclonal antibodies labeled with the ULight™ dye and Europium-labeled cAMP (Eu-cAMP) is utilized for time-resolved fluorescence resonance energy transfer (TRFRET)-based detection of M3R activity. When ULight™-labeled cAMP antibody binds to Eu-cAMP, there is energy transfer from the Eu-cAMP molecule to the ULight™-labeled-antibody, thereby increasing well FRET. When Gs is activated, unlabeled cellular cAMP will compete with the labeled cAMP for binding to the ULight™-labeled cAMP-antibody, thereby reducing FRET. To perform the assay, 5,000 cells in 10 μl of cell
buffer (HBSS, 25 mM HEPES and 0.1% BSA) were seeded onto 384-well microtiter plates. Then, 5μl of test compound prepared in assay buffer (0.1% BSA, 25mM Hepes and 500μM IBMX) or buffer alone was added to the appropriate wells and plates were incubated at room temperature for 30 minutes. After this incubation, 5 μl of Eu-cAMP (prepared in lysis buffer according to the manufacturer's protocol) followed by 5 μl of ULight™-labeled-anti-cAMP (also prepared in lysis buffer according to the manufacturer's protocol) was added to each well and incubated for 1 hour at room temperature. FRET measurements were performed using the Envision microplate reader (Perkin Elmer) at the following wavelengths: 340 nm (with 30 nm bandwidth) excitation, and 671 nm (with 4 nm bandwidth) emission. These data are presented as percent response, using 10μM Forskolin treated cells as 100% control and Buffer treated cells as 0% control according to the formula % Response = 100 x \[(\text{Negative control})-\text{Sample}] / [(\text{Negative control})-(\text{Positive control})].
CHAPTER 3

RESULTS

3.1 Signal transduction through M3R and its regulation by Gβ5-RGS7 in CHO-K1 cells

Previous studies in the Slepak Lab showed that Gβ5-RGS7 could attenuate M3R-mediated increases in free intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) in co-transfected CHO-K1 cells independent of its GAP activity (Narayanan et al., 2007; Sandiford and Slepak, 2009; Sandiford et al., 2010). I chose CHO-K1 cells as my model system since they do not endogenously express M3R or any other cholinergic receptors, ensuring that the Ca\(^{2+}\) signal produced by application of muscarinic agonist was initiated by transfected M3R. As expected, Gβ5-RGS7 reduced the CCh-stimulated response, however, I noticed that the effect was smaller than reported earlier in the lab and there was high variability between samples. I first hypothesized that agonist efficacy was related to sensitivity of M3R-stimulated Ca\(^{2+}\) signaling to regulation by the Gβ5-RGS7 complex. For example, a signal produced by a partial agonist may be more sensitive to attenuation by Gβ5-RGS7. To test this hypothesis I compared the responses elicited by the partial agonist pilocarpine with those stimulated by the full agonist CCh in the presence or absence of Gβ5-RGS7. Concentration dependency curves were obtained for CCh and pilocarpine in the presence or absence of Gβ5-RGS7 (Fig. 3.1). Gβ5-RGS7 reduced the Emax for CCh (Fig. 3.1A) and pilocarpine (Fig. 3.1B) responses to 56% and 37% of M3R alone, respectively. The EC\(_{50}\) for both CCh and pilocarpine in the presence of Gβ5-RGS7 were similar to the EC\(_{50}\) values in the absence of the complex, indicating that the complex does not affect agonist...
affinity. This result shows that responses elicited by a partial agonist are more sensitive to attenuation by the Gβ5-RGS7 complex.

To begin to understand how the complex is able to differentially regulate responses stimulated by pilocarpine and CCh, I more closely examined the M3R signal transduction pathway. There are two routes of Ca\(^{2+}\) entry that contribute to the overall rise in intracellular Ca\(^{2+}\) concentration initiated by muscarinic agonists: 1) Ca\(^{2+}\) release from intracellular stores and 2) Ca\(^{2+}\) influx from the extracellular space to the cytoplasm. It has been noted distinct muscarinic agonists are known to differentially activate Ca\(^{2+}\) influx and release (Hishinuma et al., 1997; Schaafsma et al., 2006).

Figure 3.1 Stimulation of M3R with pilocarpine renders it more sensitive to Gβ5-RGS7 regulation. CHO-K1 cells were grown on 12 mm glass coverslips and transfected with constructs encoding M3R or M3R + Gβ5-RGS7 and prepared for Ca\(^{2+}\) imaging as described in Subchapter 2.10. Concentration-dependencies were obtained for M3R or M3R + Gβ5-RGS7 stimulated with CCh (A) and pilocarpine (B). Non-linear regression curves were fit using GraphPad Prism sigmoidal dose-response (variable slope) equation. Each data point represents the mean amplitude of the maximal response ± SD (n=4).
Effects of Gβ5-RGS7 on M3R-induced Ca²⁺ influx and release

To investigate the effect of Gβ5-RGS7 on the different routes of Ca²⁺ entry, I first compared CCh-induced M3R activation in the presence and absence of extracellular Ca²⁺ (Fig. 3.2). In the absence of extracellular Ca²⁺, rises in [Ca²⁺]ᵢ, are released from the intracellular pool and thus, are denoted as Ca²⁺ release. I found that in the presence of extracellular Ca²⁺, co-transfection with Gβ5–RGS7 resulted in a 10-30% reduction in the amplitude of the Ca²⁺ response (Fig. 3.2A). However, attenuation of the Ca²⁺ response by the Gβ5-RGS7 complex was more pronounced in the absence of extracellular Ca²⁺, reaching 55% inhibition. This experiment demonstrates that Gβ5-RGS7 selectively attenuates Ca²⁺ release and may have a positive effect on Ca²⁺ influx.

Figure 3.2 Gβ5-RGS7 selectively attenuates M3R stimulated Ca²⁺ release. CHO-K1 cells grown on glass cover slips were transiently transfected with M3R or M3R + Gβ5-RGS7 and loaded with fura-2AM. Fluorescence images were recorded in real time using MetaFluor software as described in Chapter 2.10. The entire visual field, containing 20-40 cells responding to muscarinic agonists, was used as a region of interest. The traces represent a mean of the 340/380 ratios recorded in at least two independent experiments, with 3-6 replicates per experiment. (A) CCh (1 μM) was applied for 30 s as indicated by the horizontal bar above the traces in the presence or absence of co-transfected Gβ5-RGS7 in complete HBSS buffer containing 1.26 mM CaCl₂. (B) Cells were stimulated with CCh in Ca²⁺-free HBSS. (C) The mean amplitude ± SD of the maximal responses (n=9) with and without extracellular Ca²⁺. Black bars represent cells transfected with M3R and pcDNA3 plasmid. Tan bars represent M3R co-transfected with Gβ5 and RGS7.
Experimentally isolating the M3R-stimulated Ca\(^{2+}\) influx response is not as straightforward as Ca\(^{2+}\) release. I wanted to use an approach that would not affect M3R signaling or cell viability. CHO-K1 cells endogenously express ionotropic and Gq-coupled purinergic receptors (Iredale and Hill, 1993; Marcet et al., 2004). In Ca\(^{2+}\)-free buffer, I applied 10 \(\mu\)M ATP, which stimulates Ca\(^{2+}\) release through a Gq-

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**Figure 3.2 G\(\beta\)5-RGS7 augments M3R stimulated Ca\(^{2+}\) influx.** Transfected CHO-K1 were cells loaded with fura-2 and superfused with 10 \(\mu\)M ATP in Ca\(^{2+}\)-free buffer to deplete intracellular Ca\(^{2+}\) stores. (A) Subsequent stimulation with CCh (time 260 s) in Ca\(^{2+}\)-free buffer resulted in no measurable rise in [Ca\(^{2+}\)]. (B) Following ATP-stimulated Ca\(^{2+}\) depletion as in A, cells were treated with Ca\(^{2+}\) containing buffer (gray box) in the absence of CCh. (C) Cells were treated with ATP as in A and B, but 1 mM CCh was delivered in Ca\(^{2+}\)-containing HBSS buffer. The traces in A, B and C represent an average of three independent experiments with 3 (A and B) and 5 (C) replicates per experiment. (D) The mean amplitude ± SD of the maximal responses for total Ca\(^{2+}\) response (shown in Fig. 3.2A), Ca\(^{2+}\) release (Fig. 3.2B) and Ca\(^{2+}\) influx (Fig. 3.3C).
mediated mechanism, leading to depletion if the intracellular Ca\(^{2+}\) pool (Fig. 3.3). As expected, ATP-pretreated cells did not respond to CCh in Ca\(^{2+}\)-free medium (Fig. 3.3A), demonstrating complete depletion of Ca\(^{2+}\) stores. After ATP-induced Ca\(^{2+}\) depletion, treatment of cells with Ca\(^{2+}\)-containing buffer did not result in any significant signal (Fig. 3.2B), indicating that the plasma membrane Ca\(^{2+}\) channels opened by ATP treatment were now closed. However, CCh in Ca\(^{2+}\) containing HBSS buffer induced a marked increase in [Ca\(^{2+}\)]_i, showing that stimulation of M3R induces Ca\(^{2+}\) influx after ATP-induced Ca\(^{2+}\) store depletion (Fig. 3.2C). Under these conditions, G\(\beta\)5-RGS7 augmented the CCh-mediated Ca\(^{2+}\) influx response. Taken together with the finding in Fig. 3.2, this result demonstrates that the G\(\beta\)5-RGS7 complex has contrasting effects on the two routes of Ca\(^{2+}\) entry to the cytoplasm: it inhibits release from intracellular stores (Fig. 3.2) and stimulates influx across the plasma membrane (Fig. 3.3). The positive effect of G\(\beta\)5-RGS7 was not known and is perhaps the most significant finding in my dissertation work.

Evidently, when Ca\(^{2+}\) is present in both extracellular medium and intracellular stores, the net effect of G\(\beta\)5-RGS7 on CCh-induced increases in [Ca\(^{2+}\)]_i is slightly negative (Fig. 3.2A; Narayanan et al., 2007; Sandiford and Slepak, 2009; Sandiford et al., 2010). Since Ca\(^{2+}\) influx is essential in many cellular processes including neurotransmitter release, the novel effect of G\(\beta\)5-RGS7 on M3R-stimulated Ca\(^{2+}\) influx was particularly interesting. Thus, I focused on delineating the mechanisms involved in this pathway.
The action of Gβ5-RGS7 involves Gq, but not Gi

It is well established that M3R couples to Gq, however Gi-coupling has been observed in CHO-K1 overexpressing M3R (Burford et al., 1995a). To rule out the possibility that Gβ5-RGS7-enhanced Ca²⁺ influx is mediated through GAP activity on Gi, I used three separate approaches. It is important to note that only the influx component was assayed for sensitivity to inhibitors and mutants. First, I blocked Gi with pertussis toxin (PTX) prior to stimulating Ca²⁺ influx with CCh. As a positive control for PTX, I assessed the activity of endogenous 5-HT1B receptors, which couple to Gi and mobilize Ca²⁺ though a βγ-mediated mechanism ((Dickenson and Hill, 1996); (Fig. 3.4A)). Cells transfected with pcDNA or Gβ5-RGS7 were prepared for Ca²⁺ imaging. Stimulation with 5-HT produced a Ca²⁺ transient that was inhibited in the presence of Gβ5-RGS7 (Fig. 3.4A). Inhibition of 5-HT-stimulated Ca²⁺ mobilization by Gβ5-RGS7 is expected since this complex is a GAP for Gi (Hooks et al., 2003). When cells are pre-treated with PTX

![Figure 3.4 5-HT-stimulated Ca²⁺ response in CHO-K1 cells is sensitive to PTX.](image-url)
as described in Subchapter 2.15, the 5-HT-stimulated Ca\(^{2+}\) response is dramatically inhibited, demonstrating that PTX is active (Fig. 3.4B).

Cells expressing M3R or M3R and G\(\beta\)5-RGS7 were depleted of intracellular Ca\(^{2+}\) as in Fig. 3.3 and stimulated with CCh (Fig. 3.5). Pre-treatment with PTX had no effect on M3R-stimulated Ca\(^{2+}\) influx or the ability of the G\(\beta\)5-RGS7 complex to augment M3R-stimulated Ca\(^{2+}\) influx (Fig. 3.5A, B). Taken together, these results indicate that M3R-stimulated Ca\(^{2+}\) influx is not mediated by Gi and this mechanism of G\(\beta\)5-RGS7 action does not involve Gi activation.

**Figure 3.5** CCh-stimulated Ca\(^{2+}\) influx is not sensitive to PTX. CHO-K1 cells were transfected with M3R or M3R + G\(\beta\)5-RGS7 and treated with PTX as described in Figure 3.4. Transfected cells were stimulated with ATP in Ca\(^{2+}\) free buffer to deplete intracellular Ca\(^{2+}\) before stimulation with CCh in the presence of extracellular Ca\(^{2+}\). The traces shown are after ATP-stimulated Ca\(^{2+}\) depletion. (A) Cells were not pre-treated with PTX prior to Ca\(^{2+}\) imaging. (B) Cells were pre-treated with PTX for 4 h before Ca\(^{2+}\) imaging. Each trace is the mean ratio of three coverslips from two independent experiments.
Next, I deleted the RGS domain (RGS7ΔΔRGS) to determine if GAP activity of the complex was required (Fig. 3.6). I inserted a stop codon into the linker region between the GGl and RGS domains using PCR. Prior to my work in the lab, the only antibody that detected RGS7 was raised against the RGS domain, and therefore would not detect the RGS7ΔΔRGS mutant. Fortunately, as part of my earlier attempts to characterize a commercially acquired “RGS7 knockout” mouse, I raised an antibody against the DEP domain if RGS7 (Materials and Methods, Chapter 2.1). As seen in Fig. 3.6A, the DEP antiserum is able to detect full-length RGS7 which has a molecular weight of about 55 kD. As expected, the RGS7ΔΔRGS runs at a molecular weight of about 39 kD and is expressed at the same level as WT (Fig. 3.6A). When this mutant was used in the M3R-stimulated Ca2+ influx assay, the Ca2+ response was not significantly different from WT RGS7 response (Fig. 3.6B). These results indicate that the RGS domain of RGS7 is not necessary for the complex to enhance M3R-stimulated Ca2+ influx.

Figure 3.6 The RGS domain is not necessary for the complex to enhance M3R-stimulated Ca2+ influx. To determine the necessity of the RGS domain in facilitating CCh-stimulated Ca2+ influx, I constructed a RGS7 RGS domain deletion mutant, RGS7ΔΔRGS. (A) I assessed transfection efficiency and expression of RGS7ΔΔRGS in transiently transfected CHO-K1 cells by western blot analysis with a DEP antibody I made for another project. The RGS7ΔΔRGS mutant runs at a molecular weight of about 39 kD which is expected with deletion of 130 amino acids and expression level is comparable to WT. (B) Cells transfected with M3R, M3R + Gβ5-RGS7 or M3R + Gβ5-RGS7ΔΔRGS were prepared for Ca2+ imaging of CCh-stimulated Ca2+ influx. Traces shown are after depletion of intracellular Ca2+ with ATP. Each trace is the mean of three coverslips from two independent experiments.
required for enhancing M3R-stimulated Ca\(^{2+}\) influx and it is therefore unlikely this effect is facilitated by a Gi GAP mechanism.

Next, I pharmacologically inhibited Gq with UBO-QIC, a cyclic depsipeptide also known as FR900359 (Zaima et al., 2013). This plant depsipeptide is closely related to the better described compound YM254890 from Chromobacterium sp, which blocks Gq signaling by direct binding to Gq and inhibition of GDP release (Takasaki et al., 2004). UBO-QIC completely blocked the Ca\(^{2+}\) response to M3R stimulation (Fig. 3.7B). Together, these data confirm that the effect of G\(\beta\)5-RGS7 on M3R-induced Ca\(^{2+}\) signaling is dependent on a Gq/11- but not a Gi-mediated mechanism.

![Figure 3.7 CCh-stimulated Ca\(^{2+}\) signaling in CHO-K1 cells overexpressing M3R is solely dependent on Gq.](image)

Sometimes in overexpression systems, coupling specificity of a GPCR can be lost. To investigate the magnitude of the Ca\(^{2+}\) signal that is mediated by a Gq mechanism in my system, I used the Gq inhibitor, UBO-QIC. Cells were transfected with M3R or M3R + G\(\beta\)5-RGS7 and prepared for Ca\(^{2+}\) imaging. (A) Control cells were not pre-treated with UBO-QIC and stimulated with 1 µM CCh. (B) Transfected cells were incubated with 100 nM UBO-QIC for 30 m during fura-2AM loading and stimulated as in A. Each trace is the mean of three coverslips from two independent experiments.
TRP channels are involved in M3R-stimulated Ca\textsuperscript{2+} influx, but are not enhanced by G\textbeta{}5-RGS7

![Figure 3.8](image)

**Figure 3.8** 

\textit{CCh-stimulated Ca\textsuperscript{2+} influx is mediated by a 2-APB-sensitive channel.} TRP channels play a major role in capacitive Ca\textsuperscript{2+} entry and some TRP channels are blocked by 2-APB. To test whether TRP channels mediate CCh-stimulated Ca\textsuperscript{2+} influx in my system I applied 2-APB before and during stimulation of Ca\textsuperscript{2+} influx. All traces shown are after cells were stimulated with ATP to deplete intracellular Ca\textsuperscript{2+}. (A) Cells transfected with M3R were stimulated with CCh in the absence (broken line) or presence (solid line) of 2-APB. (B) Cells expressing M3R and G\textbeta{}5-RGS7 were stimulated with CCh in the absence (broken line) or presence (solid line) of 2-APB. Each trace is the mean of three coverslips from two independent experiments.

Multiple channels can mediate M3R-stimulated Ca\textsuperscript{2+} influx, including several transient receptor potential (TRP) channels (Montell, 2005). There are seven subfamilies of TRP channels which are activate by diverse stimuli including ions, temperature, shear-stress, DAG and changes in the level of phosphoinositidylibisphosphate (PIP2) (Hardie, 2007). First, I tested the effect of the non-selective TRP channel inhibitor 2-APB on M3R-stimulated Ca\textsuperscript{2+} influx (Fig. 3.8). 2-APB is known to inhibit several TRP channels and also store-operated Ca\textsuperscript{2+} entry through blockade of IP3 receptors (Iwasaki et al., 2001). It is important to remember that in my Ca\textsuperscript{2+} influx assay involvement of store-operated Ca\textsuperscript{2+} channels is unlikely because all...
Ca\textsuperscript{2+} stores are emptied prior to M3R stimulation. 2-APB blocked Ca\textsuperscript{2+} influx by more than 90% irrespective of the presence of G\textbeta{5}-RGS7 (Fig. 3.8A, B). This experiment suggests that M3R-stimulated Ca\textsuperscript{2+} influx and the action of G\textbeta{5}-RGS7 are mediated by a/some TRP channel(s).

Next, I wanted investigate whether G\textbeta{5}-RGS7 could enhance TRP channel-mediated Ca\textsuperscript{2+} conductance in the absence of M3R-stimulation. If the complex could enhance Ca\textsuperscript{2+} influx under these conditions, this would strongly suggest that the G\textbeta{5}-RGS7 complex is working at the level of an ion channel.

To stimulate TRP channel opening, I used the DAG analog phorbol 12-myristate 13-acetate, PMA (Fig. 3.9). After Ca\textsuperscript{2+} stores were depleted with ATP, addition of PMA enhanced Ca\textsuperscript{2+} influx (Fig. 3.9, black line) and the presence of the G\textbeta{5}-RGS7 complex had no effect on the Ca\textsuperscript{2+} signal (Fig. 3.9, tan line). These results demonstrate that the G\textbeta{5}-RGS7 complex does not enhance DAG-activated Ca\textsuperscript{2+} channel conductance.
**Gβ5-RGS7-enhanced Ca\(^{2+}\) influx is sensitive to nifedipine, but is not voltage-dependent**

In cells where only Gq-coupled muscarinic receptors are expressed activation of voltage-dependent L-type Ca\(^{2+}\) channels is preceded by muscarinic stimulation (Boschero et al., 1995; Wuest et al., 2007). Although CHO-K1 cells are non-excitable, evidence suggests that these cells express a Ca\(^{2+}\) channel that has some characteristics of an L-type channel (Skryma et al., 1994a, b). To determine if an L-type channel is involved in M3R-stimulate Ca\(^{2+}\) influx in CHO-K1 cells, I used the dihydropyridine nifedipine. Nifedipine had little effect on M3R-stimulated influx in the absence of Gβ5-RGS7 (Fig. 3.10A, B; black lines). However, nifedipine abolished the positive effect of Gβ5-RGS7 on Ca\(^{2+}\) influx (Fig. 3.10A, B; tan lines). This set of experiments indicates that M3R-coupling to a nifedipine-sensitive channel is enhanced by the Gβ5-RGS7 complex.

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**Figure 3.11 Gβ5-RGS7 enhances coupling of M3R to a nifedipine-sensitive channel.** To investigate involvement of L-typed Ca\(^{2+}\) channels in mediated M3R-stimulated Ca\(^{2+}\) influx, I used the dihydropyridine nifedipine to block these channels. Intracellular Ca\(^{2+}\) was depleted before application of nifedipine and stimulation with CCh. (A) Cells transfected with M3R were stimulated with 1 µM CCh in the absence (broken line) or presence (solid line) of 10 µM nifedipine. (B) Cells were transfected with M3R + Gβ5-RGS7 and stimulated with CCh in the absence (broken line) or presence (solid line) of nifedipine.
Figure 3.11 Gβ5-RGS7 does not affect membrane potential at CCh concentrations used to stimulate Ca²⁺ influx. To assess changes in membrane potential stimulated by CCh in the absence and presence of Gβ5-RGS7 I used the FLIPR membrane potential assay. Hyperpolarization is denoted by a decrease in fluorescence intensity and depolarization results in an increase in fluorescence intensity. CHO cells transfected with pcDNA, M3R or M3R + Gβ5-RGS7 were incubated with blue FLIPR® Membrane Potential Reagent, as described in Chapter 2.11. Fluorescence intensity was recorded using the Fluorometric Imaging Plate Reader (FLIPR®) Tetra. At the time of 10 s, CCh was added to a final concentrations from 100 nM – 1 mM as indicated in panels A–E. The traces represent the mean changes in fluorescence intensity collected from quadruplicate wells on the 384-well plate. (F) Concentration dependencies of the maximal change in fluorescence at the last time point recorded in A–E.

Since nifedipine inhibits the positive effect of Gβ5-RGS7 (Fig. 3.10A, B), I next wanted to test whether Gβ5-RGS7 had an effect on M3R-stimulated changes in membrane potential. In collaboration with the Wahlestedt and Brothers Labs at the Center for Therapeutic and Scientific Innovation (UMMSM), I used a FLIPR-based voltage-sensitive fluorescent dye assay (Baxter et al., 2002) to assess changes in membrane potential upon muscarinic stimulation in the presence or absence of the Gβ5-RGS7 complex (Fig. 3.11). As is evident from the reduction in the fluorescence signal, at CCh concentrations below 10 μM, M3R activation caused hyperpolarization, and addition of Gβ5-RGS7 had no effect (Fig. 3.11A–C). However, at CCh concentrations above 10 μM, M3R activity caused a notable membrane depolarization, consistent with earlier reports (Carroll and Peralta, 1998), and the Gβ5-RGS7 complex significantly
attenuated this effect (Fig. 3.11D-E). Considering that neurotransmitter in the synaptic cleft can reach millimolar concentration (Scimemi and Beato, 2009) and the differential effect of micro- and milli-molar [CCh] on M3R signal transduction (Carroll and Peralta, 1998), the effect of Gβ5-RGS7 on depolarization at 0.01-1.0 mM CCh (Fig. 3.11F) is significant and deserves further investigation.

These results are puzzling since L-type Ca^{2+} channels are activated by depolarization and as demonstrated in Fig. 3.11C, stimulation of M3R with 1 µM CCh causes hyperpolarization. This suggests that the nifedipine-sensitive channel in CHO-K1 cells is not voltage-dependent in the presence of the Gβ5-RGS7 complex. It is reasonable a reasonable assumption that such a channel exists since it has been shown that nifedipine can block other channels, a voltage-activated K^+ channel (Kv1.5) and a non-selective cation channel (Curtis and Scholfield, 2001; Zhang et al., 1997).

**Sensitivity of M3R to Gβ5-RGS7 regulation is determined by distinct agonists**

The action of the Gβ5-RGS7 complex requires the DEP domain of RGS7 and the i3 loop and C-tail of M3R, suggesting that receptor conformation is an important factor for recognition by the Gβ5-RGS7 complex (Sandiford et al., 2010). As detailed in Subchapter 1.1, GPCR ligands are capable of stabilizing a particular receptor conformation which can alter its affinity for G proteins and other accessory proteins. This, along with my finding that pilocarpine-stimulated Ca^{2+} signaling is more sensitive to Gβ5-RGS7 (Fig. 3.1), led to my hypothesis that an agonist may stabilize a receptor conformation that is more or less sensitive to regulation by the Gβ5-RGS7 complex.
To test this hypothesis I stimulated M3R with muscarinic agonists of varying efficacies (Richards and van Giersbergen, 1995) in the absence or presence of Gβ5-RGS7 (Figs. 3.12-3.16). As in Figures 3.2-3.3, I dissected the Ca^{2+} release and influx pathways stimulated by each agonist. I found that Ca^{2+} influx and release responses elicited by the tested compounds exhibited varying degrees of sensitivity to Gβ5-RGS7. First, I used the endogenous ligand acetylcholine (ACh), which is endowed with nearly the same efficacy as CCh and is structurally very similar (Fig. 3.16; Table 3.1). Application of ACh produced a Ca^{2+} signal with a magnitude that was similar that of CCh, but was more sensitive to Gβ5-RGS7 attenuation (Fig. 3.12A). The release component that ACh elicited was about half of the total response measured in Figure 3.12A and was attenuated by Gβ5-RGS7 (Fig. 3.12B). However, Gβ5-RGS7 was not able to enhance Ca^{2+} influx stimulated by ACh (Fig. 3.12C). This can explain why the total response (Fig. 3.12A) elicited by ACh is more sensitive to Gβ5-RGS7 as compared to CCh.

Figure 3.12 Gβ5-RGS7 attenuated acetylcholine-stimulated Ca^{2+} release, but does not enhance influx. I used 10 μM ACh to elicit M3R mediated Ca^{2+} release and influx as described in Figs. 3.2 and 3.3, respectively. (A) Total ACh response in complete HBSS buffer. (B) ACh-stimulated release was measured using Ca^{2+}-free HBSS buffer. (C) ACh in complete HBSS buffer stimulates Ca^{2+} influx response after ATP-induced Ca^{2+} depletion.
Oxotermorine-M (Oxo-M) is a full agonist that has the same efficacy at M3R as CCh or ACh, but is quite different structurally (Fig. 3.16; Table 3.1). When M3R was stimulated with Oxo-M the magnitude of the total Ca\(^{2+}\) response was similar to CCh and ACh (Fig. 3.13A). The Ca\(^{2+}\) release response elicited by Oxo-M was about two thirds of the total response and it was not inhibited by G\(\beta\)5-RGS7 (Fig. 3.13B). Likewise, Ca\(^{2+}\) influx stimulated by Oxo-M was insensitive to G\(\beta\)5-RGS7. These results indicate that in CHO-K1 cells, Oxo-M-stimulated Ca\(^{2+}\) signaling is insensitive to G\(\beta\)5-RGS7.

As I have shown in Figure 3.1, pilocarpine-stimulated Ca\(^{2+}\) signaling is much more sensitive to attenuation by the G\(\beta\)5-RGS7 complex. I found that pilocarpine-elicted Ca\(^{2+}\) signals were comprised of strong release and modest influx components (Fig. 3.14B, C). While G\(\beta\)5-RGS7 strongly attenuated pilocarpine-stimulated release (Fig. 3.14B), it had no measurable effect on influx (Fig. 3.14C). The modest influx signal stimulated by pilocarpine and the fact that it is not sensitive to G\(\beta\)5-RGS7 can explain why the complex
These findings led me to initially hypothesize that sensitivity of M3R to the $G_{\beta 5}$-RGS7 complex is correlated with agonist efficacy. To test this hypothesis, I chose the partial agonist McN-A-343 which has a unique structure (Fig. 3.16). My experiments with this agonist did not support this idea; rather it revealed a much more complex relationship between agonist and sensitivity to $G_{\beta 5}$-RGS7 (Fig. 3.15). Surprisingly, instead of inhibiting the net $Ca^{2+}$ response, $G_{\beta 5}$-RGS7 augmented it $G_{\beta 5}$-RGS7 (Fig. 3.15A). The release component of the M3R response to McN-A-343 was much smaller than the other tested compounds (Fig. 3.15B). Nevertheless, this small release component was still sensitive to $G_{\beta 5}$-RGS7 inhibition. At the same time, the $G_{\beta 5}$-RGS7 complex robustly enhanced $Ca^{2+}$ influx (Fig. 3.15C). The strong positive effect the complex has on McN-A-343-stimulated $Ca^{2+}$ influx apparently results in the positive effect on the total $Ca^{2+}$ response elicited by McN-A-343.

**Figure 3.14** $G_{\beta 5}$-RGS7 strongly inhibits pilocarpine-elicited responses through its action on $Ca^{2+}$ release. (A) Total $Ca^{2+}$ response to 10 μM pilocarpine in complete HBSS buffer. (B) Pilocarpine-stimulated $Ca^{2+}$ release in $Ca^{2+}$-free buffer. (C) $Ca^{2+}$ influx response to pilocarpine in complete HBSS buffer following ATP-induced $Ca^{2+}$ depletion.
Figure 3.15 McN-A-343-stimulated Ca\(^{2+}\) response is enhanced by G\(\beta\)5-RGS7 (A) Total response of M3R to 100 \(\mu\)M McN-A-343 in complete HBSS buffer, in the absence and presence of G\(\beta\)5-RGS7. (B) McN-A-343 stimulated Ca\(^{2+}\) release. (C) Ca\(^{2+}\) response to McN-A-343 in complete HBSS following ATP depletion in Ca\(^{2+}\)-free buffer.

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<th>Net</th>
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<tr>
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<td>113±20</td>
<td>95±11</td>
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<td>Acetylcholine</td>
<td>49±12</td>
<td>107±55</td>
<td>51±16</td>
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<tr>
<td>Carbachol</td>
<td>44±18</td>
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<td>77±20</td>
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<td>Pilocarpine</td>
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<td>101±10</td>
<td>36±5</td>
</tr>
<tr>
<td>McN-A-343</td>
<td>48±3</td>
<td>179±19</td>
<td>360±79</td>
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The magnitude of the effect of G\(\beta\)5-RGS7 is expressed as a percentage ± SD of the maximal amplitude of the Ca\(^{2+}\) response stimulated in the absence of G\(\beta\)5-RGS7.

Together, these results (Table 3.1) indicate that the action of G\(\beta\)5-RGS7 on M3R-stimulated Ca\(^{2+}\) influx involves a specific receptor state that can only be induced by some ligands. To my knowledge, this is the first account of ligand bias toward an RGS protein and merits further investigation. Comparison of the ligand structures reveals that both agonists which stimulate a Ca\(^{2+}\) influx response that is augmented by the G\(\beta\)5-RGS7 complex, CCh and McN-A-343, have carbamate groups which are not present in the other compounds (Fig. 3.16). Future experiments could utilize this information to choose...
other agonists to test for identification of a pharmacophore that stimulates a Gβ5-RGS7-sensitive response.

3.2 The role of Gβ5-RGS7 in regulation of hormone secretion

Expression of Gβ5 in adrenal gland and pancreatic β-cells

Complexes of Gβ5 and R7-RGS proteins were originally discovered in neuronal tissue, notably cortical and cerebellar brain regions and photoreceptors, but not other peripheral tissues (Witherow et al., 2000a; Zhang et al., 2000). Characterization of the Gβ5 knockout mouse in our lab suggested that the complex may play a role in the regulation of hormone secretion (Wang et al., 2011). In the knockout animal, which lacks all R7 family RGS proteins, our lab reported decreased plasma insulin when challenged with glucose and increased basal plasma epinephrine levels as compared to WT animals (Wang et al., 2011). The phenotype of the knockout indicated that Gβ5 may play a role in hormone secretion. These findings led us to the hypothesis that Gβ5-R7RGS complexes may be regulating hormone secretion at the level of the target organ.

To begin to answer this question, I first probed for Gβ5 protein expression in adrenal and pancreatic glands. First, I isolated adrenal glands from WT and Gβ5 knockout mice and separated cortex from medulla. Western analysis revealed that Gβ5
is present in both cortex and medulla, with higher expression in cortex of mouse adrenal
gland (Fig. 3.17). Taken together, these findings suggest that Gβ5-R7RGS complexes
may a role regulating epinephrine secretion from the adrenal gland.

Another neuroendocrine system that we have started assessing for Gβ5 expression and
activity is Islets of Langerhans. Some advantages to working with this system include
high levels of insulin secretion which are easily measured by ELISA and the high level of
expertise in this field here at the Diabetes Research Institute (UMMSM). Pancreatic islets
are composed of α-, β-, Δ-, PP- and ε-cells which work in symphony to maintain glucose
homeostasis. β-cells are stimulated to secrete insulin by plasma glucose, other nutrients
and parasympathetic nerves. Gβ5 is detected in isolated islets and insulinoma cells by
Western, albeit at much lower concentrations than brain (Nini et al., 2012; Wang et al.,
2011). To investigate the subcellular localization of Gβ5, I prepared pancreatic tissue
from WT and Gβ5 knockout mice for cryo-sectioning as described under Materials and
Methods. Immunofluorescence detection of Gβ5 by the ATDG and SGS antibodies
directed against the N- and C-termini, respectively, of Gβ5 displayed punctate staining,
but was also present in the knockout samples, indicating this staining was non-specific
(Fig. 3.12A, B). Another antibody against the N-terminus of Gβ5, CT215, which differed
from the SGS epitope by only one amino acid, appeared to produce a specific signal as it
was not detected in the knockout sample (Fig. 3.18C). However, the staining pattern was
identical to that of insulin (Fig. 3.18D). When laser intensity was set so that fluorescence
could not be detected in the knockout sample (Fig. 3.18E), the signal was likewise not
detected in the WT sample (Fig. 3.18F). Together, these results suggest that the signal
seen with the CT215 antibody was nothing more than bleed-through from insulin
Fig 3.18 Detection of Gβ5 in pancreatic islets. Pancreata from WT and Gβ5 knockout (Gβ5-/-) mice were isolated and prepared for cryostat sectioning as detailed in Chapter 2.8. Two antibodies that recognize the N-terminus, SGS (A) and ATDG (B), and one against the C-terminus, CT215, were used to detect Gβ5 in the fixed tissue slices. (D-F) Pancreas tissue slices were co-stained with the CT215 and insulin antibodies. (E-F) The Gβ5-/- sample was used to calibrate the laser intensity so that there was no signal, then these settings were used to capture the images in E.
staining. I made various modifications to the protocol, i.e. fixation solution and time, antigen unmasking and blocking buffer composition, but the fluorescence signal from Gβ5 did not improve (data not shown). This set of experiments indicated that although Gβ5 is present in pancreatic β-cells, the signal-to-noise ratio was too low for subcellular localization studies with the available antibodies, evidently due to the very low expression level.

Modulation of insulin secretion and Ca^{2+} signaling by the Gβ5-RGS7 complex

The unpublished data from our lab on isolated islets from WT and knockout animals demonstrated that loss of Gβ5 results in impaired insulin secretion when stimulated with glucose and the muscarinic agonist Oxo-M together (Fig. 3.19A). Interestingly, there was no difference between WT and knockout when stimulated with just glucose (Fig. 3.19A), suggesting that a Gβ5-R7RGS complex positively regulates

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Figure 3.19 Loss of Gβ5-RGS7 results in impaired insulin secretion and overexpression of Gβ5-RGS7 enhances Ca^{2+} signaling in insulinoma cells. (A) Pancreatic islets were isolated from WT and Gβ5 KO mice, stabilized in between two layers of Bio-Gel P-4 (BioRad) in minicolumns and perfused with a solution containing 3 mM glucose, then stimulated with 16.7 mM glucose (Wang, Qiang; unpublished at time of print). (G) with or without 1 μM muscarinic agonist oxotremorine-M (OXO-M). Fractions were collected from the columns and insulin concentration was determined with a commercial ELISA kit. The data points are the mean from 3 different animal cohorts, total of 9 animals/ genotype. (B) Min6 cells grown on glass coverslips were transfected with pcDNA (black) or Gβ5-RGS7 (tan) and prepared for Ca^{2+} imaging as described in Chapter 2.10. Baseline values were recorded for 80 s prior to stimulation with 10 μM Oxo-M. Traces shown are the average change in fluorescence for two independent experiments with 3 replicates in each.
cholinergic-stimulated insulin secretion. Prior to my findings that Gβ5-RGS7 enhances M3R-stimulated Ca$^{2+}$ influx in CHO-K1 cells, we could not explain how loss of Gβ5 resulted in less insulin secretion considering that earlier, our lab reported Gβ5 as a negative regulator of Ca$^{2+}$ signaling (Narayanan et al., 2007; Sandiford and Slepak, 2009; Sandiford et al., 2010).

To better understand the mechanism by which Gβ5 can be augmenting insulin secretion, I used an overexpression approach in mouse insulinoma cells (Min6). Min6 cells were transfected with pcDNA or Gβ5-RGS7 and prepared for Ca$^{2+}$ imaging as described under Materials and Methods. I found that overexpression of the Gβ5-RGS7 complex augmented Ca$^{2+}$ signaling as compared to pcDNA alone (Fig. 3.19B, tan vs. black lines). This result suggests that the positive effect Gβ5-RGS7 has on Ca$^{2+}$ signaling in Min6 cells could be the underlying mechanism of enhanced insulin secretion. Indeed, other unpublished data in the lab show that overexpression of Gβ5 in Min6 cells enhances insulin secretion.

Previous experiments with RGS7 domain deletion mutants in the lab demonstrated that the RGS domain is not required, but rather the DEP is necessary for regulation of M3R in CHO-K1 (Sandiford et al., 2010). I applied that same approach to Min6 cells to determine if those findings are relevant to a system which endogenously expresses M3R and Gβ5. This system also allows us to investigate the significance of L-type Ca$^{2+}$ channels in this pathway as they are endogenously expressed and known to mediate muscarinic-stimulated Ca$^{2+}$ signaling and insulin secretion (Boscherio et al., 1995).
First, to determine which domain of RGS7 might be involved in enhancing Oxo-M-stimulated Ca\(^{2+}\) signaling, I used two RGS7 mutants: RGS7\(^{R249}\), which is missing the DEP/DHEX domain and RGS7\(^{ΔRGS}\), which lacks the catalytic RGS domain (Fig. 3.20). Deletion of the RGS domain has no effect, suggesting this action is not mediated through Gi. However, deletion of the DEP/DHEX domain abolishes the positive effect of the complex. These results suggest that M3R regulation by G\(β5\)-RGS7 in CHO-K1 cells is similar in the more physiologically relevant Min6 system.

Prior to discussion of the results, I would like to highlight a few points about Min6 cells. This cell line is one of the few insulinoma cell lines that retains glucose-stimulated insulin secretion (GSIS) in a physiological range (Skelin et al., 2010). In the course of my study, however, it became apparent that our Min6 cells lost their glucose sensitivity.

**Figure 3.20** The DEP domain is, whereas the RGS domain is not required for its positive effect on muscarinic-stimulated Ca\(^{2+}\) signaling. Oxo-M-stimulated Ca\(^{2+}\) signaling was assessed in Min6 cell expressing RGS (green) and DEP (tan) domain deletion mutants of the G\(β5\)-RGS7 complex. (A) Control cells transfected with pcDNA (black) or WT G\(β5\)-RGS7 (tan) are shown as broken lines. Each trace is the mean of three coverslips from two independent experiments. (B) The total area under the curve (AUC) was calculated for each transient recorded in A and the mean ± SD for each condition was graphed.
Identification of channels involved in Gβ5-RGS7-enhanced insulin secretion

Next, to investigate whether the mechanism of action of Gβ5-RGS7 in Min6 cells involves a nifedipine-sensitive channel, I stimulated cells with Oxo-M in the presence or absence of nifedipine. It is important to note here that this particular variant of Min6 cells is not sensitive to glucose. Hence, potentiation of the glucose-stimulated Ca^{2+} signal with muscarinic agonists is likely not present and the signal is primarily muscarinic in origin. In the presence of nifedipine, the positive effect Gβ5-RGS7 has on M3R-stimulated Ca^{2+} signaling is abolished (Fig. 3.21A, B). Surprisingly, nifedipine treatment had no significant effect on control cells, suggesting Oxo-M stimulation does not lead to L-type Ca^{2+} channel activation under these conditions. One reason for this may be due to their glucose-insensitivity, which is necessary for depolarization stimulating L-type channel activation and potentiation of the Ca^{2+} signal by muscarinic agonists.

To assess the amount of intracellular Ca^{2+} mobilized, I calculated the total area under the curve for each condition and, as seen in Fig. 3.21C, treatment with nifedipine greatly attenuated the positive effect of Gβ5-RGS7, but had no significant effect on the control pcDNA-transfected cells.

Figure 3.21 Gβ5-RGS7 enhances Ca^{2+} signaling in Min6 cells through a nifedipine-sensitive channel. The effect of the L-type Ca^{2+} channel blocker nifedipine on Oxo-M-stimulated Ca^{2+} signaling in Min6 cells was assessed. (A) Control cells transfected with pcDNA were stimulated with Oxo-M in the absence (broken line) or presence (solid line) of nifedipine. (B) Cells transfected with Gβ5-RGS7 were stimulated with Oxo-M in the absence (broken line) or presence (solid line) of nifedipine. Each trace is the mean of three coverslips from two independent experiments. (C) The total area under the curve (AUC) was calculated for each transient recorded in A and B and the mean ± SD for each condition was graphed...
Taken together, these results complement my work in CHO-K1 cells and indicate that the positive effect of Gβ5-RGS7 on M3R-stimulated Ca\(^{2+}\) influx. My study shows that this pathway involves a yet unknown nifedipine-sensitive channel which may play a significant role in hormone and/or neurotransmitter secretion. Futures studies characterizing the channel(s) involved in this mechanism will help us understand the role of Gβ5-RGS7 in insulin and/or hormone secretion and may provide evidence that targeting this complex will have clinical significance.

### 3.3 Structural basis of M3R regulation by Gβ5-RGS7

**Residues 304-390 of the i3 loop of M3R are required for its regulation by Gβ5 – RGS7**

To extend earlier findings that the M3R i3 loop is required for the interaction with Gβ5-RGS7 (Sandiford and Slepak, 2009), I created a deletion of M3R lacking 85 amino acids in its middle, M3R\(^{Δ304-390}\) and tested this construct in the Ca\(^{2+}\) mobilization assay (Figs. 3.22-3.23). Concentration-response curves for M3R and M3R\(^{Δ304-390}\) to CCh were obtained in the presence and absence of Gβ5 – RGS7. Consistent with previous findings (Karpinsky-Semper et al., 2014; Sandiford and Slepak, 2009; Witherow et al., 2003), Gβ5 – RGS7 reduced the E\(_{max}\) of the M3R-stimulated Ca\(^{2+}\) response to 65% of M3R alone, with no effect on EC\(_{50}\) (about 5 µM for both M3R and M3R + Gβ5 – RGS7) (Fig. 3.22A). In contrast, Gβ5 – RGS7 could not attenuate CCh-stimulated Ca\(^{2+}\) responses from the M3R\(^{Δ304-390}\) mutant (Fig. 3.22B). Compared to WT, the E\(_{max}\) from M3R\(^{Δ304-390}\) was reduced by 25%, however the EC\(_{50}\) remained unchanged. Cell surface staining of
mutant and WT M3R with the HA-epitope on their N-termini, revealed that M3RΔ304-390 expression was similar to WT (Fig. 3.22C). These results indicate that deletion of residues 304-390 did not alter affinity of CCh to M3R, but slightly reduced coupling to Gq and abolished interaction with Gβ5-RGS7.

One potential explanation why M3RΔ304-390-stimulated Ca^{2+} signaling is insensitive to Gβ5-RGS7 is that the complex only slightly attenuated Ca^{2+} release but still enhances Ca^{2+} influx. To test this possibility, I experimentally isolated each of the Ca^{2+} release and influx components stimulated by M3RΔ304-390 to examine the effects, if any, of Gβ5-RGS7 on each of these Ca^{2+} entry pathways. CCh-stimulated Ca^{2+} release was measured in a Ca^{2+}-free buffer (Fig 3.23B), and Ca^{2+} influx was isolated by depleting intracellular Ca^{2+} stores with ATP through endogenous Gq-coupled purinergic receptor activation (Fig. 3.3.23C). Both the Ca^{2+} release and influx components were insensitive
to Gβ5-RGS7 regulation in this M3RΔ304-390 mutant. These findings demonstrate that the 304-390 region of the i3 loop is required for Gβ5-RGS7 regulation of both Ca\textsuperscript{2+} entry pathways.

The integrity of M3R C-terminus secondary structure is required for its interaction with Gβ5 – RGS7

Previous studies in our lab revealed that along with the i3 loop, regulation by Gβ5-RGS7 involves the C-terminus of M3R, with both Gβ5 and DEP moieties binding to recombinant M3R C-tail \textit{in vitro} (Sandiford et al., 2010). To gain insights into the structural features of the C-tail required for this interaction, I generated four GST-fusions of the C-tail and tested them in a pull-down assay with the Gβ5 – RGS7 complex (Fig. 3.24-3.25). I first focused on a unique polybasic sequence, 565-KKKRRKK-570 in the middle of the C-tail, which was identified as a region necessary for anti-apoptotic effects.
Figure 3.24 In vitro binding of the Gβ5-RGS7 complex to smaller fragments of the M3R C-tail is reduced as compared to the full length C-tail. Three GST-fusion proteins of the c-tail of M3R were purified from bacteria and tested for interaction with Gβ5-RGS7 in a GST pull-down assay as described in Subchapter 2.7. (A) Sequence of the C-tail fragments showing the underlined polybasic region and the Ala substituted Lys residues in bold. To assess DEP or Gβ5 binding, fresh lysate from CHO-K1 cells expressing YFP-DEP or Gβ5-RGS7R239, respectively, was applied to sepharose beads with immobilized GST-fusion protein. The total (T) unbound (U) and eluted (E) fractions were separated by SDS-PAGE and detected by western blot with antibodies against YFP and Gβ5 for DEP and Gβ5, respectively. (B) The average ± SD relative density (n=3) of DEP detected in the eluted fraction of each sample divided by the relative density of DEP in the WT eluted fraction. (C) Quantification of the average relative density ± SD (n=3) of Gβ5 in each sample divided by the relative density of Gβ5 in the WT sample.

of the M3R (Budd et al., 2004). In the GST-M3CT K→A construct, the first three Lys residues were replaced with Ala (Fig. 3.24A). I also made two smaller fragments, GST-M3CT NT, which included the three Lys residues, and M3CT CT, which starts with the second half of the polybasic region and contains the remaining C-tail (Fig. 3.24A). I used lysates from CHO-K1 cells overexpressing YFP-DEP or the Gβ5 complex with the
RGS7$^{R249}$ construct missing the DEP/DHEX domains, to probe for DEP or Gβ5 interaction with the GST fusions, respectively (Narayanan et al., 2007; Sandiford and Slepak, 2009). YFP-DEP exhibited reduced binding to all three GST-fusions as compared to the full-length C-tail (Fig. 3.24B). For Gβ5 interaction, the K→A mutation had no effect, while both the NT and CT fragments exhibited reduced binding (Fig. 3.24C). Thus, the entire C-tail including the polybasic stretch is essential for interaction with the DEP domain. Interaction with Gβ5 is not dependent on the polybasic region, but like the DEP domain, requires the complete C-tail. One explanation why the smaller fragments still retained some affinity for the Gβ5-RGS7 complex is that part of the secondary structure was intact in each of the CT and NT fragments. I therefore explored the idea that secondary structure of the C-tail was mediating the interaction with the Gβ5-RGS7 complex.

Crystallographic analysis of M3R confirms that the proximal region of its C-tail adopts an α-helical conformation, commonly referred to as helix 8 (Kruse et al., 2012). To test the idea that Gβ5-RGS7 binding is dependent on secondary structure of helix 8, I introduced two Pro residues in place of Thr and Leu at positions 553 and 558, respectively (Fig. 3.25A). I then expressed this mutant, TP/LP, as a GST-fusion protein and found that the TP/LP mutation nearly abolished binding of recombinant M3R C-tail to both DEP (Fig. 3.25B) and Gβ5 (Fig. 3.25C). These results demonstrate that substitution with two Pro residues in helix 8 inhibits interaction with Gβ5-RGS7, presumably due to structural changes introduced by the Pro residues.
Figure 3.25 Substitution of two Pro residues in helix 8 of M3R abolishes binding to the Gβ5-RGS7 complex. To test the significance of helix 8 in Gβ5-RGS7 interaction, two proline residues were substituted for T453 and L458, TP/LP. (A) The amino acid sequence of the M3R C-tail with helix 8 underlined and the two substituted Pro residues in red. The GST pull-down assay was performed and the results were quantified as in Fig. 3.24. (B) Binding of the DEP domain was analyzed by SDS-Page and western blot with a GFP antibody. The average ± SD relative density (n=3) of DEP detected in each sample divided by the relative density of DEP in the WT C-tail sample. (C) Binding of Gβ5 to the TP/LP was assessed as in B. Quantification of the average relative density ± SD (n=3) of Gβ5 in each sample divided by the relative density of Gβ5 in the WT sample.

Biophysical analysis of Pro substituted M3R helix 8

In order to understand if the TP/LP mutation indeed affects secondary structure, I collaborated with the lab of Dr. Amjad Farooq (Biochemistry Depeartment, UMMSM) to perform biophysical analyses on WT and mutant synthetic peptides. We conducted far-UV CD analysis on 20-mer wildtype (WT) and mutant (TP/LP) peptides spanning helix 8 (Fig. 3.26B, C). Our analysis reveals that the spectra of both WT and TP/LP peptides in aqueous solution are characterized by a negative band centered around 200 nm (Fig. 3.26B-C, black line), characteristic of peptides predominantly harboring random coil
Figure 3.26 Pro destabilizes the helical fold in M3R helix 8. (A) Ribbon representation of the structural models of helix 8 of WT (left) and TP/LP peptides (right). For each structural model, helix 8 is colored green, the terminal loops gray, and the sidechain moieties of T553/L558 residues and their proline counterparts are depicted in red. (B) Far-UV spectra of WT peptide (40mM) in the absence (black) and presence of 5mM DDPC (red). (C) Far-UV spectra of TP/LP peptide (40mM) in the absence (black) and presence of 5mM DPC (red). (D-E) Molecular dynamics simulations for each peptide, root mean square deviation (RMSD) of backbone atoms (N, Ca and C) within each simulated structure relative to the initial modeled structure of helix 8 of M3R WT (D) and TP/LP (E) as a function of simulation time is shown.

conformation (Rabanal et al., 1993; Woody, 2009). However, in the membrane-like environment of DPC bicelles, the WT peptide spectrum exhibits a positive band centered around 190 nm and two negative bands around 208 nm and 222 nm (Fig. 3.18B, red line). Such a spectral signature is hallmark of α-helical peptides, which strongly suggests that
the WT peptide adopts an α-helical conformation in an apolar membrane-like environment. In sharp contrast, the addition of DPC bicelles to TP/LP peptide does not alter its spectrum (Fig. 3.26C, red line), implying that double Pro substitution disrupts the α-helix.

Structural models of WT and TP/LP peptides were based on the partially solved crystal structure of M3R and MD simulations were conducted in water solvent (Fig. 3.26D, E). The predicted structure of the TP/LP peptide is slightly uncoiled as compared to the WT peptide (Fig. 3.26A). Interestingly, MD analysis reveals that the WT peptide implies that it is extremely unstable in water due to its intrinsic structural flexibility (Fig. 3.26D). This observation is consistent with our CD data showing that the WT peptide adopts a random coil conformation in aqueous solution and only becomes α-helical in the presence of apolar membrane. Likewise, the stability of the TP/LP peptide in water is comparable to the WT peptide (Fig. 3.26E). In the future, MD simulations carried out in a lipid solvent would give us a better understanding of structural instability introduced into the TP/LP mutant in a more physiologically relevant environment.

![Figure 3.27 Pro substitutions in helix 8 of M3R severely impair ability of M3R to stimulate Ca2+ signaling.](image-url)

To investigate the function of the M3RTP/LP mutant I used the FLIPR Ca2+ signaling assay (Subchapter 2.11). CH-K1 cells were transfected M3R (black), M3R + Gβ5-RGS7 (tan), M3RTP/LP (solid red) or M3RTP/LP + Gβ5-RGS7 (broken red) in a 10-cm dish. At 24 h post-transfection, cells were seeded in a 384-well plate and returned to the incubator for another 24 h, after which Fluo-8 Ca2+ dye was added. Concentration-dependencies were acquired for CCh using the FLIPR Tetra system. Each point (n=4) is the mean ± SD change in fluorescence of the maximal response for a given CCh concentration.
**Helix 8 is required for M3R trafficking, signaling and Gβ5-RGS7 interaction**

Next, I introduced the TP/LP mutation in the full-length M3R (M3R\textsuperscript{TP/LP}) and tested its sensitivity to Gβ5-RGS7 in the Ca\textsuperscript{2+} signaling assay. Ca\textsuperscript{2+} responses to CCh were nearly undetectable in cells transfected with the M3R\textsuperscript{TP/LP} mutant (Fig 3.27). Since helix 8 is necessary for GPCR trafficking (Feierler et al., 2011; Spomer et al., 2014; Thielen et al., 2005), I compared the localization of M3R\textsuperscript{TP/LP} to WT M3R (Fig. 3.28). I used WT and mutant M3 receptors tagged with the HA-epitope on their N-terminals, allowing me to detect receptors expressed on the membrane in non-permeabilizing conditions. Under phase-contrast light, cells transfected with M3R or M3R\textsuperscript{TP/LP} appeared morphologically similar; indicating that transfection with mutant M3R did not have any appreciable deleterious effects on cell viability (Fig. 3.28A, D). I found that membrane

![Figure 3.28 Membrane expression of the trafficking-defective M3R\textsuperscript{TP/LP} mutant is improved by pharmacological chaperone treatment.](image)

Localization of N-terminal HA-tagged WT and mutant M3R was analyzed by fluorescence microscopy under non-permeabilizing or permeabilizing conditions. Cells were grown on glass coverslips and transfected with M3R (A-D) or M3R\textsuperscript{TP/LP} (E-H). (A, E) Phase-contrast light overlaid with images from the DAPI channel (blue) for nuclei and FITC channel (green) for HA-tagged M3R. (B, F) Fluorescence microscopy of HA-tagged WT (B) or mutant (D) M3R in non-permeabilized cells. (C, G) Cells expressing WT (C) or mutant (G) receptor (red) were permeabilized with Triton X-100 prior to incubation with antibodies. (D, H) Cells expressing WT (D) or mutant (H) M3R were treated with 100 nM atropine for 18 h before staining under non-permeabilizing conditions to promote membrane expression.
expression of M3R$^{TP/LP}$ was severely compromised as compared to WT (Fig. 3.28B, E). It has been documented that membrane-permeable antagonists can improve membrane expression of helix 8 muscarinic M1 receptors (M1R) (Kaye et al., 2011) and vasopressin V2 receptor (V2R) (Morello et al., 2000) mutants by stabilizing the receptors in the endoplasmic reticulum (ER), thereby allowing them to pass the quality control system of the early secretory pathway (Wuller et al., 2004). Hence, the term molecular chaperone or pharmacological chaperone is used to describe such ligand action. Here, I attempted to promote membrane trafficking of M3R$^{TP/LP}$ using the muscarinic antagonist atropine as a pharmacological chaperone. Treatment of cells with 100 nM atropine for 18 h greatly enhanced membrane expression of M3R$^{TP/LP}$ (Fig. 3.28F) and had a negligible effect on WT (Fig. 3.28C).

To quantify membrane expression of M3R and M3R$^{TP/LP}$ with or without atropine treatment I used a flow cytometry approach. I used the same receptors with an HA-tag on their extracellular tails as in Fig 3.28, which was stained with fluorescently conjugated anti-HA antibodies as described in Subchapter 2.14 (Fig. 3.29). Consistent with findings in Figure 3.28, atropine treatment did not have a significant effect on membrane expression of WT receptor (Fig. 3.29A-B). However, membrane expression of M3R$^{TP/LP}$ was greatly enhanced with atropine treatment (Fig. 3.29C-D). HA-positive cells were expressed as a percentage of the gated population and as seen in Fig. 3.29E membrane expression of M3R$^{TP/LP}$ increased from approximately 15% of WT M3R without atropine treatment to 70% of WT with atropine treatment (Fig. 3.29E). Median fluorescence intensity was increased by 35% and over 200% with atropine treatment for WT and
Figure 3.29 Pharmacological chaperone treatment increases membrane expression of M3R<sub>TP/LP</sub> to 70% of WT M3R. Cells were transfected and treated with atropine as in Fig. 3.28, then were labeled with phycoerythrine-conjugated anti-HA antibody under non-permeabilizing conditions and analyzed by flow cytometry as described in Subchapter 3.14. Histograms show the number of HA-positive cells as a function of fluorescence intensity with the percentage of HA-positive cells in the total population in the top right corner. For each condition 10,000 events were counted and the gated HA-positive population is shown in between the vertical blue lines. Dark grey peaks on each graph are control cells not expressing M3R but stained with the fluorescent HA-antibody to control for background fluorescence. (A, C) Cells expressing WT (A) or mutant (C) M3R were not treated with atropine. (B, D) Cells transfected with WT (B) or mutant M3R (D) were treated with 100 nM atropine for 18 h prior to antibody staining. (E) HA-positive cell count expressed as a percentage of the gated population, mean ± SD (n=2). (F) Median fluorescence intensity (MFI) of HA-positive cells, mean ± SD (n=2).
mutant M3R, respectively (Fig. 3.29F). Taken together, these results demonstrate that atropine treatment increases the number of cells expressing M3R<sup>TP/LP</sup> and the amount of receptor expressed in each cell.

I next tested the ability of the atropine treated cells to elicit a Ca<sup>2+</sup> response. I seeded CHO-K1 cells transfected with M3R or M3R<sup>TP/LP</sup> in a 384-well plate for imaging with FLIPR and treated them with 100 nM atropine. Before incubation with the Ca<sup>2+</sup> indicator, I washed the cells twice to ensure atropine was removed. When cells were stimulated with CCh in the absence of extracellular Ca<sup>2+</sup>, the signal from WT M3R was reduced by more than 50% in the presence of G<sub>β5</sub>-RGS7 with no change in the EC<sub>50</sub> (Fig. 3.30). This is consistent with earlier findings (Fig. 3.2B) and demonstrates that atropine did not affect the amplitude of the Ca<sup>2+</sup> response. However, atropine treatment did not enhance the Ca<sup>2+</sup> signal from M3R<sup>TP/LP</sup> as anticipated (Fig. 3.30). Based on these results, I hypothesized that agonist affinity and/or G protein-coupling is diminished in the M3R<sup>TP/LP</sup> mutant.

Many lines of evidence suggest that ligands stabilize distinct receptor conformations. For example, experiments using disulfide cross-linking with recombinant M3R shows that distinct muscarinic agonists could produce different cross-linking patterns, suggesting that ligands are capable of stabilizing different receptor conformations (Wess et al., 2008). Therefore, it is plausible that affinity to some agonists, like CCh, is compromised in M3R<sup>TP/LP</sup>, while other agonists may be able to elicit a full response. To explore the idea that this mutant could possibly be activated by other agonists, I tested atropine treated cells in the Ca<sup>2+</sup> signaling assay using the FLIPR tetra system to compare multiple ligands simultaneously. I used CCh, ACh, Oxo-M,
pilocarpine, arecoline, bethanechol and methacholine at concentrations ranging from 100 nM – 5 mM to stimulate Ca$^{2+}$ signaling from cells expressing WT or mutant M3R in the absence or presence of G$\beta_5$-RGS7. None of the tested agonists were able to elicit a response from M3R$^{TP/LP}$ that was greater than the background signal (data not shown). It is noteworthy that WT M3R Ca$^{2+}$ responses stimulated by agonists in the millimolar range were mitigated by the application 200 µM atropine, demonstrating that this is indeed a specific M3R-stimulated event. Taken together, this set of experiments indicates that introduction of Pro residues into helix 8 of M3R not only impedes receptor trafficking, but renders it insensitive to many ligands and G$\beta_5$-RGS7, suggesting this region is crucial for stabilizing an active conformation of the receptor and interaction with the G$\beta_5$-RGS7 complex.

It has been shown that the high receptor density produced in overexpression systems allows M3R to couple to other G proteins, including Gs (Burford et al., 1995a, b). To test whether M3R$^{TP/LP}$ could activate Gs in transfected CHO-K1 cells, I collaborated with the Wahlestedt lab (Center for Therapeutic and Scientific Innovation, UMMSM) to measure cAMP accumulation using the Perkin Elmer LANCE Ultra cAMP

Figure 3.30 Pharmacological chaperone treatment does not restore signaling deficits introduced with the TP/LP mutation. Cells were transfected and treated with atropine as in Fig. 3.28, and prepared for FLIPR Ca$^{2+}$ imaging as in Fig. 3.26. Cells were washed in Ca$^{2+}$-free buffer to remove atropine before incubation with Fluo-8. All measurements were carried out in the absence of extracellular Ca$^{2+}$. The mean ± SD maximal well fluorescence stimulated by the indicated CCh concentration is shown as a percentage of the M3R $E_{\text{max}}$ under these conditions.
competitive immunoassay kit. In this assay, the interaction between anti-cAMP monoclonal antibodies labeled with the ULight™ dye and Europium-labeled cAMP (Eu-cAMP) is utilized for time-resolved fluorescence resonance energy transfer (TRFRET)-based detection of M3R activity. When ULight™-labeled cAMP antibody binds to Eu-cAMP, there is energy transfer from the Eu-cAMP molecule to the ULight™-labeled-antibody, thereby increasing well FRET. A decrease in the FRET signal indicates an increase in unlabeled cellular cAMP.

I treated cells expressing M3R or M3RTP/LP in the presence or absence of Gβ5-RGS7 with atropine as in Figs. 3.28-3.30 to enhance membrane expression of M3RTP/LP. Forskolin was used as a reference control and cAMP accumulation was similar in cells expressing WT or mutant M3R, regardless of the presence of Gβ5-RGS7 when stimulated with forskolin (Fig. 3.31A). For WT M3R, CCh-elicted cAMP accumulation...
had an Emax that was 85% of the forskolin-stimulated response (Fig. 3.31B). Gβ5-RGS7 had no effect on CCh-stimulated Emax and the EC50 values, approximately 10 µM, were similar to those obtained in the Ca2+ imaging assay (Fig. 3.31B). These results suggest that CCh-stimulated cAMP accumulation is specific for M3R and Gβ5-RGS7 does not regulate this pathway. However, the Emax for M3RTP/LP reached only 10% of WT receptor regardless of the presence of Gβ5-RGS7 (Fig. 3.23B). Collectively, my data shows that atropine enhances membrane expression of M3RTP/LP, indicating this mutant is capable of binding ligand, but coupling of this mutant to either Gq or Gs is nearly abolished.
CHAPTER 4

DISCUSSION

In the course of my dissertation work I discovered a novel mechanism mediated by Gβ5-RGS7 which couples M3R to Ca^{2+} influx. My work is significant because having a fundamental understanding of GPCR-mediated signaling is essential for devising new approaches to modulate receptor activity. The study of GPCRs and their signal transduction pathways is a steadily growing field (Fig. 1.1). However, much of the therapeutic potential of GPCRs is still largely untapped because only a small fraction of GPCRs is currently targeted by therapeutic agents. Thus, there is a great interest in developing new approaches pertaining not only to targeting GPCRs traditionally with drugs that are analogs to their natural ligands, but altering activity of the molecules that regulate GPCR activity.

When RGS proteins were discovered there was great excitement that they would druggable because they show selectivity for GPCR subtypes and they have a more discrete expression pattern than many traditionally targeted GPCRs (Neubig and Siderovski, 2002). However, this idea has yet to be materialized due to the obstacles that accompany targeting protein-protein interactions (PPI). The PPI interface can span over a large area and is usually featureless making it difficult to design a small molecule that shows a high degree of selectivity (Buchwald, 2010). Notwithstanding, there has been clear success with some PPI inhibitors, mainly, the biologics which target proteins from the extracellular space (Colombel et al., 2010; Quinn et al., 2005; Sandborn and Targan, 2002; Saurat et al., 2008).
While our understanding of the molecular basis of ligand bias progresses, it becomes increasingly apparent that exploiting this attribute of GPCR signaling may prove to be successful in developing new drugs (Kahsai et al., 2011; Lamberts and Traynor, 2013; MacKinnon et al., 2001; Pradhan et al., 2011). Therefore, the study of the nature of protein-protein interactions in GPCR signal transduction pathways and how they relate to a particular ligand-stabilized receptor conformation is highly significant. Some progress has already been made by identifying ligands preferentially activate β-arrestin signaling for several receptor subfamilies (Allen et al., 2011; Boerrigter et al., 2011; Leduc et al., 2009; Rajagopal et al., 2010; Seifert et al., 2011; Violin and Lefkowitz, 2007). GPCRs interact with several known accessory proteins expanding the therapeutic potential of ligand bias.

Identifying a drug that alters the receptor’s affinity to another GPCR-interacting protein thereby producing a desirable biological effect is highly probable. My work on Gβ5-RGS7 provided evidence for ligand bias towards an RGS protein; for the first time I showed that the extent of GPCR regulation by an RGS protein can be directed by intrinsic properties of an agonist.

4.1 Gβ5-RGS7 has a dual effect on M3R-stimulated Ca^{2+} signaling

Earlier work in our lab demonstrated that the Gβ5-RGS7 complex attenuated M3R-stimulated increases in intracellular [Ca^{2+}]i (Narayanan et al., 2007; Sandiford and Slepak, 2009; Sandiford et al., 2010; Witherow et al., 2000b). When I started working on this project I first hypothesized that sensitivity of M3R to Gβ5-RGS7 could be related to
agonist efficacy. To test this idea, I stimulated cells with the partial agonist pilocarpine and found that this agonist produces a response that is more sensitive to Gβ5-RGS7 attenuation than those elicited by CCh (Fig. 3.1). To better understand how the action of Gβ5-RGS7 could be agonist directed, I dissected the two routes of Ca^{2+} entry.

It is well documented that activated M3R initiates both release from intracellular stores and influx across the plasma membrane (Carroll and Peralta, 1998; Felder et al., 1992; Parekh and Brading, 1992). To study the effect of Gβ5-RGS7 on each component M3R-stimulated Ca^{2+} signaling, I experimentally isolated these constituents of Ca^{2+} entry. This allowed me to discover a novel regulatory mechanism mediated by Gβ5: the Gβ5-RGS7 complex inhibits the release pathway (Fig. 3.2B), but augments Ca^{2+} influx (Fig. 3.3). Apparently, this dual action produces a net negative effect on CCh-stimulated increases in [Ca^{2+}]_i observed in Fig. 3.2A and in our earlier studies (Narayanan et al., 2007; Sandiford and Slepak, 2009; Sandiford et al., 2010).

Ca^{2+} release is mediated by the canonical pathway involving Gq, PLC-β, and the second messenger IP3, and the inhibitory action of Gβ5-RGS7 on M3R-stimulated Ca^{2+} release may be explained by its competition with G proteins for the receptor (Blin et al., 1995; Sandiford and Slepak, 2009) or PLC-β (Posner et al., 1999b). Elucidating the role of Gβ5-RGS7 in M3R-stimulated Ca^{2+} influx is more complex as this pathway involves multiple channels that can be expressed and regulated in a cell- and even species-specific manner (Felder et al., 1992; Singer-Lahat et al., 1996; Wuest et al., 2007). The first questions I wanted to answer were whether this new mechanism was a consequence of RGS7 GAP activity and whether this pathway was initiated by another G protein, i.e. Gi (Fig. 3.4-3.7).
First, I used the Gi inhibitor pertussis toxin (PTX) to determine if M3R-stimulated Ca\(^{2+}\) influx required Gi (Fig. 3.4-3.5). PTX treatment had no effect on Ca\(^{2+}\) influx indicating this pathway is not Gi-coupled (Fig. 3.5). Next, to rule out involvement of the RGS domain I constructed a deletion mutant, RGS7\(^{\Delta RG5}\) (Fig. 3.6A). This mutant of the G\(\beta\)5-RGS7 complex was capable of stimulating Ca\(^{2+}\) influx, establishing that the RGS domain is not necessary for enhancing influx (Fig. 3.6B). Finally, my experiments with the novel Gq inhibitor, UBO-QIC, clearly demonstrated that stimulation of Ca\(^{2+}\) influx by G\(\beta\)5-RGS7 is solely dependent upon a Gq-mediated mechanism (Fig. 3.7).

Receptor-induced Ca\(^{2+}\) influx involves two major pathways: Ca\(^{2+}\)-dependent, i.e. store-operated, and Ca\(^{2+}\)-independent (Putney, 2010). In my experiments, contribution of store-operated channels is unlikely since I depleted intracellular Ca\(^{2+}\) stores prior to M3R stimulation (Fig. 3.3). As G\(\beta\)5-RGS7 can potentiate Ca\(^{2+}\) influx under these conditions, it stands to reason that these channels are not store-operated Ca\(^{2+}\). My results with the L-type Ca\(^{2+}\) channel blocker nifedipine and the non-selective TRP channel blocker 2-APB indicate that both these types of channels participate in this pathway. While nifedipine had no significant effect on M3R-stimulated Ca\(^{2+}\) influx, it reduced the response in the presence of G\(\beta\)5-RGS7 (Fig. 3.10). This finding indicates that G\(\beta\)5-RGS7 facilitates coupling of M3R to a nifedipine-sensitive channel. Since G\(\beta\)5-RGS7 did not influence M3R-stimulated changes in membrane potential at CCh concentrations below 10 \(\mu\)M (Fig. 3.11), this channel does not appear to be voltage-dependent. Indeed, it has been shown that nifedipine can influence activity of channels other than the L-type family of VDCCs (Curtis and Scholfield, 2001).
M3R-stimulated Ca\(^{2+}\) influx was nearly abolished by the non-selective TRP channel inhibitor 2-APB regardless of the presence of G\(\beta\)S-RGS7 (Fig. 3.8), suggesting that TRP channel activation is necessary in this M3R-initiated pathway. Since some TRP channels can be activated by the membrane-delimited second messenger DAG (Montell, 2005), I tested if G\(\beta\)S-RGS7 could influence PMA-induced Ca\(^{2+}\) influx, and found no such effect (Fig. 3.9). These findings suggest the G\(\beta\)S-RGS7 complex does not directly influence a DAG-activated TRP channel, and possibly acts upstream of the DAG-generating M3R effector enzyme, PLC-\(\beta\).

In the past decade it has become increasingly apparent that phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) can either facilitate or inhibit pore opening of various ion channels (Suh and Hille, 2008). For example, ubiquitously expressed TRPM7 channels, which are Ca\(^{2+}\) permeant, are inhibited by activation of Gq and depletion of PIP\(_2\) (Runnels et al., 2002). Therefore, if G\(\beta\)S-RGS7 inhibits M3R- Gq coupling or Gq activation of PLC-\(\beta\), it should result in potentiation of Ca\(^{2+}\) conductance through TRPM7. This idea could explain an early observation by Carroll and Peralta (Carroll and Peralta, 1998) that at low [CCh] the Ca\(^{2+}\) responses primarily consist of influx, and as M3R occupancy increases PLC activity, there is less influx because depletion of PIP\(_2\) reduces Ca\(^{2+}\) channel activity.

It is also reasonable to speculate that G\(\beta\)S-RGS7 participates in a macromolecular complex that couples M3R to a nifedipine-sensitive channel, analogous to the complex containing G\(\beta\)S-RGS7 and some GIRK channels (Xie et al., 2010). Future experiments should include investigation of the effect of G\(\beta\)S-RGS7 on PLC-\(\beta\) activity. Measuring IP3 accumulation would be the first place I would start because this assay is reliable and
directly assesses PLC-β activity. The three possible outcomes and their interpretations are listed in Table 4.1.

<table>
<thead>
<tr>
<th>Possible effects of Gβ5-RGS7 on IP3 accumulation</th>
<th>Interpretation</th>
<th>Next step</th>
</tr>
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<tr>
<td>Inhibit</td>
<td>The action of Gβ5-RGS7 on M3R-stimulated Ca^{2+} signaling is at the level of Gq-GPCR or PLCβ</td>
<td>Replenish the PIP2 pool by overexpressing the PI4-5 kinase, the enzyme that catalyzes this reaction. It would be interesting to see if the positive effect that Gβ5-RGS7 has on Ca^{2+} influx is inhibited under these conditions.</td>
</tr>
<tr>
<td>Enhance</td>
<td>This outcome is unlikely and difficult to interpret since Gβ5-RGS7 has a profound negative effect on Ca^{2+} release, which is dictated by the activity of PLC-β. Nevertheless, if this is the case it would indicate that Gβ5 is inhibiting the IP3 receptor.</td>
<td>The next question would be how can the complex selectively inhibit M3R-initiated activation of IP3 receptor, but enhance IP3 accumulation?</td>
</tr>
<tr>
<td>No change</td>
<td>This scenario would indicate that the action of the Gβ5-RGS7 complex is downstream of PLC-β possibly at the target channel(s). The same question of selectivity for M3R would arise here.</td>
<td>It is plausible that Gβ5-RGS7 participates in a macromolecular complex. To test this idea, a chemical cross-linking/immunoprecipitation strategy followed my mass spectrometry may help identify binding partners of the complex and M3R.</td>
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**TABLE 4.1 The possible effects of Gβ5-RGS7 and future experiments**

**4.2 Possible role of Gβ5-RGS7 in hormone secretion**

In addition to elevated plasma glucose, pancreatic islets respond to cholinergic inputs from the sympathetic nervous system to secrete insulin. The mechanisms by which cholinergic stimulation potentiates glucose stimulated insulin secretion are not fully
understood, but involve the Gq-PLC-β pathway and Ca$^{2+}$ influx (Boschero et al., 1995; Gautam et al., 2007; Kong et al., 2010; Love et al., 1998; Ruiz de Azua et al., 2012). My discovery that the Gβ5-RGS7 complex has a positive effect on M3R-stimulated Ca$^{2+}$ influx in CHO-K1 cells for the first time was able to explain our observations of impaired insulin secretion in the Gβ5 knockout mouse (Wang et al., 2011). Hormone secretion and neurotransmitter release are always preceded by a rise in [Ca$^{2+}$]. It has been demonstrated in various secretory cells, including pancreatic β-cells, that oscillations in intracellular Ca$^{2+}$ drives rhythmic hormone/neurotransmitter release (Gilon et al., 1993; Hales et al., 1994; Haydon and Carmignoto, 2006; Tse et al., 1993). As illustrated in Fig. 4.1, Ca$^{2+}$ influx accounts for much of the muscarinic-stimulated increases in [Ca$^{2+}$], in Min6 cells. In these cells, Gq mediated Ca$^{2+}$ release from intracellular stores is weak and very transient, rapidly returning to baseline in less than 40 s following agonist application (Fig. 4.1 red line). Ca$^{2+}$ release is therefore not sufficient to drive rhythmic hormone secretion. Rather, it plays a role in activating second messengers and membrane channels which are capable of producing such a necessary signal.

The necessity of Ca$^{2+}$ influx in insulin secretion is illustrated by an experiment with rat islets where muscarinic potentiated insulin secretion was nearly abolished in Ca$^{2+}$-free buffer (Boschero et al., 1995). Thus, intracellular Ca$^{2+}$ release is not sufficient
to stimulate insulin secretion. For this reason, the finding that I made regarding the positive effect of Gβ5-RGS7 on M3R-stimulated Ca$_{2+}$ influx in CHO-K1 cells may be highly significant in a physiological setting. Indeed, my experiments showed that this mechanism is present in insulinoma cells, a more physiologically relevant system since M3R is not overexpressed (Fig. 3.19B). The precise molecular events still remains to be determined, but likely involves a nifedipine-sensitive Ca$_{2+}$ channel (Fig. 3.21). Furthermore, the RGS domain does not appear to be essential, but like CHO-K1 cells, the action of Gβ5-RGS7 on M3R-stimulated Ca$_{2+}$ signaling requires the DEP domain of RGS7 (Fig. 3.20).

Some clues regarding this mechanism of action on Ca$_{2+}$ influx may found in previous studies. In a study using purified proteins, Gβ5-RGS7 has been shown to inhibit PLC-β2 activated by Gβγ (Posner et al., 1999a). Thus, it is reasonable to speculate that the Gβ5-RG7 complex competes with Gβγ for PLC-β, which can explain why M3R-stimulated Ca$_{2+}$ release is attenuated by the complex. Another interesting concept is that in cells which express multiple MR subtypes, Gi-, through Gβγ, and Gq- coupled receptors can synergistically activate PLC-β3 (Philip et al., 2010; Rebres et al., 2011). In has been suggested that both M3R (Gq) and M4R (Gi) contribute to excitation secretion coupling in chromaffin cells (Olivos and Artalejo, 2008). Therefore, it stands to reason that in these cells both classes of G protein can equally contribute to the oscillatory rises in [Ca$_{2+}$]i needed for rhythmic neurotransmitter release.

I have shown that Gβ5 is expressed in both layers of the adrenal gland, cortex and medulla (Fig. 3.17) and loss of Gβ5 results in increased urine catecholamine levels (Wang et al., 2011). This effect is the opposite of what is happening in islets: loss of Gβ5
results in **reduced** plasma insulin. Some possible explanations for the effect of Gβ5-RGS7 on circulating catecholamine levels are of the following:

- Since Gβ5 is expressed in all levels of the nervous system, it is possible that increased plasma epinephrine is a consequence of loss of inhibition at postganglionic sympathetic fibers, either by loss of cholinergic tone or an intrinsic property of the sympathetic neurons. Like our experiments with primary islets, measuring secretion from isolated adrenal tissue would start to unravel this pathway.

- Since chromaffin cells express Gi and Gq coupled muscarinic receptors, the effect of Gβ5 on catecholamine secretion can involve GAP activity towards Gi and competitive inhibition of PLCβ activation. Future experiments in catecholamine-secreting cells, i.e. PC12, employing the RGS7 mutant I constructed and my pharmacological inhibitor strategy could start answering these questions.

For pancreatic β-cells, though, it is clear that M3R is the only MR that facilitates potentiation of the glucose stimulated insulin secretion response (Wess et al., 2007). As suggested above (Subchapter 4.1, paragraph 4), pore opening of many membrane ion channels can be regulated by PIP2 in β-cells (Rohacs and Nilius, 2007). Therefore, it is reasonable to hypothesize that an inhibitory action of Gβ5 on PLC-β could have a positive effect on ion channels which are either closed when PIP2 is depleted or opened when PIP2 is present.
Another finding to consider is that Gβ5-R7RGS complexes have been shown to participate in macromolecular complexes to regulate G protein activated inwardly rectifying K⁺ (GIRK) channel activity (Xie et al., 2010). In β-cells, ATP-sensitive K⁺ (K_{ATP}) channels play an important role in regulating glucose-stimulated insulin secretion. Under normal plasma glucose concentrations, K_{ATP} channels are open which hyperpolarizes the cell. When plasma glucose is elevated there is an increase in ATP and a fall in MgADP in the immediate vicinity of the K_{ATP} channel resulting in K_{ATP} channel closure, membrane depolarization, opening of voltage-gated Ca^{2+} channels, Ca^{2+} influx, and exocytosis of insulin granules (Ashcroft, 2005). My experiments with the L-type Ca^{2+} inhibitor in Min6 cells suggested that Gβ5-RGS7 selectively couples M3R to a nifedipine-sensitive channel (Fig. 3.21B). On the contrary, in the absence of Gβ5-RGS7, nifedipine did not significantly reduce Ca^{2+} signaling (Fig. 3.21A) Again, it is important to note that these cells were stimulated with muscarinic agonist in the absence of glucose, which is likely the reason why nifedipine does not have an effect on cells transfected with pcDNA (Fig. 3.21A). Although it is too speculative to infer any relationship between Gβ5-RGS7 and K_{ATP}, this would be something to investigate in the future. Such an experiment would be fairly straightforward with the FLIPR membrane potential assay I used on CHO-K1 cells (Fig. 3.11; (Karpinsky-Semper et al., 2014)). I have done a preliminary experiment with non-transfected Min6 cells in the FLIPR membrane potential assay and they respond nicely to KCl and Oxo-M (Fig. 4.2).

4.3 Agonist conferred functional selectivity of M3R regulation by Gβ5-RGS7

A single GPCR can control multiple signaling pathways within the same cell. The pleiotropic nature of GPCR signal transduction may be explained by
compartmentalization (Halls, 2012; Ostrom and Insel, 2004), GPCR oligomerization (Terrillon and Bouvier, 2004), functional selectivity and receptor conformation (Kelly et al., 2008; Kobilka and Deupi, 2007; Li et al., 2008; Wacker et al., 2013). Previous reports showed that various muscarinic agonists can preferentially activate Ca\(^{2+}\) influx or release (Hishinuma et al., 1997; Karpinsky-Semper et al., 2014; Schaafsma et al., 2006; Wang et al., 1992).

Consistent with this concept, I found that agonists used in my study were biased toward one of the two routes of the Ca\(^{2+}\) entry (Figs. 3.12-3.16, Table 3.1). Acetylcholine, oxotremorine-m and CCh stimulated both influx and release. Pilocarpine strongly stimulated Ca\(^{2+}\) release, but had little impact on influx. Another partial agonist, McN-A-343, generated strong influx and rather weak Ca\(^{2+}\) release constituents. G\(\beta\)5-RGS7 inhibited Ca\(^{2+}\) release in the presence of all agonists except Oxo-M, suggesting that Oxo-M stabilizes an M3R conformation unfavorable to G\(\beta\)5-RGS7 binding in CHO-K1 cells. The positive effect on influx was only observed when M3R was stimulated with CCh and McN-A-343. Pilocarpine-evoked influx was not augmented by G\(\beta\)5-RGS7 (Fig. 3.15C). These results indicate that the action of G\(\beta\)5-RGS7 on influx involves a specific receptor state that can only be induced by some ligands.

Developing sub-type specific muscarinic agonists is a major goal in muscarinic pharmacology. Tissue distribution and function of M3R is diverse, so M3R-specific ligands may not be as clinically significant as other sub-type specific GPCR agonists. For example, M3R is found in endothelial cells, skeletal and smooth muscles, CNS and glands, but G\(\beta\)5 has only been found in the CNS, endocrine pancreas and pace-maker cells of the heart. Molecules like G\(\beta\)5-RGS7 could provide an additional platform of
selectivity. Understanding the structural basis of ligand bias for M3R sensitivity to Gβ5-RGS7 regulation may provide fundamental information useful for developing selective therapeutic agents.

4.4 The central 85 amino acids of the M3R i3 loop may dictate selectivity for Gβ5-RGS7

Some of my thesis focused on expanding previous studies in the Slepak lab investigating the structural basis of the interaction between Gβ5-RGS7 and M3R. With a GST pull-down approach our lab showed that recombinant fragments of M3R can bind to the Gβ5-RGS7 complex and/or portions of the dimer (Sandiford et al., 2010). Specifically, the i3 loop fragment spanning amino acids 304-390 displayed higher affinity to the DEP domain than the full-length i3 loop (residues 253-492). The i3 loop of several GPCRs is recognized as the coupling site for G proteins (Chakir et al., 2003; Conchon et al., 1997; Degraff et al., 2002). M3R is no exception, but its i3 loop is unusually big accounting for half of the receptor’s molecular weight. Over the past decade or so, many labs showed that several regulatory proteins bind to this region and alter the activity of M3R (Budd et al., 2001; Hu et al., 2012; Novi et al., 2005; Simon et al., 2012; Wu et al., 2000). G protein binding sites have been mapped to the juxtamembrane region of the i3 loop (Wu et al., 2000), but we found that Gβ5-RGS7 binds in the middle (Sandiford et al., 2010). It is therefore reasonable to speculate that binding of Gβ5-RGS7 to the center of the i3 loop could reduce coupling efficiency to Gq.
One of the original goals of this project was to identify Gβ5-RGS7 binding motif to possibly help identify other GPCRs, if any, that directly interact with the complex. In order to narrow down the Gβ5-RGS7 binding site in M3R I produced shorter fragments of the i3 loop region 304-390. However, this approach did not produce consistent results (data not shown), presumably because the short fragments lose their native conformation. To test the significance of the interaction of i3 loop 304-390 region with Gβ5-RGS7, I deleted this region within the context of the full-length receptor (Figs. 3.22-3.23). As expected, this deletion rendered M3R-stimulated Ca^{2+} signaling insensitive to regulation by Gβ5-RGS7 (Figs. 3.22-3.23). Furthermore, my results suggest that deletion of amino acids 304-390 did not change the affinity of receptor to CCh, but only slightly impaired signaling, as is evident by the reduction in $E_{\text{max}}$ (70% of WT) and absence of significant change in EC_{50} (Fig. 3.22, Table 3.3).

Some future experiments to validate my findings with the M3R\textsuperscript{Δ304-390} mutant would be to insert this region into other Gq-coupled receptors and determine if they become sensitive to Gβ5-RGS7. M1 and M5 would be good to start with and this work could be expanded to include other receptor families. It would be interesting if the M3R-304-390 insertion sensitized the other muscarinic receptors but had not effect on other Gq-coupled receptors. This would indicate that another region which is common amongst muscarinic receptors, possibly helix 8, is required.
4.5 Critical role of M3R helix 8 in receptor trafficking, signal transduction and regulation by Gβ5-RGS7

In addition to the i3 loop of M3R, previous studies identified that the carboxyl-terminus of the receptor (M3R CT) is required for interaction with Gβ5-RGS7 (Sandiford et al., 2010). I investigated the importance of two characteristic features of M3R CT, the central polybasic stretch, 565-KKKRRK-570, and helix 8. My pull-down data shows that the polybasic region is not essential for the interaction (Fig. 3.24). In contrast, my results show that a double Pro substitution in helix 8 abolishes interaction with the complex (Fig. 3.25). Helix 8 is a common structural motif present in many GPCRs and has been demonstrated to be involved in several processes including membrane trafficking and G protein recognition and activation (Aratake et al., 2012; Choi et al., 2005; Kirchberg et al., 2011; Wess et al., 2008). Data from circular dichroism (CD) spectroscopy and molecular dynamics (MD) simulations support my hypothesis that substitution of two helix 8 residues with Pro destabilizes its helical fold (Fig. 3.26). Given that Pro is known to disrupt α-helices, due to its inability to participate in backbone hydrogen bonding, this result is not be surprising. However, the destabilization of helix 8 with the double Pro substitution only became apparent in the phospholipid environment (Fig. 26C). The dependence of helix 8 folding on the presence of membrane mimetics was previously observed for peptides from cannabinoid and β-2 adrenergic receptors (Choi et al., 2005; Choi et al., 2002; Katragadda et al., 2004; Tiburu et al., 2009). Such a behavior thus appears to be common amongst rhodopsin-like GPCRs. Taken together, my results indicate that residues 548-567 of the carboxyl-terminus of M3R form an α-helix and its structural integrity is necessary for interaction with Gβ5-RGS7.
In the course of this study I found that membrane expression of full-length M3R\textsuperscript{TP/LP} was severely impaired (Fig. 3.28). This observation is consistent with previous work with helix 8 mutants of V2R and M1R (Kaye et al., 2011; Morello et al., 2000; Wuller et al., 2004). To address this problem, I applied the pharmacological chaperone approach, treating cells expressing M3R\textsuperscript{TP/LP} with atropine. This treatment increased M3R\textsuperscript{TP/LP} membrane expression from approximately 20% to nearly 70% of WT (Fig.3.29). Despite such dramatic improvement, M3R\textsuperscript{TP/LP} \(\text{Ca}^{2+}\) signaling remained severely impaired, with the \(E_{\text{max}}\) for CCh reaching only 10% of WT and its EC\textsubscript{50} in the millimolar range.

Helix 8 is known to participate in conformational changes of M3R which are required for receptor activation (Wess et al., 2008). My findings that sensitivity of M3R to G\(\beta\)5-RGS7 may be coded in its agonists implies that some ligands may stabilize a M3R conformation more conducive to G\(\beta\)5-RGS7 interaction (Karpinsky-Semper et al., 2014). This led me to hypothesize that an agonist other than CCh may overcome the structural instability introduced by the Pro substitutions in helix 8 of M3R. However, all six tested agonists elicited a response that was less than 10% of the WT M3R signal (data not shown). My data also indicates that alteration of helix 8 abolishes M3R coupling not only to Gq to another G protein, Gs (Fig 3.30). In the course of these experiments I found that G\(\beta\)5-RGS7 does not affect AC activation by WT M3R, showing that G\(\beta\)5-RGS7 does not compete with Gs for interaction with the receptor (Fig.3.30). This suggests that action of G\(\beta\)5-RGS7 on the Gq-mediated activation of PLC-\(\beta\) involves not only competition with Gq for M3R, but could also involve another mechanism.
Indeed, previous studies with purified proteins showed that Gβ5-RGS7 had no effect on AC, but reduced PLC-β2 activity stimulated by Gβγ (Posner et al., 1999a). Since the Gβ5-RGS7 complex is capable of inhibiting PLC-β2, then it is reasonable to speculate that Gβ5-RGS7 can inhibit numerous metabotropic PLC-β activation events. However, this is not the case since our lab has shown that the effect of the Gβ5-RGS7 complex on M3R-stimulated Ca\(^{2+}\) signaling is selective for M3R, presumably due to unique structural features of this receptor (Sandiford and Slepak, 2009). This, taken together with my finding, one can hypothesize that regulation of M3R signaling by Gβ5-RGS7 could involve two mechanisms: 1) localization of the complex to M3R by its unique i3 loop and 2) competitive inhibition of Gq and/or PLC-β.

**Summary**

My work on the Gβ5-RGS7 complex led to a deeper understanding of the novel action of the complex on M3R-stimulated Ca\(^{2+}\) signaling, ultimately solving the discrepancy between the negative effect of the complex seen *in vitro* and the inferred positive effect observed in the Gβ5 knockout mouse. This discrepancy can be explained by my discovery that Gβ5-RGS7 has a dual effect on the M3R-stimulated Ca\(^{2+}\) response: while it inhibits Ca\(^{2+}\) release from the ER, which was the predominant event observed in CHO-K1 cells, it can also enhance Ca\(^{2+}\) influx across the plasma membrane, which is needed for insulin secretion. This novel regulatory mechanism can explain why Gβ5 knockout mice have impaired insulin secretion. Thus far, I have shown that this mechanism requires Gq and involves activation of 2-APB-sensitive channel(s) and selective coupling of M3R to a nifedipine-sensitive Ca\(^{2+}\) channel by Gβ5-RGS7. Based on my findings here, it stands to reason that Gβ5-RGS7 acts at the level of PLC-β altering...
its activity and leading to changes in ion channel(s) opening by the membrane delimited pathway. To date, only a handful of Gq-coupled receptors have been tested for sensitivity to Gβ5-RGS7 and the M3R-stimulated Ca\textsuperscript{2+} response was the only one inhibited (Sandiford and Slepak, 2009). However, at that time the concept of Gβ5-RGS7 having a dual effect on Ca\textsuperscript{2+} signaling was not known. Therefore, it is possible that the effects of Gβ5-RGS7 on the other tested Gq-mediated responses may not have been apparent due to a canceling effect (i.e. the magnitude of the effects of Gβ5-RGS7 on Ca\textsuperscript{2+} influx and release are equal but opposite). To explore this possibility, other Gq-coupled receptors should be tested in the Ca\textsuperscript{2+} influx and release assays in the absence and presence of Gβ5-RGS7. On the other hand, selectivity of the M3R-Gβ5-RGS7 interaction is, in part, conferred by the unique i3 loop of M3R. One strategy to substantiate the importance of this receptor region in selectivity towards Gβ5-RGS7 is to insert the 304-390 i3 loop region, which I have shown here to be necessary for this interaction, into another muscarinic receptor, for example M5, and determine whether its Ca\textsuperscript{2+} signal is sensitive to Gβ5-RGS7.

While the concept of biased agonism is established for G protein- and arrestin-mediated signaling, my finding that the effects of Gβ5-RGS7 can be agonist-directed implicates an RGS protein complex in this phenomenon for the first time. Since Gβ5-RGS7 expression is more discrete than M3R expression patterns, development of Gβ5-biased ligands may have some clinical significance in treating metabolic diseases such as diabetes or obesity. Such ligands would also be instrumental in extending my studies of the structural basis of Gβ5-RGS7-regulated M3R signaling.


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