Spring 2015

Constitutive activity in orphan G protein coupled receptors

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CONSTITUTIVE ACTIVITY IN ORPHAN G PROTEIN COUPLED RECEPTORS

by

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A DISSERTATION

Presented to the Faculty of the Graduate School of the

MISSOURI UNIVERSITY OF SCIENCE AND TECHNOLOGY

In Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

2015

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ABSTRACT

The goal of this research was to use a distal signaling pathway analysis to evaluate the extent of agonist independent constitutive signaling among orphan class-A G protein coupled receptors (GPCRs). These receptors translate extracellular signals via conformational change into intracellular activation of different G proteins and subsequent second messenger synthesis. These small molecules regulate cellular biochemistry, eventually leading to nuclear signaling that results in changes in gene expression. Some GPCRs are capable of signaling in the absence of an activating ligand, a phenomenon called constitutive activity that is inhibited via an "inverse-agonist". The use of cAMP dependent Luciferase expression is used to compare the canonical signaling of all five wild-type Muscarinic Acetylcholine receptors and their constitutively active (CA) mutant counterparts. All five members, both wild-type and CA, signaled via cAMP dependent pathways, although only the CA mutants do so in the absence of an agonist. This technique is then applied to 40 different orphan GPCRs for which an agonist is unknown/not-present. This resulted in 75% (30 out of 40) scoring as constitutively active, grouped into five different categories based on their response. The largest and most significant group of 17 orphans inhibited cAMP dependent expression, both basal and forskolin stimulated, by more than 40%, indicating activation of $G\alpha_i$. In total, novel findings of constitutive activity were found in 23 of the 40 Orphan receptors with results otherwise in agreement with literature in most cases. Orphan receptors that were closely related based on amino acid homology tended to have similar effects on gene expression. These results suggest that identification of inverse agonists may be a fruitful approach for categorizing these orphan receptors and targeting them for pharmacological intervention.

ACKNOWLEDGMENTS

Dr. Ercal, thank you for allowing me to join your lab group and for mentoring me through the completion of this PhD program. I have grown personally and professionally from my experiences in your lab. Chemistry is something that I avoided in my early education as something that held little interest for me. This has changed- and I owe a lot of that to you.

Dr. Aronstam, thank you for your mentorship over not just the last few years as my co-advisor, but also as the chair of the Biological Sciences Department, my boss, and mentor. I have relied on you rather heavily throughout this process and could not have finished without your help and advice.

Dr. Klaus Woelk, Dr. Yinfa Ma, Dr. Prakash Reddy, thank you all for being willing to join my PhD advisory committee so late in the game and for such a non-traditional student. I appreciate your presence and input on this dissertation.

My wife and children. My friends. Thank you for putting up with me. For listening to my boring stories about chemistry and biology. For dealing the late nights and distracted weekends, especially near the end as I was finishing the 100 yard dash.

Thank you to the cDNA Resource Center, affiliated with the Biological Sciences Department at Missouri University of Science & Technology for the funding for this research.

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LIST OF ABBREVIATIONS

Abbreviation	Description
CA	Constitutively Active; agonist independent signaling
WT	Wild-type; native version of a protein
CRE	Cyclic AMP Response Element (DNA Sequence)
GPCR / GPR	G Protein Coupled Receptor
RLU	Relative Luminescent Units; light emitted by Luciferase enzyme
$G\alpha_q$	G Protein Alpha Q
$G\alpha_s$	G Protein Alpha S
$G\alpha_i$	G Protein Alpha I
$G\alpha_{12}$	G Protein Alpha 12
α	Alpha sub-unit of the heterotrimeric G protein complex
$\beta\gamma$	Beta/Gamma sub-units of the heterotrimeric G protein complex
IP3	Inositol tri-phosphate; 2^{nd} messenger
DAG	Diacylglycerol; 2^{nd} messenger
cAMP	Cyclic Adenosine Mono Phosphate; 2^{nd} messenger

1. INTRODUCTION

1.1. BACKGROUND

G protein coupled receptors (GPCR or GPR) comprise a large superfamily of receptors characterized by a seven transmembrane domain structure and an ability to activate intracellular transducer G proteins. Over 800 GPCRs in five main families have been identified in eukaryotes on the basis of genomic sequence analysis [1]. These receptors can be activated by hormones, neurotransmitters, odorants, light, or pheromones (Figure 1.1). Orphan GPCRs are a group of receptors for which the endogenous agonist is not known or remains unclear or in dispute. The application of drug screening, binding assays, and second messenger profiling techniques has decreased the numbers of orphan G protein coupled receptors from 150 in 2004 [2] and to as few as 77 in 2014 [3]. The first receptor to be "de-orphanized" (or "adopted") was the serotonin (5-HT1A) receptor [4]. This process continued with new methods that allowed high throughput screening for endogenous activating molecules for the remaining orphan receptors [5]. However, in recent years the rate of de-orphanization appears to be slowing [6].

Muscarinic receptor subtypes (M1-M5) differ in the coupling to cellular effector mechanisms. M2 and M4 receptors generally couple most efficiently to inhibition of certain isoforms of adenylate cyclase lowering cytosolic cAMP levels as a consequence of interactions with $G\alpha_i$ transducer proteins, while M1, M3 and M5 couple most efficiently to the stimulation of phospholipase Cβ causing a mobilization of intracellular calcium as a consequence of interactions with $G\alpha_{q/11}$ proteins [7-9]. This specificity is not absolute and a myriad of signaling variations have been reported [10-13]. Thus, while

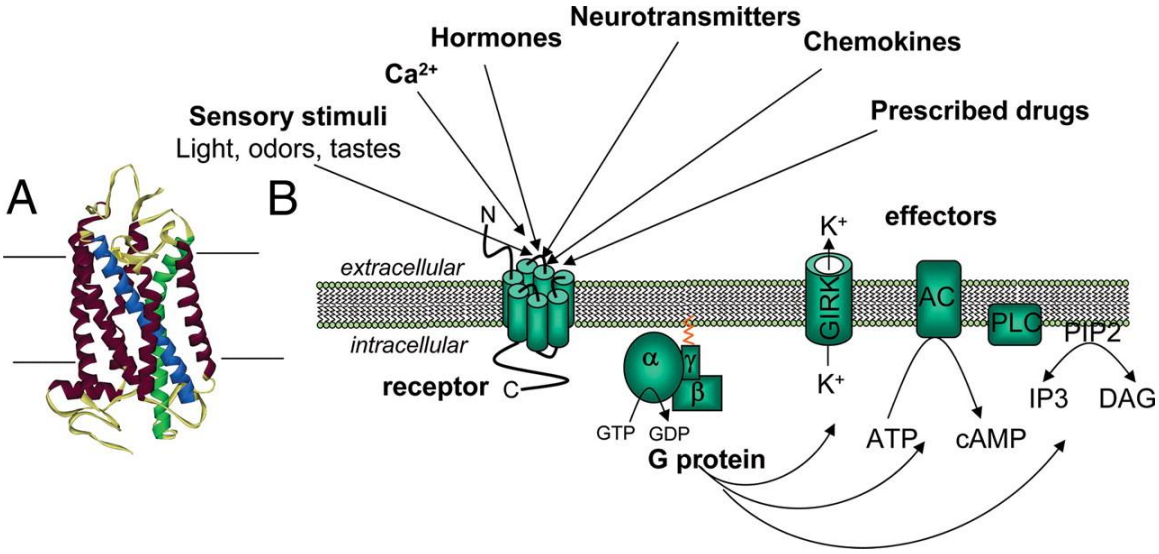

Figure 1.1. Basic principle of GPCR-mediated signal transduction. A, Molecular representation of a GPCR based on the x-ray crystal structure of rhodopsin. Helices 3 and 6 are shown in *blue* and *green*, respectively. B, Basic pattern of ligand-mediated GPCR signal transduction. After ligand agonist binding, the receptor undergoes conformational changes, which promotes the coupling with heterotrimeric G proteins (Gαβγ) and catalyzes the exchange of GDP by GTP on the α-subunit. This event engages conformational and /or dissociational events between the α- and βγ-subunits, and both GTP-bound Gα-subunit and the Gβγ-dimer can then modulate the activity of various effectors. For example, stimulation or inhibition of adenylyl cyclase (AC)-mediated cAMP synthesis by the α-subunit of the G_s or G_i families, respectively; production of inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG) after cleavage of phosphatidylinositol 4,5-biphosphate (PIP2) by $G\alpha_q$ stimulation of phopholipase C (PLC); activation of G_i and G_o also mediated most of the Gβγ-mediated signaling processes such as activation of GIRK. Directly from [14].

M2 receptors inhibit adenylate cyclase, they can (in appropriate systems and under certain conditions) stimulate adenylate cyclase and phospholipase Cβ [11,12]. M3 receptors can also stimulate adenylate cyclase at high agonist concentrations.

This multiplicity of actions reflects multiplicity of signal transduction effector isoforms (e.g., 10 isoforms of adenylate cyclase), crosstalk between signaling pathways, and cell type specific differences in the expression of signaling proteins. Crosstalk

between pathways can take place at different levels: at the receptor level, signaling potential is affected by the level of receptor expression [15]; at the transduction level, M2 receptors can directly activate multiple transducer G proteins ($G\alpha_i$ and $G\alpha_s$) [11]; at the second messenger level, cAMP, inositol trisphosphate, Ca^{2+}, and diacylglycerol can activate or inhibit protein and enzymes that in turn regulate both receptors and effector molecules.

Signaling mediated by second messengers transiently regulates cell processes, many of which lead to changes in transmembrane potential and thus electrical excitability of the cell [16]. However, long term changes in cell behavior require the integration of second messenger activity (proximal signaling) into changes in gene expression (distal signaling) (Figure 1.2). Measurement of these second messengers can sometimes be problematic, especially in the case of inhibitory pathway activation (ie. $G\alpha_i$). Under these conditions it is useful to artificially elevate second messenger (cAMP) levels in order to more easily visualize the inhibition of that pathway. Forskolin (Figure 1.3), a diterpene derivative, has been used previously for this purpose [17]. Acting as a potent stimulator of adenylate cyclase, this compound increases the frequency and length of time that the enzymatic site is formed via the binding of the two catalytic domains [18] (Figure 1.4). While these two domains are in contact, the active site can catalyze the formation of cAMP via Mg^{2+} assisted ring formation [19] (Figure 1.5). This activity is directly opposed by the binding of inhibitory G proteins via a decrease in catalytic domain affinity, thus decreasing the stimulatory effect of Forskolin [20].

The phenomenon of agonist-independent “constitutive” signaling (Figure 1.6) was first observed with the delta opioid receptor in 1989 [21]. This was followed by the

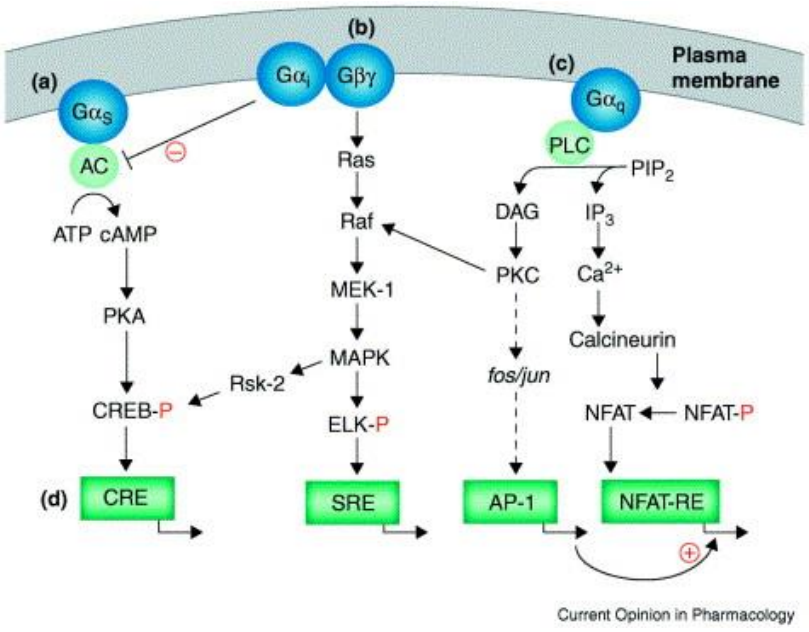

Figure 1.2. G protein signaling pathways. A schematic diagram showing how, after stimulation of the GCPR and dissociation of the G-protein subunits, the major G-protein families signal via the different intracellular second messenger pathways to communicate with nuclear promoter elements. **(a)** $G\alpha_s$-coupled receptors stimulate adenylyl cyclase (AC), which synthesises cAMP from ATP. In contrast $G\alpha_i$-coupled receptors inhibit AC and so reduce cAMP formation. **(b)** The βγ subunits from $G\alpha_i$ and other G proteins are able to activate the MAP kinase pathways and PLCβ. **(c)** GPCRs coupled to the $G\alpha_q$ family of G proteins stimulate PLCβ, which cleaves membrane phospholipids to produce IP_3, which mobilises intracellular calcium, and DAG, which activates PKC. **(d)** Second messenger pathways then activate a range of effector systems to change cell behaviour; in many cases this includes the regulation of gene transcription. Dotted line shows a more indirect pathway. MAPK, MAP kinase, MEK, MAP kinase kinase; P, phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate. Taken directly from [22].

discovery of mutant versions of other native GPCRs that signal in a similar manner [23]. Constitutive activity is now known to be present in a large number of GPCRs. As of this publication, a PubMed search for “GPCR” and “constitutive” reveals 132 references since 2010. Constitutive activity can be created in most Class-A GPCRs by slightly

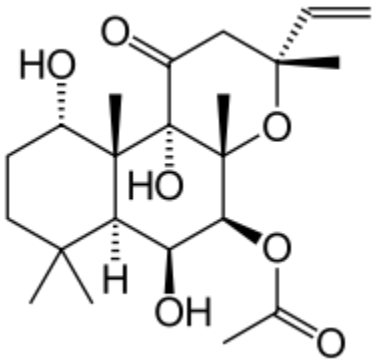

Figure 1.3. Structure of Forskolin. This molecule interacts directly with both catalytic subunits of Adenlyate Cyclase to form the active site and increase cAMP [17].

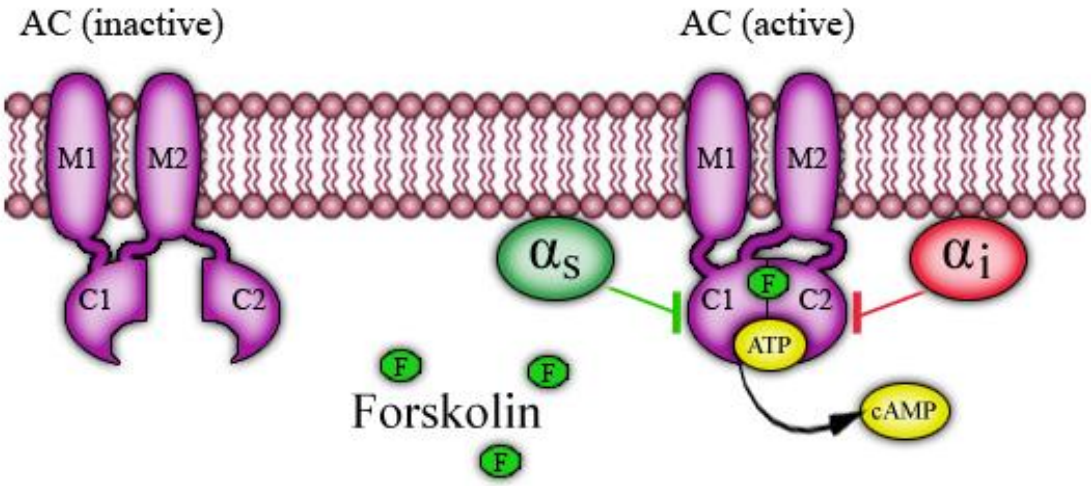

Figure 1.4. Activity of Forskolin, $G\alpha_i$ and $G\alpha_s$ in regulating Adenylate Cyclase. The two catalytic domains of Adenylate Cyclase normally have very low affinity for each other. Formation of cAMP can only occur when these two domains bind together, forming the active site. Forskolin, a diterpene binds to both C1 & C2 domains via hydrogen bonding and hydrophobic interactions increasing their affinity. $G\alpha_s$ and $G\alpha_i$ work in antagonistic fashion to increase or decrease affinity for the two catalytic domains respectively.

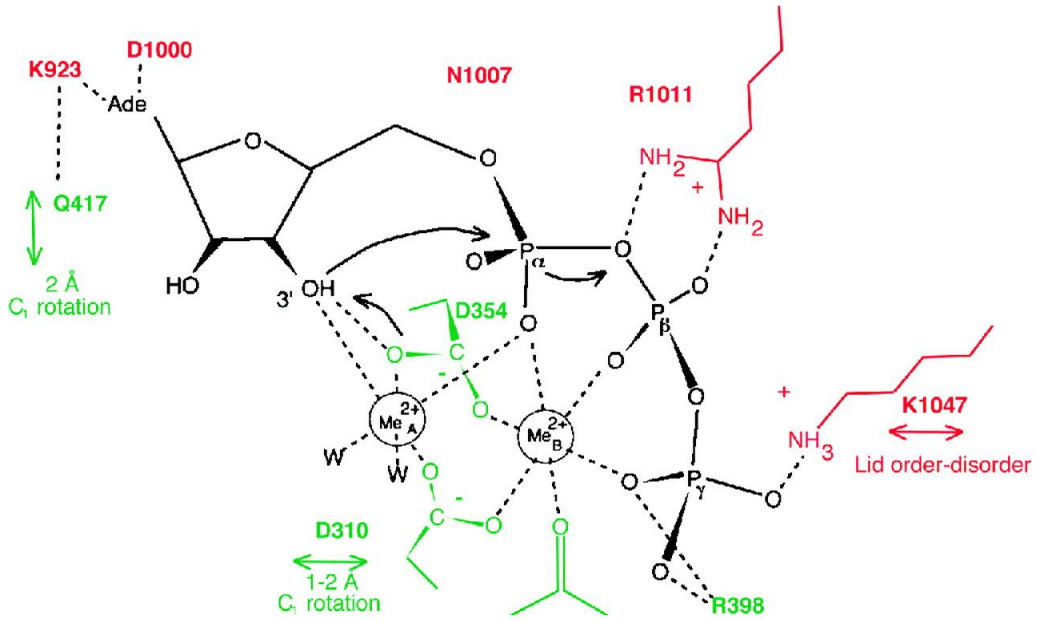

Figure 1.5. Formation of cAMP is dependent on both catalytic domains. Coordination of metal ions (Mg^{2+}) allow for interactions between the two catalytic domains (C1 in green, C2 in red). Conformational changes of individual residues during catalysis are marked. Adapted from [19].

lengthening the sixth transmembrane domain [24]. Structural analyses of some receptors suggest that this mutation eliminates interactions between hydrophobic amino acids on the third and sixth transmembrane, leading to the formation of a water filled pore [25] [26]. This led to a "unifying" theory on the biochemical mechanisms that regulate GPCR activation, including the changes that may lead to constitutive signaling [27].

The use of constitutive signaling poses certain challenges. There is the risk that endogenous ligands or activating conditions may be present in the testing media, thereby confounding data interpretation, as was the case for the ADORA2 receptor [28].

Receptors can also respond differently under different conditions, either due to promiscuous interactions with transducer elements, cellular conditions [29], or even

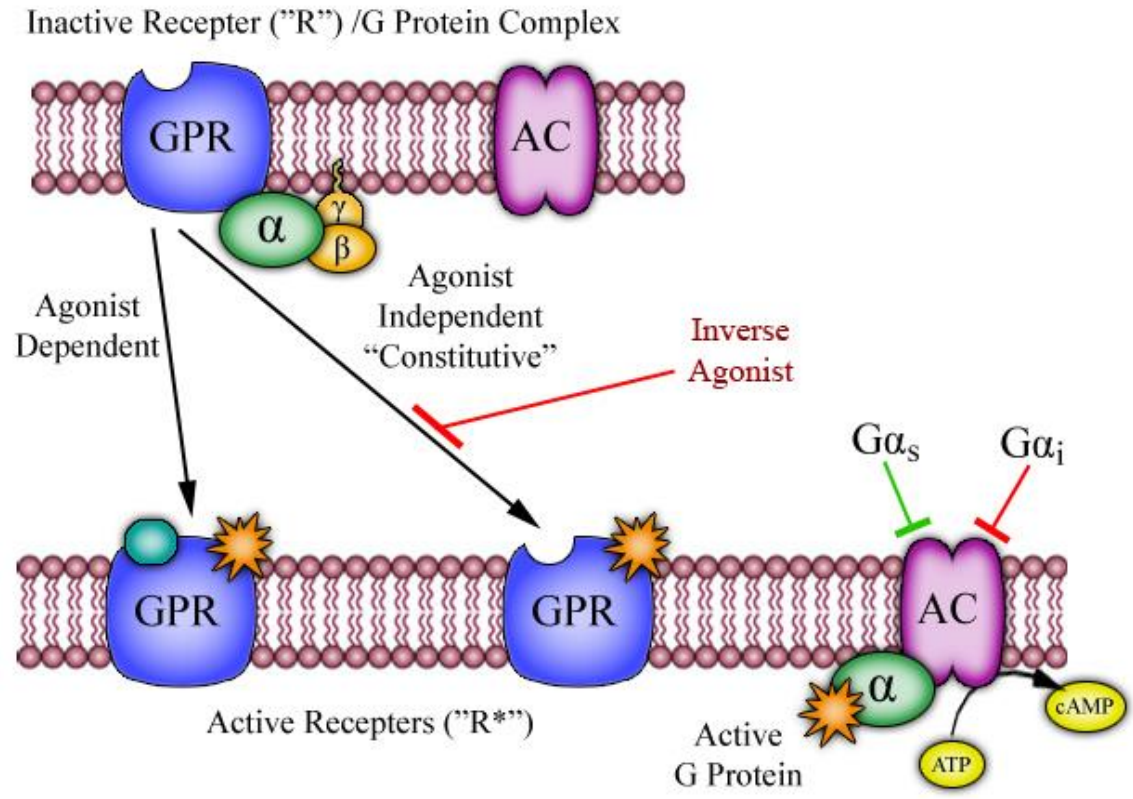

Figure 1.6. Agonist vs. Constitutive activation of GPCR signaling. G protein coupled receptors (GPCR, or GPR) are in a constant equilibrium between the inactive (R), and active (R*) forms of the protein. The ratio of the two (R*/R) can be expressed as an equilibrium constant. "Agonists" are ligands that act to destabilize the inactive form of the receptor shifting the equilibrium to the active conformation, thus recruiting and activating intracellular G proteins, which in turn activates downstream second messenger synthesis/release. Constitutive activity occurs when the K_{eq} of the native receptor is large enough that the GPCR can activate signal cascades in the absence of a ligand. Inverse Agonists are molecules that bind to the agonist active site but act as stabilizing agents for the inactive form, thus shifting equilibrium away from the active state and preventing activation of G proteins, even- if not especially, in constitutively active receptors.

hetero-dimerization with other native receptors [30]. Nevertheless, constitutive signaling has been useful in the discovery of native ligands [31], and is a required for the systematic search for inverse agonists.

Constitutive signaling as a tool in orphan receptor characterization was reviewed in 2006 [32]. The history of inverse-agonists (i.e., compounds that inhibit constitutive activity) as a therapeutic approach has also been reviewed [33]. While the use of constitutive signaling for drug discovery (notably, for inverse-agonists) has been discussed [5], the use of constitutive signaling to de-orphanize or to understand potential signaling pathways in GPCRs has not been widely exploited. Accordingly, this work was developed to experimentally establish that distal signaling via a Luciferase linked reporter vector could be used to measure and characterize cAMP dependent constitutive activity among the largest collection of orphan class-A G protein coupled receptors to date.

1.2. FORMAT

1.2.1. Section 2: Establishing the Assay. Experiments performed in this section compared cross talk patterns in receptor signaling at distal levels of the signaling cascade. Specifically, gene activation by muscarinic receptors (M1 – M5; GenBank AF498915-9) possessing a wild type (WT) or constitutively active (CA) phenotype were examined. Comparing signaling pathways of known receptors (M1-M5) with known responses vs. the characterization of constitutively active mutant (CAM) receptors of the same family established the validity of the assay and supported its use in the characterization of unknown orphan receptors.

1.2.2. Section 3: Constitutive Signaling Among Orphan Receptors. The prevalence of constitutive activity among Class-A orphan GPCRs has not been comprehensively examined. Experiments performed in this section examined 40 class-A orphan G protein coupled receptors to determine the prevalence of cAMP dependent constitutive signaling (i.e., signaling that is generally mediated by $G\alpha_i$ and $G\alpha_s$ transducer proteins), using receptor activation or inhibition of gene expression under control of the cAMP-dependent response element (CRE) as the indicator of pathway activation.

1.2.3. Section 4: Summary, Discussion, and Impacts. Considering the nature of the subject under evaluation, an initial "Review of Literature" option would not be appropriate. This section provides an individual review of the literature on each orphan GPCR examined within this work and discusses the results of experiments performed. Indications of agreement/disagreement with established receptor behavior is noted, as well as the contribution of this work towards that body of knowledge.

2. ESTABLISHING THE ASSAY

2.1. METHODS

2.1.1. Cell Culture. CHO-K1 wild-type Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (ATCC). CHO cells stably transfected with the coding sequences for M2 or M3 muscarinic receptor (CHO-M2 and CHO-M3; GenBank AF498916; GenBank AF498917, respectively) were obtained from the cDNA Resource Center (www.cdna.org). Cells were maintained at 37^{o}C with 5% CO_2. Culture media consisted of 90% HyClone DMEM (without phenol red or additional L-glutamine) supplemented with 10% HyClone FetalClone II (bovine serum product). Plates were allowed to reach 80% confluency before being split for growth or use in subsequent experiments.

2.1.2. CRE Regulated Gene Expression. A luciferase coupled reporter vector (Promega) was used to monitor CRE regulated gene expression in CHO cells. CHO cells in (200,000 in 100 µl media) were plated onto 96-well plates and incubated until they reached 80% confluency. Cells were mixed with firefly luciferase reporter vector (Promega) at a final concentration of 250 ng per well and Lipofectamine 2000 Reagent (Invitrogen), following manufacturer's suggested protocols. In some experiments, CHO cells were co-transfected with clones for a specific subtype of muscarinic receptor (M1 – M5) possessing either a wild type (WT) or constitutively active (CA) phenotype in a pcDNA3.1+ vector (Invitrogen) obtained from the cDNA Resource Center (www.cdna.org), and incubated for 24 hours. Transfection with an empty pcDNA3.1+ vector served as a control. In other experiments, the reporter gene vector was transfected

into CHO cells stably expressing a muscarinic receptor cDNA clone (obtained from the cDNA Resource Center; www.cdna.org).

Transfection media was removed and replaced with complete media. The plate was then incubated for an additional 6 hours. Immediately prior to visualization, each well was rinsed with PBS and replaced with 50ul of DMEM (-phenol red) without serum. Controls and treatments intended to test $G\alpha_i$ signaling (i.e., inhibition of adenylate cyclase) were exposed to 3 µM forskolin (Sigma Aldrich) to activate the catalytic subunit of the cyclase. Imaging was performed using a FLUOstar Omega (BMG Labtech) 96-well plate reader. Auto-injection of 25ul of Bright-Glo Luciferase Reagent (Promega) was followed by 2 minutes of rotary incubation. Relative Light Units (RLUs) were measured in each well for 1 minute. Each plate was repeated in triplicate and contained 12 treatment groups with 4 replicates in each group, separated by a row of unused wells to minimize light pollution. Control treatments (3 µM forskolin stimulated cells with CRE-Luciferase and empty pcDNA3.1+) were used to standardize RLU values between plates.

2.1.3. Data Analysis. Experiments were performed 3-8 times in triplicate or quadruplicate. Data is expressed as the mean and standard deviation from the independent experiments. Measurements from two populations (e.g., wild type vs. constitutively active receptors) were compared using Student's *t*-test. Values from experiments with multiple independent variables (e.g., concentration curves) were compared by ANOVA and Tukey's test using GraphPad Prism software. Significant differences were indicated by P values of < 0.05.

2.2. RESULTS

2.2.1. Activation of Gene Expression Under Control of the cAMP Response Element (CRE) in CHO-M2 Cells.

Dose-response curves for forskolin induced gene expression mediated by CRE were determined. Direct enzymatic activation by forskolin was not affected by receptor expression (i.e., was identical in wild type, M2-expressing and M3-expressing CHO cells). The concentration relationship for forskolin activation depended on the length of exposure to forskolin (Fig 2.1). When exposed to forskolin for 15 minutes followed by removal by washing, gene expression 6 hours later was increased by forskolin only at concentrations above 10 μM (Figure 2.1.A). When the forskolin was not removed from the incubation medium, the threshold was 30 fold lower (≈ 0.3 μM). These effects of forskolin were independent of receptor expression. Thus, emphasizing that gene expression assays provide a valid and reliable means of characterizing distal GPCR signaling.

CHO-M2 cells potentiate adenylate cyclase at 100uM of Carbamylcholine, showing increased expression of luciferase under control of the CRE. The increase in reporter gene expression in response to 100 μM carbamylcholine was significant within 2.5 hours and peaked after 8 hours (Figure 2.1.B). In subsequent expressions, a 6 hour incubation period was routinely used.

2.2.2. CRE-Mediated Gene Expression Stimulated by each WT and CA Muscarinic Receptor Subtype; Distal Receptor Signaling is Potentiated by Constitutive Activity.

Luciferase reporter assays were used to compare second messenger signaling with signaling patterns integrated at the gene expression level in each of the five muscarinic receptor subtypes with either a wild type or constitutively active phenotype.

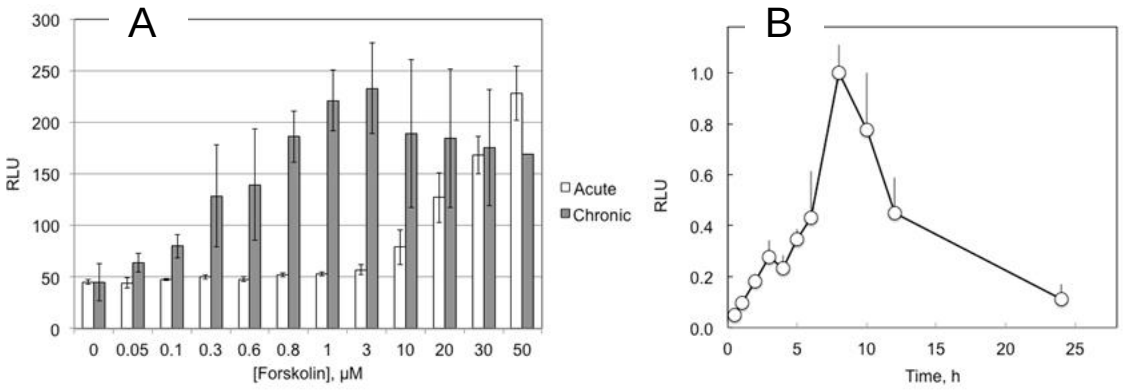

Figure 2.1. Carbamylcholine and forskolin activation of CRE-mediated expression of a reporter gene in CHO-WT cells. RLU, relative luminescent units.
Figure 2.1.A. CHO wt cells were incubated with the indicated concentrations of forskolin. In the "Acute" condition, the cells were exposed to forskolin for 15 min and then the forskolin was removed by replacing the media; in the "Chronic" condition, forskolin was present throughout the 6 hour incubation.
Figure 2.1.B. CHO-M2 cells were incubated with 100 µM carbamylcholine for the indicated times before measuring luminescence catalyzed by reporter gene generated luciferase. Subsequent experiments were performed using a 6 h incubation.

Constitutively active muscarinic receptors were created by inserting two amino acids into the sixth transmembrane domain. This alteration conveys a constitutively active phenotype in many G protein coupled receptors [24], possibly by disrupting a hydrophobic lock structure that involves elements of the 6^{th} and 3^{rd} transmembrane domains[25-27]. Constitutive activity was associated with a higher level of basal CRE-mediated gene expression with all 5 receptor subtypes, although M3 receptors showed the greatest fractional response (≈ 700%) and M2 and M4 showed the lowest fractional responses (≈ 100%) (Figure 2.2). Carbamylcholine increased expression mediated by all CA receptors subtypes except M4. The greatest increases (and lowest basal activities) were seen with M1, M3 and M5 CA receptors.

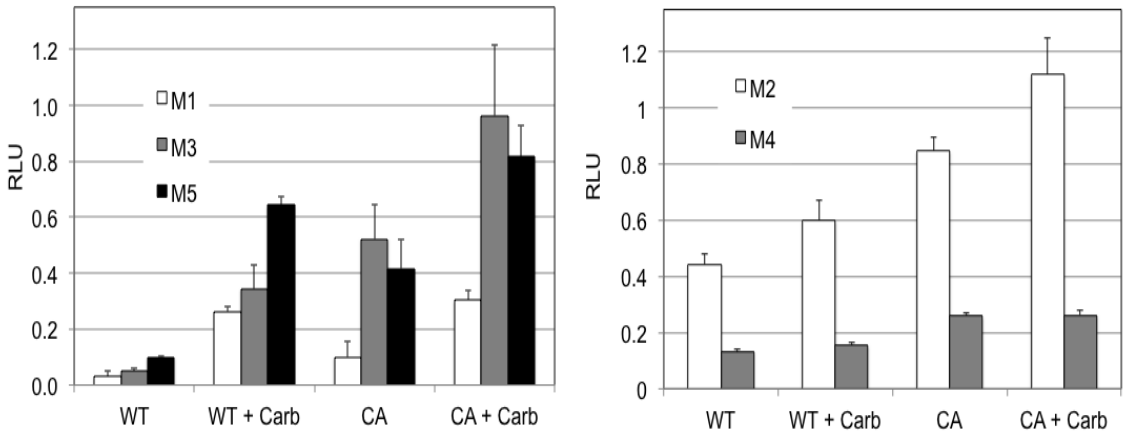

Figure 2.2. Stimulation of CRE-mediated gene expression by activation of the five muscarinic receptor subtypes with either a wild type (WT) or constitutively active (CA) phenotype. CHO cells were transiently transfected with genes for each of the muscarinic receptors subtypes (M1 – M5, WT or CA). Activity was measured 24 h later in the absence or presence of 100 µM carbamylcholine and is expressed as relative luminescence units normalized to activity measured in the presence of 3 µM forskolin. Mean ± SD; N = 4. Carbamylcholine increased expression mediated by all WT receptors subtypes except M4; receptors with constitutive activity had greater activity than wild type receptors of the same subtype; carbamylcholine further increased the activity of all subtypes with a CA phenotype, except M4 (all comparisons by Student's *t*-test; $p < 0.05$).

2.2.3. Agonist Concentration Dependence of CRE-Mediated Gene Expression Stimulated by WT and CA M2 and M3 Muscarinic Receptors.

CRE mediated gene expression assays were used to further characterize the signal transduction potential of M2 and M3 (WT and CA) receptors, since these two receptors exhibit the strongest activation in the classes of receptor subtypes (i.e., $G\alpha_i$- and $G\alpha_{q/11}$-coupled receptors). Basal levels of CRE-mediated gene expression were more than doubled in the CHO cells expressing M2 or M3 receptors with a CA phenotype (Figure 2.2). CRE-mediated activity was further increased by carbamylcholine in cells expressing either the M2 or M3 CA receptors; dose responses curves for these relationships are presented in Figure 2.3.

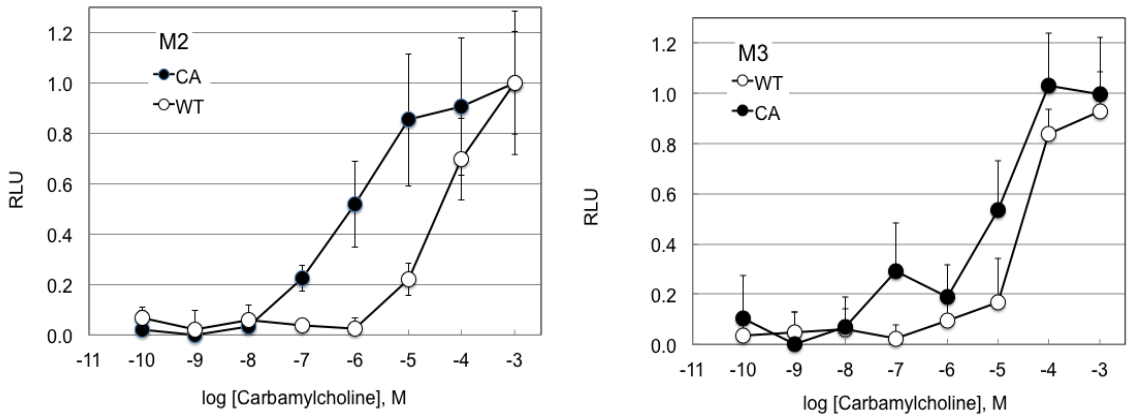

Figure 2.3. Influence of constitutively active (CA) phenotype on stimulation of CRE-mediated expression in cells transiently expressing M2 (left) and M3 (right) receptors. Activity is expressed as relative luminescent units normalized to background luminescence (sham reporter gene transfection) and the response obtained at the highest agonist concentration. Basal levels of CRE-mediated gene expression were 123 ± 21% and 115 ± 26% greater in the cells expressing M2 and M3 receptors with a CA phenotype, respectively ($p < 0.05$; N = 3; Student's *t*-test). Mean ± SD from 4 determinations from a typical experiment repeated 5 (M2) or 3 (M3) times with essentially similar results.

CA M2 receptors, but not CA M3 receptors responded to carbamylcholine with a lower threshold than the corresponding WT receptors.

2.2.4. Concentration-Dependent Inhibition and Activation of CRE Mediated Gene Expression by M2 Receptors. It is generally recognized that M2 receptors preferentially activate $G\alpha_i$ proteins thereby attenuating adenylate cyclase activity[7-9]. It is readily apparent form the experiments depicted in Figures 2.1, 2.2, and 2.3 that M2 receptor subtypes can also stimulate adenylate cyclase activity and CRE-mediated gene expression at high concentration of agonists. As shown in Figure 2.4 the nature of these responses illustrates the concentration dependence of carbamylcholine stimulated gene

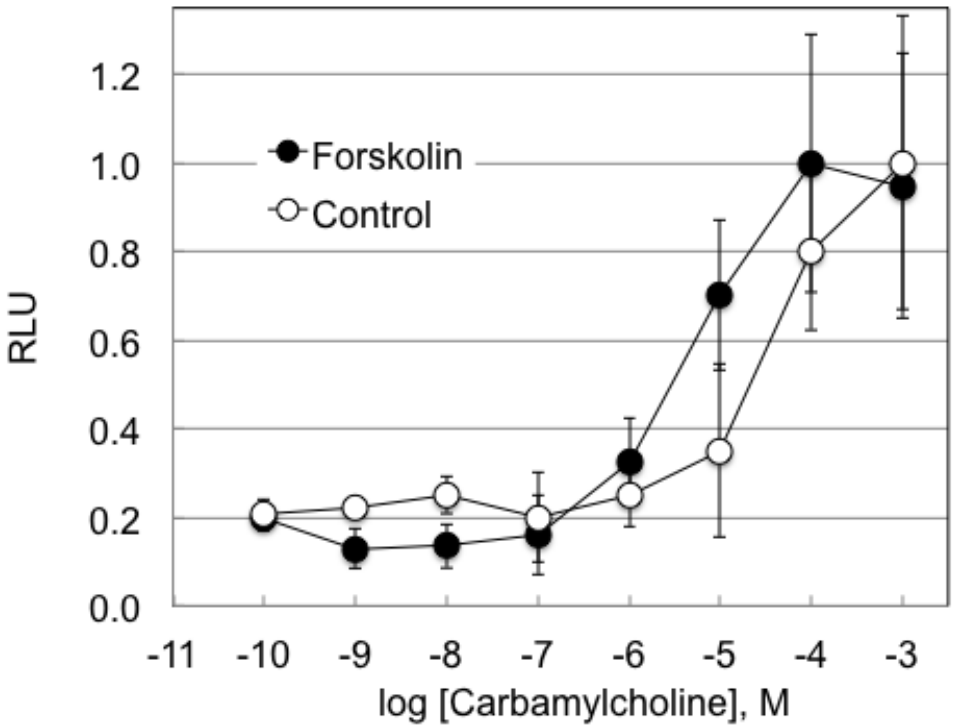

Figure 2.4. Carbamylcholine inhibition/stimulation of CRE-mediated gene expression of luciferase in CHO-M2 cells. Activity is expressed as relative luminescent units normalized to the response produced by a maximally effective concentration of carbamylcholine. Measurements were made in the absence (Control) and presence of 0.6 μM forskolin, as indicated. Mean ± SD from 4 determinations from a typical experiment repeated 4 times with essentially similar results. Carbamylcholine attenuated gene expression in the presence of forskolin at 1 and 10 nM (ANOVA; $p < 0.05$), but the EC50 values for carbamylcholine activation of response in the two conditions were not different. Inhibition of forskolin stimulation under low concentrations of carbamylcholine show Gα-i signaling.

expression under control of CRE in CHO-M2 cells was determined. Carbamylcholine activated gene expression following a 6 hour incubation with an EC50 of ≈ 30 μM. While inclusion of 0.6 μM forskolin in the incubation medium increased the response at higher agonist concentrations by ≈ 40%, an inhibition of response was revealed at very low concentrations of carbamylcholine (1-10 nM), presumably reflecting $G\alpha_{\iota}$ mediated inhibition of adenylate cyclase. This result is consistent with previous observations in

literature cited above and further demonstrates the close correlation between M2 receptor alterations of CRE mediated gene expression and cAMP production.

2.3. DISCUSSION

Activation of the G protein-coupled muscarinic receptors leads to relatively rapid changes in transmembrane potential through modulation of ion channels and transporters, either directly or through second messenger-mediated events [16]. Activation of the same receptors also leads to changes in gene expression mediated by second messenger activation of kinase cascades whose targets include transcription factors. The purpose of these experiments was to increase understanding of crosstalk signaling in muscarinic systems and to establish the efficacy of this experimental assay in characterization of constitutively signaling receptors. Specifically, this work compared crosstalk in distal signaling events (second messenger production causing altered gene expression), to evaluate the persistence of cross talk signaling in receptors with a constitutively active phenotype, and to evaluate the potential for these effects in all 5 receptor subtypes which represent multiple signaling profiles.

2.3.1. Canonical Muscarinic Signaling in CHO Cells. CHO cells are a widely used model system in biomedical research, including cellular signaling pathways, due to their robust growth and amenability to transfection and expression of recombinant proteins. In wild-type CHO cells, acetylcholinesterase activity, muscarinic receptor binding, or muscarinic signaling is not detected in either calcium mobilization or alteration of cAMP synthesis in response to the muscarinic agonist carbamylcholine. However, CHO cells express components of both the phospholipase Cβ (influencing AP-1 and NFAT) [34,35] and adenylate cyclase (CRE) [36] signaling pathways, and CHO

cells transfected with transgenes for the different muscarinic receptor subtypes respond to muscarinic agonists in pharmacologically and physiologically appropriate manners: Activation of human M1, M3 or M5 receptors expressed in CHO cells leads to the production of IP3, release of calcium from the ER, activation of store-operated calcium entry, and modulation of the expression of genes under their control. Activation of human M2 and M4 receptors expressed in CHO cells leads to the inhibition of forskolin-stimulated cAMP formation and alteration of the expression of genes under the control of the cAMP response element (CRE) [36].

2.3.2. Crosstalk in Muscarinic Signaling. A myriad of studies have shown that M2 and M4 receptors couple efficiently to Gα–i transducer proteins to inhibit adenylate cyclase, while M1, M3 and M5 couple more efficiently to the stimulation of phospholipase Cβ as a consequence of interactions with $G\alpha_{q/11}$ proteins[7-10]. Crosstalk at this level of the signal transduction cascade encompasses the ability of specific receptors to interact with different transducer G proteins, thereby activating different pathways. The factors that affect receptor/G protein coupling status are incompletely understood, but in experimental systems include identity and concentration of the agonist as well as receptor/G protein stoichiometry [11,12,15].

M2 receptor activation inhibits forskolin-stimulated cAMP formation at relatively low (EC50 ≈ 0.1 μM) but stimulated adenylate cyclase activity at high concentrations (> 100 μM). This is consistent with earlier studies [11]. Clearly, M2 receptors have an intrinsic ability signal through either pathway. As a consequence of this crosstalk signaling, M2 activation increases gene expression under the control of the CRE, even at concentrations at which an increase in cAMP production is not apparent. In the presence

of forskolin, a decrease in CRE-mediated gene expression is evident [11], paralleling the inhibition of cAMP production. Thus, the major features of M2 – $G\alpha_i$ crosstalk seen at the level of adenylate cyclase regulation are also evident at the level of regulation of gene expression.

Many class A GPCRs can be endowed with constitutive activity by slightly lengthening the sixth transmembrane domain [24]. Structural analyses of muscarinic receptors suggest that this mutation eliminates interactions between hydrophobic amino acids on the 3rd and 6th transmembrane, leading to the formation of a water filled pore [25-27]. M2 receptors with constitutive activity thus conferred mediate enhanced crosstalk signaling through the $G\alpha_s$ pathways.

A CA-inducing mutation increases CRE-mediated gene expression activity of all muscarinic receptor subtypes. This is of course expected with M1, M3 and M5 receptors, but was equally evident with M2 and M4 receptors. Moreover, carbamylcholine further activated CRE-mediated gene expression of all muscarinic receptors subtypes except M4. This suggests that the degree of receptor activation by CA-inducing mutation is less that that produced by a receptor agonist. These measurements were preformed following transient expression of the CA receptor variants. Attempts to produce a stably transfected cell line constitutively expressing increased cAMP levels were met with no success.

2.4. CONCLUSIONS

Muscarinic receptors activate both preferred and secondary signaling pathways through activation of different G proteins. Both M2 (preferred signaling through $G\alpha_i$) and M3 (preferred signaling through $G\alpha_{q/11}$) activated adenylate cyclase ($G\alpha_s$ signal) at high agonist concentrations, and these increases in cAMP resulted in upregulation of distal

reporter gene expression under control of the cAMP-dependent response element. These results demonstrate that gene expression assays are a viable and reliable means to characterize receptor-signaling pathways, and reveal similar promiscuity of receptors with respect to signaling pathway.

3. CONSTITUTIVE SIGNALING AMONG ORPHAN RECEPTORS

3.1. METHODS

Histamine, Muscarinic and Orphan GPCR receptor genes, cloned into pcDNA3.1+ (Life Technologies) were acquired from the MS&T cDNA Resource Center (www.cdna.org). These constructs were transiently co-transfected with Luciferase coupled reporter vectors to monitor CRE dependent gene expression. Each experimental treatment involved 4 wells seeded with 40,000 CHO-K1 cells in 96-well plates and incubated for 24 hours. Experiments were repeated 4 to 8 times. An "empty" plasmid (pcDNA3.1+) was used as a transfection negative control. Forskolin (3µM) mediated stimulation of adenylate-cyclase served as a positive control for the assay and additionally was used to normalize responses across experiments. Forskolin was administered 6 hours prior to measurements concurrently with sham dosing (media) where appropriate. Receptor activity was reflected by induction of luciferase expression under the control of the cAMP response element (CRE).

3.1.1. Cell Culture. CHO-K1 wild-type Chinese hamster ovary cells were obtained from the American Type Culture Collection (ATCC) and maintained at 37^{o}C with 5% CO_2 as previously described [37]. Culture media consisted of 90% HyClone DMEM (without phenol red or additional L-glutamine) supplemented with 10% HyClone FetalClone II (bovine serum product). Plates were allowed to reach 80% confluency before splitting or for use in subsequent experiments.

3.1.2. Transfection. Approximately 40,000 CHO-K1 cells in 100 µl of media were plated onto a 96-well plate and allowed to attach overnight and incubated until they had reached 80% confluency. Firefly Luciferase reporter vector (pGL4.29, Promega) was mixed with plasmid DNA containing orphan receptor GPCR coding sequences (Missouri S&T cDNA Resource Center, www.cdna.org) or an empty pcDNA3.1+ vector (Life Technologies) at a final concentration of 250 ng each per well. Transfection was carried out following manufacturer's suggestions (Lipofectamine 2000 Reagent, Life Technologies), followed by an 18 h incubation before use.

3.1.3. Luciferase Assay. Transfection medium was removed and replaced with complete medium. Controls and treatments intended to evaluate inhibition of the cAMP pathway (i.e., putative Gα-i signaling) were treated with 3.0 µM Forskolin (Sigma Aldrich). The plate was then incubated for an additional 6 hours. Immediately prior to visualization, the medium within each well was replaced with 25 µl of DMEM (-phenol red) without serum.

Imaging was performed using a FLUOstar Omega (BMG Labtech) 96-well plate reader. Auto-injection of 25ul of Bright-Glo Luciferase Reagent (Promega) was followed by 2 minutes of rotary incubation. Relative Light Units (RLUs) were obtained for each well in series over 1 minute. Each 96-well plate consisted of 12 treatment groups with 4 replicates in each group. Each treatment group was separated by a row of unused wells to minimize light pollution.

3.1.4. Data Analysis. Statistical analysis was performed in MiniTab, version 17, using a randomized complete block design. This variant of an ANOVA analysis takes differences between experiments (plates/blocks) into account and also allows for examination of treatment-block interaction. This statistical analysis allows for a strong isolation of treatment effect within the experiments.

Each experimental group (n=4) was divided by the average of the positive control treatments (3 μM Forskolin stimulated cells with CRE-Luciferase and empty pcDNA3.1+, n=4) to normalize results between plates. Each experimental treatment was then divided by its control (+/- 3 μM Forskolin) to determine the fractional stimulation or inhibition. Data was graphed as the average percent change over control between 4 to 8 plates with the over-all treatment p-value for each comparison indicated via either a single star for a threshold of 0.05, or a double star indicating a threshold of 0.01. Treatment effects that did not meet either of these thresholds were displayed individually and in red.

3.2. RESULTS & DISCUSSION

Orphan receptors were judged to be constitutively active if they significantly affected cAMP dependent signaling ($p < 0.05$, according to a randomized complete block ANOVA , 4 - 8 experiments) and additionally fulfilled at least one of the following criteria: 1) 200% elevation over baseline reporter gene expression, 2) 40% inhibition of baseline expression, or 3) 40% inhibition of expression stimulated by 3 μM forskolin. These criteria were chosen to reflect thresholds large enough to minimize false-positives due to receptor over-expression. Among the 40 orphan receptors evaluated, 75% (30) met criteria for constitutive activity.

GPCR's are characterized by their interaction with specific transducer G proteins. $G\alpha_s$ and $G\alpha_i$ play opposing roles in modulating cAMP levels in response to external stimuli by mediating the activation and inhibition of adenylate cyclase respectively. While this work did not directly measure association with either $G\alpha_s$ or $G\alpha$, changes in gene expression under control of the cAMP-dependent response element (CRE) were considered to indicate the involvement of pathways mediated by these transducers. Five patterns of signaling were noted.

3.2.1. Group A: Constitutive Inhibition of Baseline and Forskolin Stimulated CRE-Dependent Expression. As shown in Figure 3.1, the largest group of receptors (17 of 40) exhibited significant constitutive inhibition of CRE-mediated gene expression under both baseline and forskolin-stimulated conditions. This group is comprised of GPR15, GPR17 variant 3, GPR18, GPR20, GPR25, GPR27, GPR31, GPR32, GPR45, GPR55, GPR57 variant 1, GPR68, GPR83, GPR84, GPR132, GPR150, and GPR176. In all cases, the statistical significance level was less than 0.01.

This behavior is similar to results obtained with the histamine receptor 4 (HRH4), a receptor with known constitutive signaling through the $G\alpha_i$ pathway [38] using this experimental design. While all 17 of these receptors inhibited gene expression by over 40%, five of them inhibited cAMP dependent gene expression by over 80%.

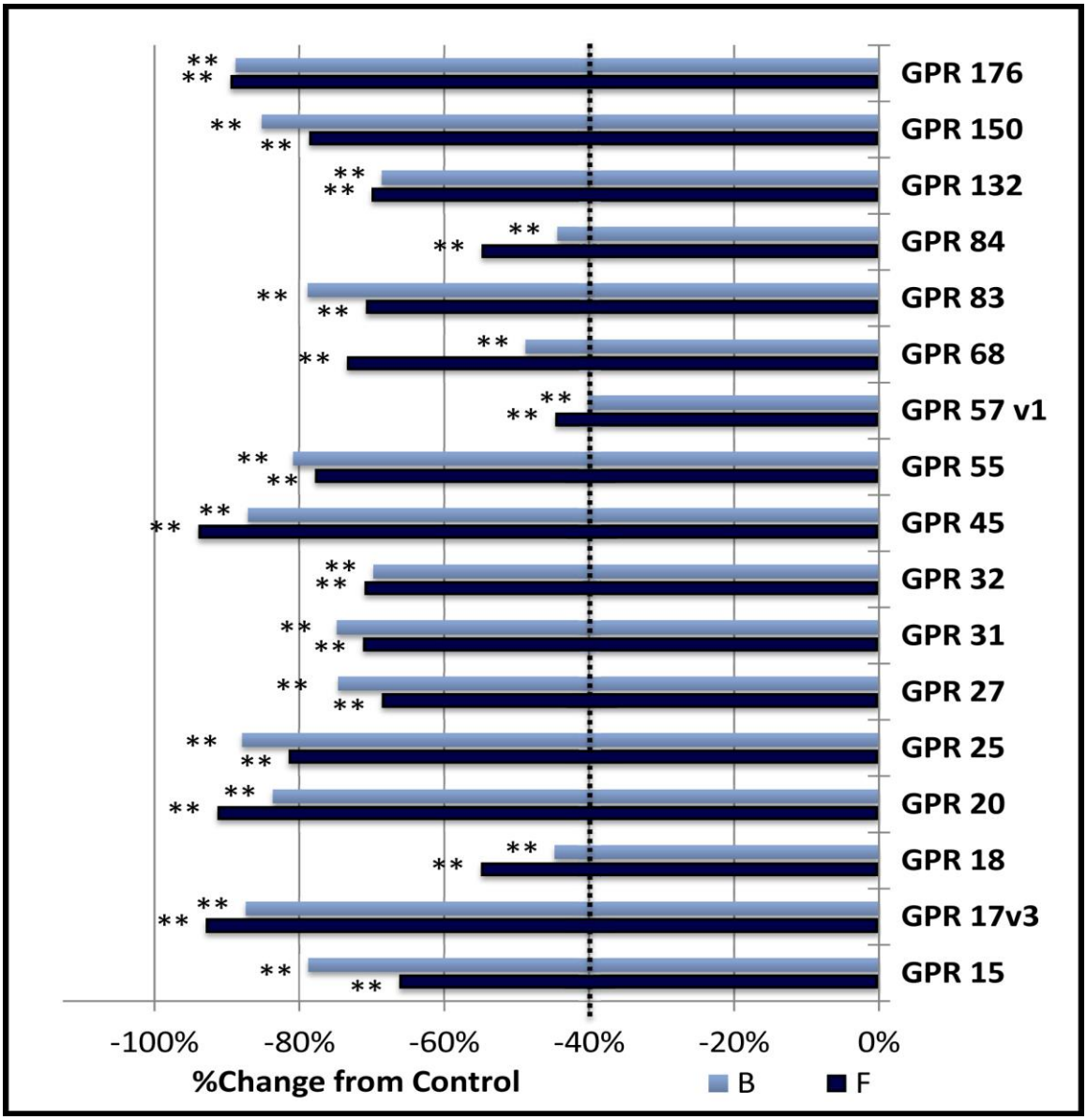

Figure 3.1. Group A: Constitutive inhibition of baseline (B) and forskolin stimulated CRE-dependent expression (F). The percent change of cAMP-dependent signaling in CHO-K1 cells transfected with CRE-Luciferase reporter vector and an orphan G protein coupled receptors is shown. Activity was measured as relative light units (RLU) and normalized between experiments by dividing by the average of the 3 µM Forskolin stimulated control within each plate. This value was then divided by the control for each condition to obtain the fractional change, with a value of zero indicating no change from control levels. Vertical dotted lines represent the minimum signaling threshold to be scored as constitutively active within this study. Changes in basal cAMP dependent expression are indicated by the light blue bars labeled "B". Changes in expression in the presence of 3 µM Forskolin (6 hour exposure) is indicated by the dark blue bars labeled "F". All receptors presented in this figure showed a significant treatment effect (** = $p < .01$) and met the criteria adopted in this work to define constitutive activity.

3.2.2. Group B: Constitutive Stimulation of Baseline and Inhibition of Forskolin Stimulated Expression. As shown in Figure 3.2, this group is comprised of receptors that are closely related in terms of amino acid homology: GPR6 and GPR12 [39]. A third member of this family (GPR3) produced extremely variable effects on forskolin stimulated expression and its inhibition within these experiments was not statistically significant ($p = 0.072$). Accordingly, it was included in group D.

Receptors in this group exhibited constitutive stimulation of CRE-mediated gene expression under baseline conditions while inhibiting CRE-mediated gene expression stimulated by 3 μM forskolin. Thus, these GPCRs can constitutively stimulate at least one aspect of baseline cAMP-mediated signaling (i.e., CRE mediated gene expression) while inhibiting high levels of cAMP-mediated signaling induced by an exogenous agent (forskolin). It is possible that these receptors act to maintain an elevated but controlled homeostatic level of cAMP by this pathway, a function known to be present in maintenance of meiotic arrest in oocyte development [40].

3.2.3. Group C: No Effect on Baseline Expression but Inhibit Forskolin Stimulated Expression. As shown in Figure 3.3, this group is comprised of GPR4, GPR26, GPR61, GPR62, GPR78, GPR101, and GPR119. These receptors did not alter baseline signaling enough to meet criteria for constitutive activity, although they all inhibited CRE mediated gene expression stimulated by 3 μM forskolin by at least 40%. In this way, they are similar to results obtained with a constitutively active mutant version of the M2 acetylcholine receptor, which is capable of signaling through both the $G\alpha_s$ and $G\alpha_i$ pathways [12,13].

While baseline stimulation did not meet criteria for constitutive activity as defined in this study, many of the receptors in this group produced a very significant "block" and "treatment by block" effect ($p < 0.01$). Further measurements may reveal constitutive activation of cAMP signaling under other conditions.

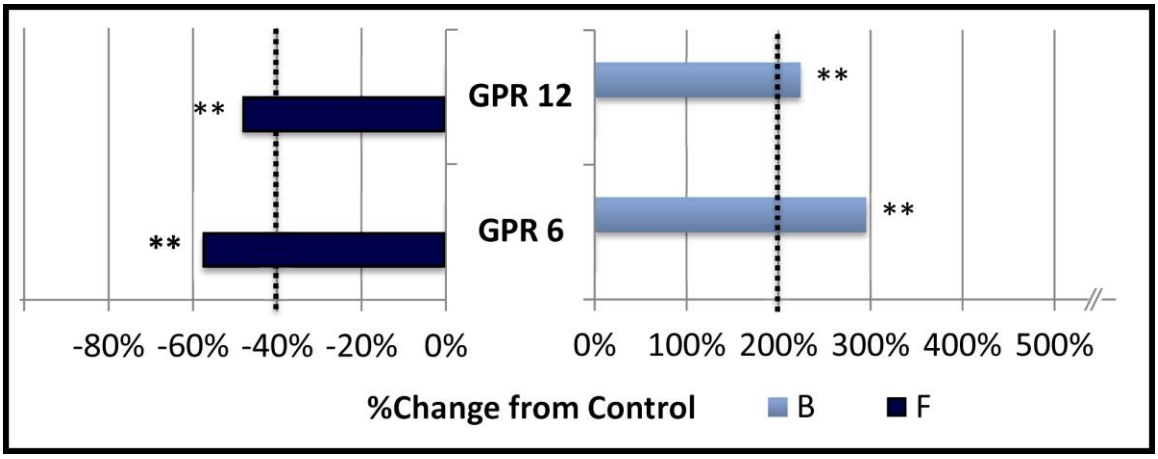

Figure 3.2. Group B: Constitutive stimulation of baseline (B) and constitutive inhibition of forskolin stimulated CRE-dependent expression (F). The percent change in cAMP dependent signaling in CHO-K1 cells transfected with CRE-Luciferase reporter vector and various orphan G protein coupled receptors is shown. Vertical dotted lines represent the minimum signaling threshold to be scored as constitutively active within this study. Activity was measured, normalized and graphed as described in the legend to Figure 3.1. Changes in basal cAMP dependent expression are indicated by the light blue bars labeled "B". Changes in expression in the presence of 3 μM Forskolin (6 hour exposure) is indicated by the dark blue bars labeled "F". Members of this group showed a significant treatment effect (** = $p < .01$) and met the criteria adopted in this study to define constitutive activity.

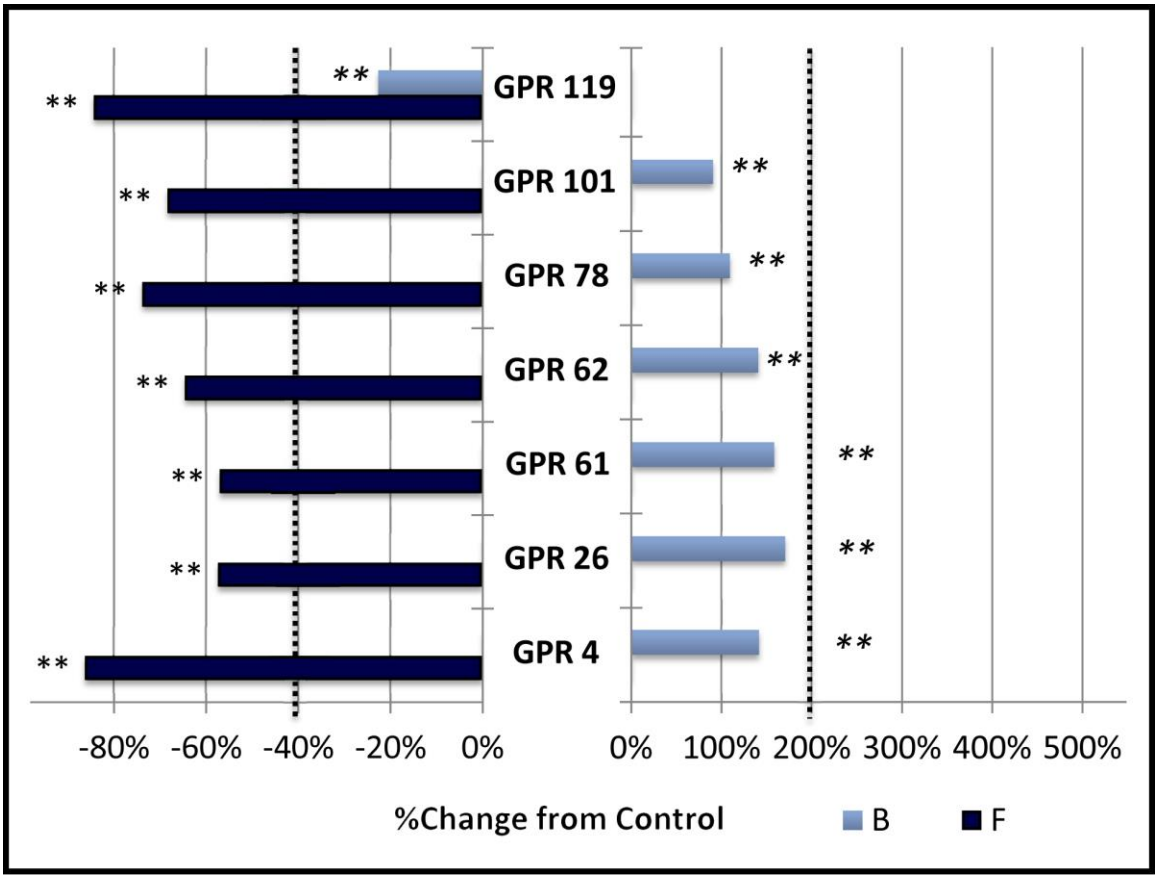

Figure 3.3. Group C: No effect on baseline (B) and constitutive inhibition of forskolin stimulated expression (F). The percent change in cAMP dependent signaling in CHO-K1 cells transfected with CRE-Luciferase reporter vector and various orphan G protein coupled receptors is shown. Activity was measured, normalized and graphed as described in the legend to Figure 3.1. Vertical dotted lines represent the minimum signaling threshold to be scored as constitutively active within this study. Changes in basal cAMP dependent expression are indicated by the light blue bars labeled "B". Changes in expression in the presence of 3 µM Forskolin (6 hour exposure) is indicated by the dark blue bars labeled "F". Members of this group showed significant treatment effect (** = $p < .01$) but did not meet criteria for constitutive activation of 200% stimulation over baseline expression levels (B). All members displayed constitutive inhibition (40% or more) of 3 µM forskolin stimulated expression (F).

3.2.4. Group D: Stimulation of Baseline Expression but No Inhibition of Forskolin Stimulated Expression.

As shown in Figure 3.4, this group is comprised of GPR3 and GPR65 along with the closely related GPR21 and GPR52 [39]. These receptors exhibited constitutive stimulation of baseline cAMP dependent signaling without any constitutive inhibition of the signaling stimulated by 3 µM forskolin. This is similar to CRE-mediated responses noted in the constitutive mutant of the M3 human muscarinic acetylcholine receptor that signals through $G\alpha_q$ and $G\alpha_s$ activation without activating the $G\alpha_i$ pathway.

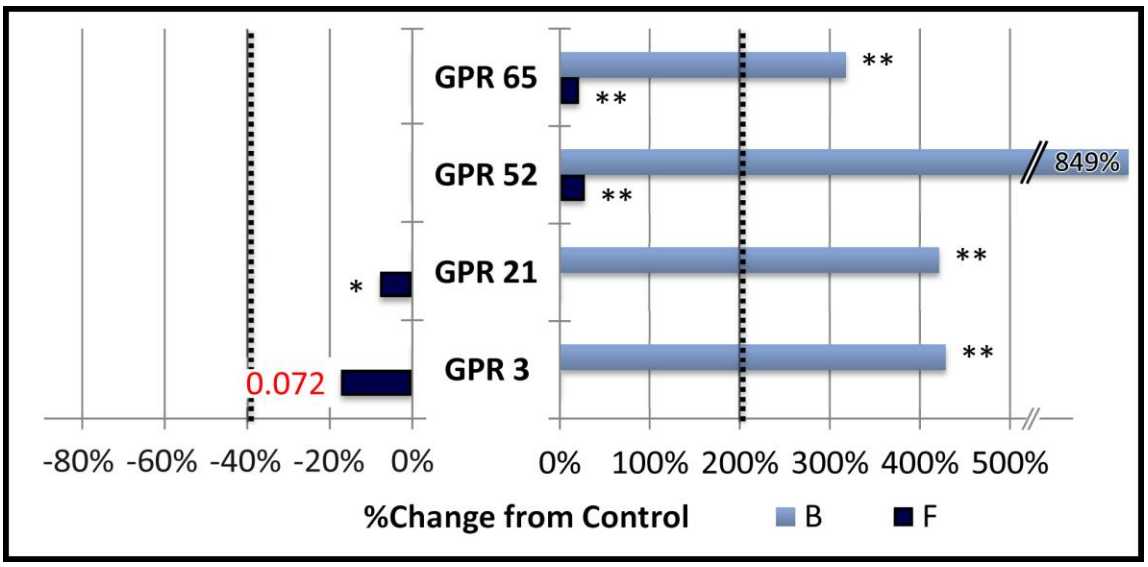

Figure 3.4. Group D: Stimulation of baseline (B) but no change of forskolin stimulated expression (F). The percent change in cAMP dependent signaling in CHO-K1 cells transfected with CRE-Luciferase reporter vector and various orphan G protein coupled receptors is shown. Activity was measured, normalized and graphed as described in the legend to Figure 3.1. Vertical dotted lines represent the minimum signaling threshold to be scored as constitutively active within this study. Changes in basal cAMP dependent expression are indicated by the light blue bars labeled "B". Changes in expression in the presence of 3 µM Forskolin (6 hour exposure) is indicated by the dark blue bars labeled "F". Members of this group showed a significant treatment effect (* = $p < .05$, ** = $p < .01$) and an increase of CRE-mediated gene expression of more than 200% under baseline conditions (B), but did not affect gene expression stimulated by 3 µM forskolin (F).

3.2.5. Group E: Non-Responders with No Constitutive Activity. As shown in Figure 3.5, this group is comprised of the remaining 10 orphan receptors: GPR1, GPR19, GPR22, GPR34, GPR35, GPR39, GPR63 variant 2, GPR82, GPR85, and GPR87.These receptors lacked constitutive activity insofar as they failed to either have a significant treatment effect or meet at least one of the three criteria for constitutive activity (i.e., 200% baseline stimulation, 40% inhibition of baseline, or 40% inhibition of forskolin-stimulated activity). Thus, not all orphan receptors exhibit constitutive signaling by criteria established in this work. Accordingly, the constitutive activity noted is unlikely to be due to an artifact arising solely from overexpression of receptor proteins in this system.

Several of the receptors in this group displayed large fluctuations in response from plate to plate, resulting in either loss of a significant treatment affect, or very significant "block" and/or "treatment by block" effect ($p < .01$). The reasons for this variability are not understood but suggest the presence of undefined variables in these multistep pathways.

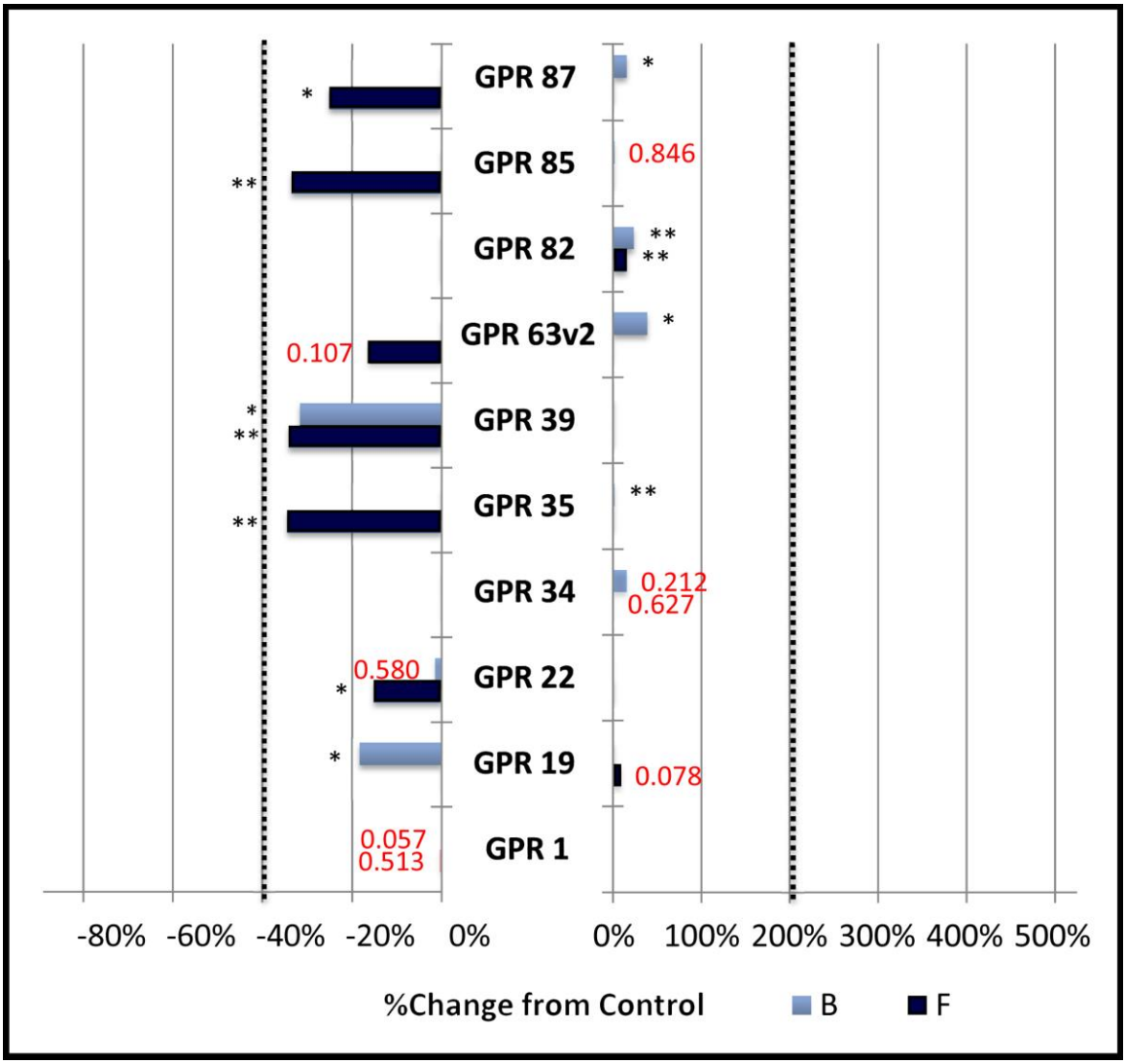

Figure 3.5. Group E: No constitutive activity. The percent change in cAMP dependent signaling in CHO-K1 cells transfected with CRE-Luciferase reporter vector and various orphan G protein coupled receptors. Activity was measured, normalized and graphed as described in the legend to Figure 1. Vertical dotted lines represent the minimum signaling threshold to be scored as constitutively active within this study. Changes in basal cAMP dependent expression are indicated by the light blue bars labeled "B". Changes in expression in the presence of 3 µM Forskolin (6 hour exposure) is indicated by the dark blue bars labeled "F". Receptors in this group failed to meet either study criteria of a significant treatment effect (p value listed in red, * = .05, ** = .01) and/or threshold for constitutive signaling.

3.3. CONCLUSIONS

The purpose of these experiments was to identify the prevalence of constitutive activity in the cAMP-dependent signaling pathway within 40 Class-A orphan GPCRs using a luciferase-linked gene expression system. The activities examined were 1) stimulation of baseline signaling, 2) inhibition of baseline signaling, and 3) inhibition of forskolin-stimulated signaling. While 10 of the 40 receptors examined did not display constitutive activity, cAMP-dependent constitutive activity was observed in 75% of the orphan class-A receptors transiently expressed in CHO-K1 cells. Five groups of receptors were defined reflecting different effects on baseline and forskolin-stimulated expression. Constitutive inhibition of cAMP-dependent signaling was much more common than stimulation (26 vs. 6 receptors), possibly reflecting cytotoxicity associated with high levels of cAMP activity.

Receptors that are closely related on the basis of amino acid homology displayed similar response patterns. For instance, the closely related GPR3, GPR6, and GPR12 all stimulated baseline cAMP-dependent signaling while GPR6 and GPR12 both inhibited forskolin activated signaling. Similarly, receptors in a second closely related group, GPR21 and GPR52, both stimulated cAMP-dependent signaling without inhibiting activity in the presence of forskolin. These results indicate that constitutive signaling is an important physiological property of most of the remaining orphan class-A GPCRs and may be a reason that many of their native ligands remain elusive. This suggests that a search for inverse agonists may be the most effective approach to understanding their physiological roles as well as selecting targets for pharmacological intervention.

4. SUMMARY, DISCUSSION, AND IMPACTS

Examination of CRE-dependent gene expression as a measure of constitutive activity in 40 different orphan class-A G protein coupled receptors is so broad a subject that a review of pertinent background would be of limited use without the inclusion of the results found within this work. Table 4.1 summarizes discussion found below, with empty cells indicating no relevant data to report. Each subsection following reviews the literature as pertaining to each individual Orphan GPCR in question, including discussion of impacts of results found here-in.

Table 4.1. Summary of results and impacts.

GPR	Agree with literature?	CA?	Impacts
1	Yes	No	
3	Yes	Yes	
4	$G\alpha_s$, but not CA	Yes	First to claim CA $G\alpha_i$
6	Yes	Yes	First to claim CA $G\alpha_i$
12	Yes	Yes	First to claim CA $G\alpha_i$
15	Yes	Yes	First to claim CA $G\alpha_i$
17v3	Yes	Yes	
18	Yes	Yes	
19	Yes	Yes ($G\alpha_q$)	
20	Yes	Yes	
21		Yes ($G\alpha_q$)	First to claim CA $G\alpha_s$
22	Yes (Removed Outlier)	Yes	
25		Yes	First to claim any CA
26	$G\alpha_s$, but not CA	Yes	First to claim CA $G\alpha_i$
27		Yes ($G\alpha_q$)	First to claim CA $G\alpha_i$
31		Yes	First to claim CA at all
32		Yes	First to claim CA at all
34	Yes		

Table 4.1. Summary of results and impacts cont…

GPR	Agree with literature?	CA?	Impacts
35	Yes		
39	Yes (Removed Outlier)	Yes ($G\alpha_{12}$)	First to claim CA $G\alpha_i$
45		Yes	First to claim CA $G\alpha_i$
52	Yes	Yes	First to claim CA $G\alpha_s$
55		Yes	First to claim CA $G\alpha_i$
57v1		Yes	First to claim CA $G\alpha_i$
61	$G\alpha_s$, but not CA	Yes	First to claim CA $G\alpha_i$
62		Yes	First to claim CA $G\alpha_i$
63v2	Yes		
65	Yes	Yes	
68	No	Yes	First to claim CA $G\alpha_i$
78	No $G\alpha_s$ CA	Yes	First to claim CA $G\alpha_i$
82	Yes		More investigation is needed
83	***No***	Yes ($G\alpha_s$)	No $G\alpha_s$; First to claim CA $G\alpha_i$
84	Yes	Yes	
85			More investigation is needed
87	Yes		
101	$G\alpha_s$, but not CA	Yes	First to claim CA $G\alpha_i$
119	***No***	Yes ($G\alpha_s$)	No $G\alpha_s$; First to claim CA $G\alpha_i$
132	No $G\alpha_i$, or CA	Yes	First to claim CA $G\alpha_i$
150		Yes	First to claim CA $G\alpha_i$
176		Yes	First to claim CA $G\alpha_i$

4.1. GPR1

A homolog of GPR1 in plants has been found to play a role in the formation and maintenance of mycorrhiza interactions [41] via increase in cAMP. Plants with this gene knocked out had very limited ability to interact with its fungal counterparts. These limitations were removed via the exogenous stimulation of cAMP. Further studies continued to implicate this orphan as involved in the cAMP dependent signaling pathways expressed under nutrient (nitrogen) starvation [42] and suggest that it may act a

glucose/sucrose sensor in yeast [43] [44]. Investigation of this orphan in animals did not reveal a specific pathway but did show an up-regulation in human-smooth muscle in response to LDL cholesterol exposure [45].

While orphan G protein coupled receptor 1 (GPR1) has been researched by multiple groups previously, no mention of specific constitutive activity was found. This is supported by this work, which did not reveal any significant impact on cAMP dependent signaling and scored this receptor in Group E: Non-responders.

4.2. GPR3

GPR3 is the first member of the "3-6-12 family", a closely related group of receptors along with its two "siblings": GPR6 and GPR12 [39]. This orphan has also shown the ability to elevate cAMP levels in multiple studies, although some go so far as to assume it is due to simple over-expression of the receptor [46]. It retains a high degree of homology between species and has been shown to act to constitutively increase cAMP levels in HEK293 cells [47] via transfection. Evidence that it may be constitutively signaling beyond the phenomenon of overexpression is found in its ability to maintain high levels of cAMP in *Xenopus laevis* oocyte development, a condition critical to their meiotic arrest [40].

The search for endogenous ligands for this receptor has led to the proposal that it and its siblings are activated by short chain free fatty acids (FFA) [48] and the proposal of an inverse-agonist with activity specific to GPR3 without affecting GPR6 and GPR12 [49].

The results of this study agree with previous findings of constitutive elevation of cAMP as indicated by a 428% increase in basal cAMP dependent expression of the

luciferase reporter vector. The response of this orphan under the influence of artificial stimulation via 3 μM Forskolin are less clear. While a majority of plates surveyed did show inhibition of the forskolin stimulation well above the 40% minimum, the range was too large to maintain statistical significance. Previous evidence of this inhibitory behavior has not been shown for this orphan and could constitute a novel finding with further investigation.

4.3. GPR4

Originally isolated in 1995 [50], GPR4 was not highly studied until a few years later. It has subsequently been shown to constitutively inhibit ERK1/2 activation [51], although a direct method was not resolved. Additional studies revealed increased activation of signaling via both the serum response element (SRE) and the cAMP response element (CRE) [52]. The search for native agonists to this receptor has led to the proposal that it may be activated by sphingosylphosphorylcholine (SPC) [53], as well as glucose and some individual amino acids [54]. Further investigation revealed that while the previous molecules do have an impact on GPR4 signaling, its primary function was as a pH sensor that coupled to $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{13}$ [55]. This was supported by the identification of specific histidine residues that undergo protonation when exposed acid conditions up to and including physiological pH [56].

While this work does reveal some stimulation of cAMP, it did not meet criteria required to be considered constitutively active. Inhibition of 3 μM Forskolin (-86%) did meet all criteria and would amount to the first evidence that this orphan receptor may couple to $G\alpha_i$ or other inhibitory mechanisms.

It is worth noting in this first example that the use of the term constitutive for this receptor is somewhat argumentative. This term is used to infer "agonist independent" signaling. It is left to the individual researcher to decide if the multiple protonation steps required to induce an active conformation of this receptor constitutes "agonist" activity.

4.4. GPR6

Another member of the 3-6-12 family, GPR6 has been shown to have a high affinity for sphingosine-1-phospate as a potential ligand and to constitutively increase cAMP in its absence as well [48]. Further study of this potential agonist has resulted in relative agreement, but in one particular case also revealed a single incident of its signaling being sensitive to pertussis toxin [57]. Studies done in-vivo and in-vitro have also implicated this orphan as having an impact in stimulation of neurite outgrowth and counteracting myelin inhibition [58] as well as being capable of signaling from internal compartments after being internalized from the cell membrane [59].

This work supports previous assertions that GPR6 constitutively stimulates cAMP dependent signaling compared to baseline (295%) but also reveals its potential to inhibit Forskolin stimulation of cAMP dependent signaling as well (-57%). This would support the findings stated above regarding pertussis sensitivity, a toxin that acts to specifically inhibit members of the $G\alpha_i$ family.

4.5. GPR12

The last member of the highly constitutively active "3-6-12 family", GPR12 behaves very similarly to its siblings with respect to elevating cAMP levels but it has

been found to have impacts on calcium mobilization as well [60]. It is upregulated in response to fluid sheer stress in vascular endothelial cells [61] and has a pertussis sensitive response to exposure of sphingosylphosphorylcholine (SPC) [62].

Results of this work agree that this receptor is capable stimulating (223%), and inhibiting (-48%) cAMP dependent signaling in a constitutive manner, although it would be the first to claim such inhibition is constitutive and not agonist dependent.

4.6. GPR15

GPR15 was first cloned in 1996 [63]. It has since been implicated in intestinal sensitivity to gp120, a small protein found in the blood of HIV positive patients. Evidence that it is capable of signaling via $G\alpha_i$ and $G\alpha_q$ pathways was found via pertussis sensitive [64], and phospholipase inhibition [65] after gp120 exposure respectively. It was suggested that high levels of expression of this orphan receptor may help target those cells for potential immune responses [66].

While this work supports these findings- with GPR4 showing strong inhibition of cAMP dependent signaling under both baseline (-79%) and 3 μM Forskolin stimulation (-66%), it is the first to reveal constitutive activity of this receptor.

4.7. GPR17 VARIANT 3

GPR17 is a putative uracil/cysteinyl-leukotrienes receptor that can signal via inhibition of cAMP and calcium mobilization [67]. It is expressed in neuronal cells in response to damage and to mediate local repair mechanisms [68]. The receptor is rather promiscuous and capable of signaling via $G\alpha_i/G\alpha_s/G\alpha_q$ pathways [69]. Inhibitory

signaling via $G\alpha_i$ has been additionally confirmed via luciferase and other methods [70], [71] with one study finding as high as 80% inhibition of forskolin stimulation in CHO cells, although they were not able to isolate the signaling to GPR17 specifically [72].

This work supports the assertion that GPR17 is a constitutively active orphan receptor with respect to $G\alpha_i$ activation with significant inhibition cAMP dependent signaling under baseline (-87%) and forskolin stimulated (-92%) conditions.

4.8. GPR18

The murine homolog of GPR18 was first isolated in 1996 [73]. A potential agonist for this orphan was proposed until 2006 when N-arachidonylglycine (NAGly) was shown to increase calcium concentration and inhibit forskolin induced cAMP production in CHO cells in a pertussis sensitive manner [74]. This orphan is known to be constitutively active in melanomas, acting as an apoptosis inhibitor [75]. It plays the reverse roll in macrophage apoptosis, signaling cell death [76]. Both of these rolls were mediated via $G\alpha_i$ pathways. Work on alternate cannabinoid compounds as potential agonist showed differential activation of $G\alpha_q$ / $G\alpha_i$ signaling suggesting biased agonism in pathway selection [77].

This work supports the assertion that GPR18 constitutively signals via the $G\alpha_i$ pathway, showing a reduction of baseline cAMP dependent signaling (-44%) and forskolin stimulated signaling (-55%) respectively.

4.9. GPR19

GPR19 was first mapped on a human chromosome in 1999 [78]. It is expressed in neuronal cells during mouse embryogenesis and plays its most significant role in early development [79]. Expression of this orphan is also increased in metastatic melanomas although its impact on the tumor cells is unknown [80]. It has been previously evaluated via a study that assumed constitutive signaling due to overexpression, where in it elevated calcium levels in the presence of a Gq/i chimeric [60]. This chimeric protein couples to receptors with an affinity for $G\alpha_i$, but signals via calcium mobilization as a native $G\alpha_q$.

This work did not reveal any significant cAMP dependent activity of GPR19 and it was scored as a "non-responder". The loss of statistical significance under forskolin stimulation was due to extreme variability of response (as shown in Figure 4.1). This, coupled with the extreme significance of the "block" and "treatment-by-block" analysis ($p << .01$ in both cases) suggests that further study of this receptor is merited.

4.10. GPR20

GPR20 is a member of the G protein coupled receptors for which exceptionally little is known. It was first cloned and mapped on a human chromosome in 1997 [81]. And it constitutively activates $G\alpha_i$ in a pertussis sensitive manner [82].

This work supports the above assertion with an inhibition of baseline signaling (-84%) and forskolin stimulated signaling (-91%).

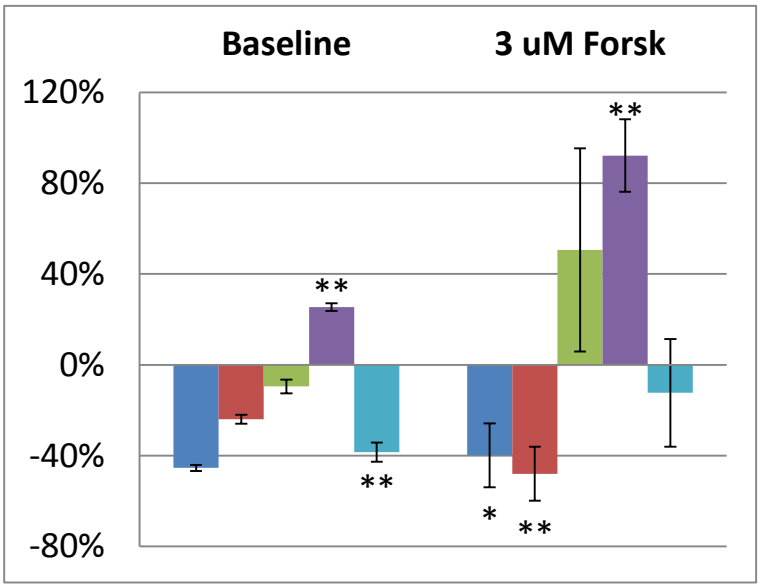

Figure 4.1. Results of five experiments with GPR19. The percent change of cAMP-dependent signaling in CHO-K1 cells transfected with CRE-Luciferase reporter vector and orphan G protein coupled receptors 19 (GPR19). Activity was measured as relative light units (RLU) and normalized between experiments by dividing by the average of the 3 µM Forskolin stimulated control within each plate. This value was then divided by the control for each condition to obtain the fractional change, with a value of zero indicating no change from control levels. Criteria for constitutive activity in this study were 200% stimulation or -40% inhibition. Changes in basal cAMP dependent expression are indicated on the left under "Baseline". Changes in expression in the presence of 3 µM Forskolin (6 hour exposure) is indicated on the right under "3 uM Forsk". Significant divergence from control expression is marked as calculated via Students T-test (n=4, * = $p < .05$, ** = $p < .01$).

4.11. GPR21

GPR21 was first cloned in 1993 [83], and then mapped on a human chromosome in 1997 [81]. It was a member of the group studied under the assumption that over-expression led to constitutive signaling. This resulted in a mobilization of calcium indicative of a $G\alpha_q$ response [60]. Subsequent work has suggested it plays a role in weight and metabolism via reducing insulin sensitivity, with GPR21 knockout mice not getting obese under high caloric diets [84] [85].

This work suggests for the first time that GPR21 is constitutively active via cAMP dependent pathway; increasing baseline cAMP dependent expression by 421%.

4.12. GPR22

GPR22 was first cloned and mapped in 1997 [81]. Evidence exists that it may couple exclusively to $G\alpha_i$ therefore inhibiting cAMP, playing a role in the regulation of cardiac function [86]. It has been implicated as a risk factor for the development of osteoarthritis based on chromosomal location [87], but other studies have called that conclusion into question [88]. It has a significant role in axis formation and knockout of GPR22 leads to defective axis formation and changes in cilia structure within the Kupffer's vesicle of zebra fish [89]. These findings suggest it functions beyond simple cardiac regulation.

This work scored GPR22 as a “Non-Responder”- although the baseline results were varied enough to loose statistical significance. This was primarily due to a single plate with highly divergent responses. If this outlier is removed, the remaining four experiments would be show an inhibition of baseline signaling (-34%) and an inhibition of forskolin stimulated signaling (-46%). These adjusted values would have a significant treatment factor ($p < 0.01$) and would meet criteria for $G\alpha_i$ activation in agreement with the previous studies above.

4.13. GPR25

GPR25 is another orphan receptor for which information is very limited. It was discovered and mapped to chromosome 1 in 1997 [90] and its expression is regulated

during exposure to LDL particles within smooth muscles [45]. No mention of a pathway or mechanism for this result is explained.

This work is the first to suggest that GPR25 is a constitutively active orphan GPCR that acts to significantly inhibit cAMP levels under both baseline (-87%) and forskolin stimulated (-81%) conditions.

4.14. GPR26

GPR26 was first cloned and identified in 2000 [91], and then mapped in 2001 [92]. It was able to elevate cAMP levels in a study that assumed overexpression would lead to constitutive activation [60]. It was found to be constitutively active in HEK293 cells where it elevated cAMP levels [93]. Another study found that GPR26 is epigenetically silenced in human glioblastomas and was capable of increasing cAMP in HEK cells in-vitro [94]. And knockout of GPR26 reduced cAMP levels in central amygdala resulting in mice showing signs of severe depression [95].

This work agrees with the previous studies asserting the ability of GPR26 to stimulate cAMP dependent signaling under baseline conditions, although it did not meet criteria to be considered “constitutive” (only 170% increase). It did meet criteria for constitutive activity via inhibition of forskolin stimulation (-57%) and this work is the first to suggest that GPR26 may also play a role in cellular metabolism via this pathway.

4.15. GPR27

GPR27 was first discovered in 1998 [96]. It is highly conserved between human, monkey, and rat homologs and may play a role in neural plasticity [97]. Overexpression

in 293T cells increases IP3 levels and siRNA knockout in MIN6 cells reduces IP3 suggesting activation of the $G\alpha_q$ pathway [98]. No other studies referencing specific mechanism, or constitutive activity outside of the $G\alpha_q$ /IP3/Calcium pathway was found.

This work is the first to suggest that GPR27 may constitutively signal through the $G\alpha_i$ pathway due to high levels of inhibition under both baseline (-74%) and forskolin stimulated conditions (-68%).

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4.16. GPR31

GPR31 was first isolated in 1997 [99]. It has been suggested that the lipid molecule 12-(S)-hydroxy-5,6,10,14-eicosatetraenoic acid (HETE) may couple with this receptor [100], but no mention of which pathway is stimulated was suggested.

This work is the first to suggest that GPR31 may constitutively signal through the $G\alpha_i$ pathway due to high levels of inhibition under baseline (-74%) and forskolin stimulated conditions (-71%).

4.17. GPR32

GPR32 was first cloned in 1998 [101]. Its expression is regulated in smooth muscles during exposure to LDL cholesterol particles [45] and may respond to "resolvins", lipid molecules that are part of inflammatory signaling [102] [103]. When triggered with these molecules it activates MapK and Nf-kB pathways [104] suggesting activation via the G12 family. One study also found possible $G\alpha_q$ activation via calcium mobilization [105]. While some study has been done on the potential impact of GPR32

activity on inflammatory signaling, no work has been done to show if this receptor is constitutively active or signals via the $G\alpha_s$ / $G\alpha_i$ cAMP dependent pathways.

This work is the first to suggest that GPR32 may constitutively signal through the $G\alpha_i$ pathway due to high levels of inhibition under baseline (-69%) and forskolin stimulated conditions (-71%).

4.18. GPR34

GPR34 was discovered in 1999 [106] and subsequently mapped in 2000 [107]. Lysophosphatidyl-L-serine (LysoPS) may be an agonist, showing a dose dependent inhibition of forskolin-stimulated cAMP in GPR34 expressing CHO cells, and possibly playing a role in mast cell degranulation [108]. Subsequent studies showed that the specificity for LysoPS is dependent on the Serine residue to activate $G\alpha_i$ signaling [109] and that it may [110], or may not be the native ligand for the human ortholog [111]. It has also been suggested that GPR34 may play a role in gastric cancer cell proliferation and migration with knockout GPR34 cancer cell lines showing considerably lower measurements under both of those metrics [112].

This work scored GPR34 as a "Non-Responder". Unlike a few other members of this group, GPR34 did not show a sizable elevation or inhibition of cAMP dependent signaling in the majority of its experiments. Removing one experiment that was a significant outlier, the remaining experiments can be seen in Figure 4.2 below. This, along with the few orphans who behaved similarly- is the basis for the assertion made in this work that the constitutive activity found here-in is not due to simple over-expression, but an inherent property of the individual receptor in question.

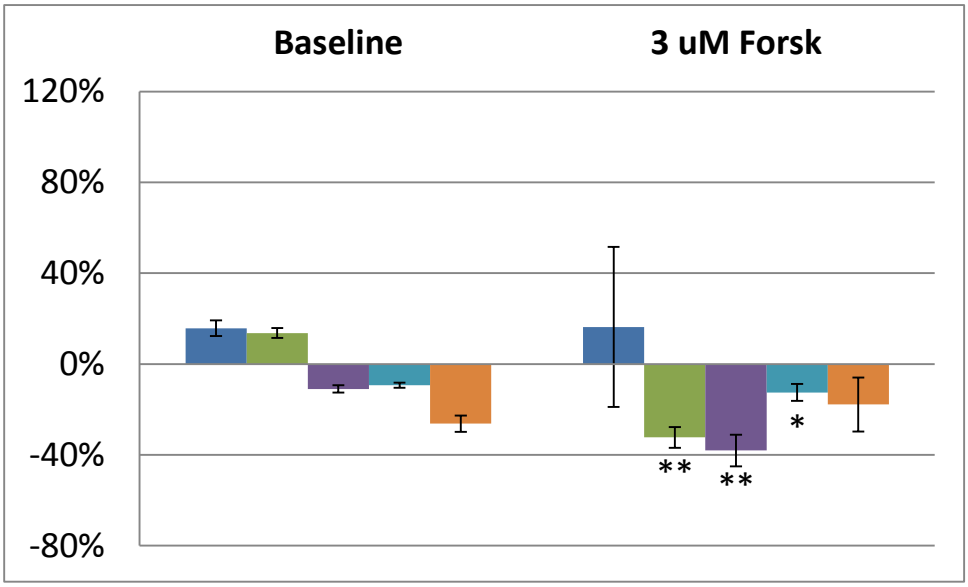

Figure 4.2. "Non-Responder" GPR34 results with single outlier removed. The percent change of cAMP-dependent signaling in CHO-K1 cells transfected with CRE-Luciferase reporter vector and orphan G protein coupled receptors 34 (GPR34). Activity was measured as relative light units (RLU) and normalized between experiments by dividing by the average of the 3 µM Forskolin stimulated control within each plate. This value was then divided by the control for each condition to obtain the fractional change, with a value of zero indicating no change from control levels. Criteria for constitutive activity in this study were 200% stimulation or -40% inhibition. Changes in basal cAMP dependent expression are indicated on the left under "Baseline". Changes in expression in the presence of 3 µM Forskolin (6 hour exposure) is indicated on the right under "3 uM Forsk". Significant divergence from control expression is marked as calculated via Students T-test (n=4, * = $p < .05$, ** = $p < .01$).

4.19. GPR35

GPR35 was discovered in 1998 [96]. Kynurenic acid was suggested as a potential ligand for the rat homolog in 2006. Exposure to this ligand inhibited calcium channels in rat sympathetic neurons, and induced a calcium response when transfected with a chimeric $G\alpha_{q/i}$ discussed previously [113]. These effects were pertussis toxin sensitive, further indicating coupling with $G\alpha_i$ [114]. It has since been found to be rather non-

specific in its agonist affinity [115] [116] including tyrphostin analogs [117], Aspirin metabolites [118], natural phenols [119].

This work scored GPR35 as a "Non-Responder". It did not meet study criteria for constitutive signaling. This is in agreement with previous studies which have all been based on different agonist dependent experiments. These findings again support the assertion of this work that the results found here-in are not the simple outcome of over-expression.

4.20. GPR39

GPR39 was first cloned in 1997 [120]. Signaling was originally thought to be due to a proposed agonist, obestatin, but was discovered to be caused instead by zinc ions [121]. Exposure to free zinc ions increased cytosolic calcium levels in cells transfected with GPR39 in a manner that was abolished by a PLC inhibitor [122]. HEK293T cells transfected with GPR39 showed constitutive activation of a SRE-Luciferase reporter which was not sensitive to obestatin [123]. These findings wound indicate that this receptor could signal through both $G\alpha_q$ and $G\alpha_{12}$ pathways. Evidence supporting this was found via disruption of the highly conserved di-sulfide bridges of this GPCR, diminishing agonist induced signaling via $G\alpha_q$ dependent calcium mobilization, but increasing constitutive SRE dependent signaling. Disruption of a second di-sulfide bridge, unique to GPR39, caused the inverse effect- greatly increasing agonist affinity and potency while diminishing constitutive SRE signaling [124]. There was a single study that found GPR39 was able to elevate cAMP levels in the cell, but in a zinc-dependent manner [125].

This work scored GPR39 as a "Non-Responder": unable to reach study criteria to be considered constitutively active (-40%). Even so, three out of four plates with GPR39 did show a significant inhibition of cAMP dependent signaling that remains statistically significant ($p < .01$). Removal of the single outlier would move this orphan into group A, significant inhibition of cAMP dependent signaling under both baseline (-63%) and forskolin stimulation (-57%). If so- it would present the first evidence of potential constitutive activation via an inhibitory pathway and in the absence of its primary indicated agonist; zinc ions.

4.21. GPR45

GPR45 was discovered in 1999 [106] and is expressed highly in the brain [126]. This work adds the first evidence of potential $G\alpha_i$ activity via the strong suppression of cAMP dependent signaling under baseline (-87%) and forskolin stimulated conditions (-94%).

4.22. GPR52

GPR52 was first identified and cloned in 1999 [127]. It is well conserved among vertebrate species, couples to $G\alpha_s$ proteins and responds to reserpine, an antipsychotic drug. In the presence of this agonist cAMP levels increased in a dose dependent manner but no evidence of constitutive basal signaling was found [128]. In the presence of a novel agonist, GPR52 was shown to inhibit D2 receptor signaling and activate D1 signaling via cAMP accumulation [129].

This work agrees with previous findings indicating activation of cAMP dependent signaling but is the first to show constitutive activation under basal signaling, let alone to the degree measured here-in (850%).

4.23. GPR55

First identified and cloned in 1999 [127], GPR55 was originally proposed as a cannabinoid receptor [130]. Pathway analysis revealed its primary function under agonist stimulation was via calcium mobilization [131]. Subsequent identification of additional cannabinoid compounds, with special affinity for delta(9)-THC, that elicited calcium mobilization solidified this as its primary mode of signaling [132]. This lead to subsequent screens for alternative agonists [133] and antagonists [134] but these were only watching the $G\alpha_q$/calcium pathways. Understandably for a receptor with such a large repertoire of suggested binding agents, the ability to recruit and signal via the $G\alpha_q$ pathway was determined to be highly agonist specific [135]. Additional information complicating the understanding of this "orphan" receptor's signaling pathway was found in its ability to hetero-dimerize with other cannabinoid GPCRs, thus shifting their signaling to its pathway [136].

This orphan receptor remains so in name only due to the lack of consensus on the primary endogenous ligand. Out of all of the research covered in this work, there was no mention of constitutive activity. The ability to significantly inhibit both baseline (-80%) and forskolin stimulated (-78%) cAMP dependent gene expression as shown here-in is the first time such evidence has been seen.

4.24. GPR57 VARIENT 1

GPR57 was first mapped in 2000 [91]. It has been suggested that it plays a role in febrile seizures due to its location on chromosome 6 [137], and it is upregulated for excretion in multiple types of human cancer cells lines [138].

This work is the first to suggest a pathway of activity for this receptor, and to show it is capable of constitutively inhibiting baseline (-40%) and forskolin stimulated (-44%) cAMP dependent gene expression.

4.25. GPR61

GPR61 was first identified in 2001 [139], and is expressed to a high degree in different areas of the brain [140]. It couples with $G\alpha_s$ constitutively and is dependent on the presence of the N-terminal 20 amino acids in order to maintain its activity, potentially acting as its own intra-molecular ligand [141]. Subsequent research suggested it plays a role in obesity [142] and Type 2 diabetes [143].

While this work does not agree with the assertion of constitutive $G\alpha_s$ coupling (157% increase did not meet thresholds for constitutive activity) it is the first to suggest that it may have an alternative role in the inhibition of cAMP via its suppression (-57%) of elevated cAMP levels due to forskolin stimulation.

4.26. GPR62

GPR62 was first discovered in 2001 and was found to be expressed highly in the brain [140]. Little else is known about this orphan receptor.

This work is the first to suggest it may be active physiologically via the constitutive inhibition of cAMP dependent signaling, shown by its ability to inhibit (-64%) forskolin dependent expression. The lack of inhibition under baseline conditions would suggest that it either couples to $G\alpha_s$ and $G\alpha_i$ as the M2 muscarinic receptor, or that its $G\alpha_i$ coupling is cAMP dependent.

4.27. GPR63 VARIANT 2

First identified in 2001 [140], it has been suggested that this orphan receptor binds to sphingosine 1-phosphate (S1P) causing an inhibition of cAMP via $G\alpha_i$ [144]. No other mention of pathway or constitutive activity was found.

This work scored this receptor as a "Non-Responder", with no significant stimulation of baseline cAMP dependent signaling and the loss of a statistically significant treatment effect under forskolin stimulation. This is further evidence that the scoring methodology used in this work is not due to simple over-expression of these receptors within this system.

4.28. GPR65

GPR65, also known as TDAG8, was first cloned in 1998 [145]. It is now known to be a "proton sensor" that stimulates cAMP under physiological pH and lower in-vitro [146]. Elevation of cAMP in cells expressing GPR65 enhanced cellular viability in mice [147]. And the proton sensor action of GPR65 may play a role in superoxide inhibition of neutrophils [148].

This study scored GPR65 as a constitutively active $G\alpha_s$ signaler due to its stimulation of baseline cAMP dependent expression (317%) and its lack of any ability to

inhibit forskolin stimulation. While conditions found within this work were controlled for pH between plates via the carbonate buffer and 5% CO2 injection during growth, as stated previously it is arguable that the signaling found here-in is not “constitutive”.

4.29. GPR68

GPR68, also known as OGR1, was first mapped in 1996 [149] and was subsequently shown to constitutively increase IP levels under physiological pH and lower [150]. Other studies found that this orphan was also able to stimulate the accumulation of cAMP, in addition to the accumulation of IP, suggesting coupling to both $G\alpha_s$ and $G\alpha_q$, but only under conditions slightly below physiological pH [151]. $G\alpha_s$ coupling was found to be unlikely during further examination, as the cAMP production was abolished in the presence of a PLC-inhibitor, suggesting it was due to cross-talk and not direct stimulation of the $G\alpha_s$ protein itself [152].

This work disagrees with previous assertions of cAMP stimulation, scoring this orphan instead as a constitutive inhibitor of cAMP dependent signaling under baseline (-48%) and forskolin stimulated (-73%) conditions. Previous studies did not examine the ability of this orphan to abolish artificially elevated cAMP levels stimulated via an exogenous agent (forskolin) but also did not mention any significant lowering of basal cAMP levels.

4.30. GPR78

GPR78 was first mapped in 2001 [92]. It was found to be constitutively active in HEK293 cells coupled to increase in cAMP as predicted in hidden Markov model method. For this reason, potential inhibition vs. $G\alpha_i$ was not assayed [93].

This work is the first to suggest constitutive activation of $G\alpha_i$ via the inhibition (-73%) of forskolin stimulated cAMP dependent expression. The lack of inhibition under baseline conditions agrees with either a competitive interaction of this orphan with a $G\alpha_s$ / $G\alpha_i$ nature, such as the human M2 muscarinic receptor, or that coupling with $G\alpha_i$ in a constitutive manner may be cAMP dependent.

4.31. GPR82

First mapped in 2001 [92], GPR82 deficient mice had a lower body weight, triglyceride level, and increased insulin sensitivity with no difference in respiratory/metabolic rates [153].

This work scored GPR82 as a "Non-Responder" in that it did not reach thresholds to be considered constitutively active. Even so, the treatment effect was very statistically significant, along with the "block" and "treatment by block" measurements ($p < .01$ in all cases). Examination of the individual experiments reveals why. Elimination of one outlier experiment, where both conditions showed stimulation above the 200% cut-off, leaves the remainder as shown in Figure 4.3. While there is usually only minor impact on baseline cAMP dependent signaling, there seems to be an intermittent phenotype that shows high levels of inhibition of forskolin stimulated cAMP dependent expression. Even with this removal, the average response still does not meet criteria for constitutive

activity (only -39% under forskolin stimulation, although still significant treatment effect, $p < 0.01$) it is worth noting that there may be something more to the story of this very unknown orphan GPCR.

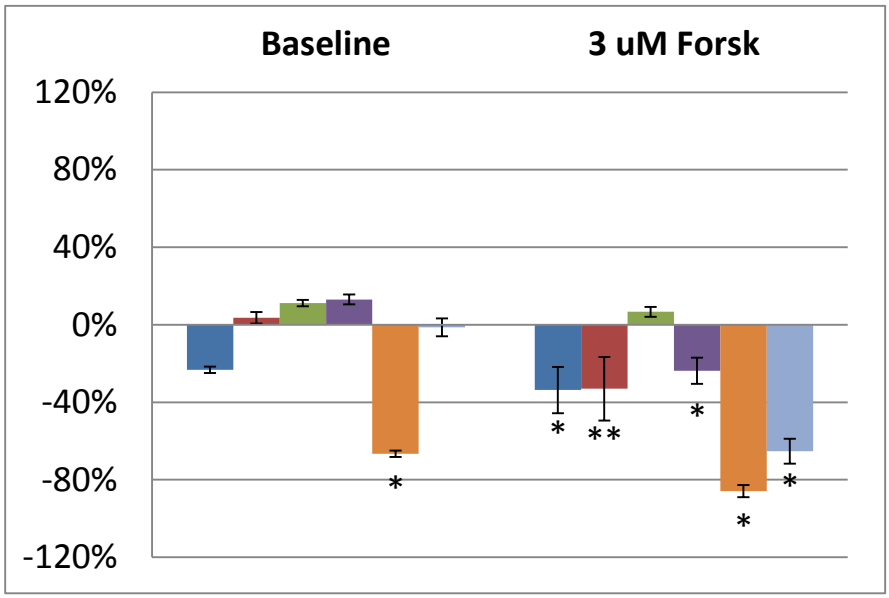

Figure 4.3. "Non-Responder" GPR82 results with single outlier removed. The percent change of cAMP-dependent signaling in CHO-K1 cells transfected with CRE-Luciferase reporter vector and orphan G protein coupled receptors 82 (GPR82). Activity was measured as relative light units (RLU) and normalized between experiments by dividing by the average of the 3 µM Forskolin stimulated control within each plate. This value was then divided by the control for each condition to obtain the fractional change, with a value of zero indicating no change from control levels. Criteria for constitutive activity in this study were 200% stimulation or -40% inhibition. Changes in basal cAMP dependent expression are indicated on the left under "Baseline". Changes in expression in the presence of 3 µM Forskolin (6 hour exposure) is indicated on the right under "3 uM Forsk". Significant divergence from control expression is marked as calculated via Students T-test (n=4, * = $p < .05$, ** = $p < .01$).

4.32. GPR83

First cloned in 2000, its expression was induced by glucocorticoid exposure [154], and is highly expressed in the forebrain [155]. It may play a role in the control of feeding behavior, regulation of stress and emotional behavior, learning and memory, and drug reinforcement and reward [156]. Pathway analysis of this orphan GPCR in mice reveals basal $G\alpha_q$ activity without any change in cAMP levels, even in the presence of forskolin. This same study also showed this orphan acts as a Zn(II) sensor via multiple extracellular histidine residues [157]. The N-terminal end of GPR83 acts as its own inverse-agonist, with deletion mutants increasing basal $G\alpha_q$ signaling via calcium mobilization but does not impact cAMP dependent signaling [158].

While this work does not agree with previous findings in mice, CHO-K1 cells transfected with GPR83 shows significant and sizable inhibition of baseline (-78%) and forskolin stimulated (-70%) cAMP dependent signaling. This would be the first suggestion of such activity.

4.33. GPR84

First discovered in 2001[159], it has been suggested as a medium chain free fatty-acid (FFA) receptor, acting via calcium mobilization and cAMP inhibition [160]. Impacts of FFA on metabolism, including consideration of GPR84 as a FFA receptor, has been reviewed twice in recent history [161], [162].

This work agrees with previous work that GPR84 acts through $G\alpha_i$ inhibiting cAMP dependent signaling of baseline (-44%) and forskolin stimulated (-54%)

conditions. It is arguable if this activity is truly "constitutive" or if it is responding to FFAs in the cell culture medium during the experiment.

4.34. GPR85

GPR85 is an orphan G protein coupled receptor expressed primarily in the brain [163], plays a role in neural plasticity and is highly conserved between human, monkey, and rat [97]. Over expression of this receptor in mice decreased brain size, and knock-out mice showed increased brain size [164].

This work scored GPR85 as a "Non-Responder", with the baseline expression not even capable of holding statistical significant treatment effect. Even so, examination of the experimental data (Figure 4.4) reveals a strong trend between two different responses- one that amounts to no effect among the first three experiments, and a significant inhibition of cAMP dependent signaling in later experiments. What may have caused this divergence in responses is unknown, but does flag this receptor as worthy of further investigation.

4.35. GPR87

Data mining of previous studies discovered GPR87 in 2001 [159]. Lysophosphatidic acid (LPA) has been suggested as a potential agonist for GPR87, via activation of a G protein fusion eliciting a calcium response [165]. It is over-expressed in many cancer cells and knockdown of GPR87 has anti-proliferative affect [166]. This work scored GPR87 as a "Non-Responder". Along with others mentioned in this group, the response of GPR87 is further evidence that this work is not measuring activity due solely to the over-expression of the receptor. This supports the claim that the criteria

used within this work as an effective means of measuring constitutive activity inherent in the receptor itself.

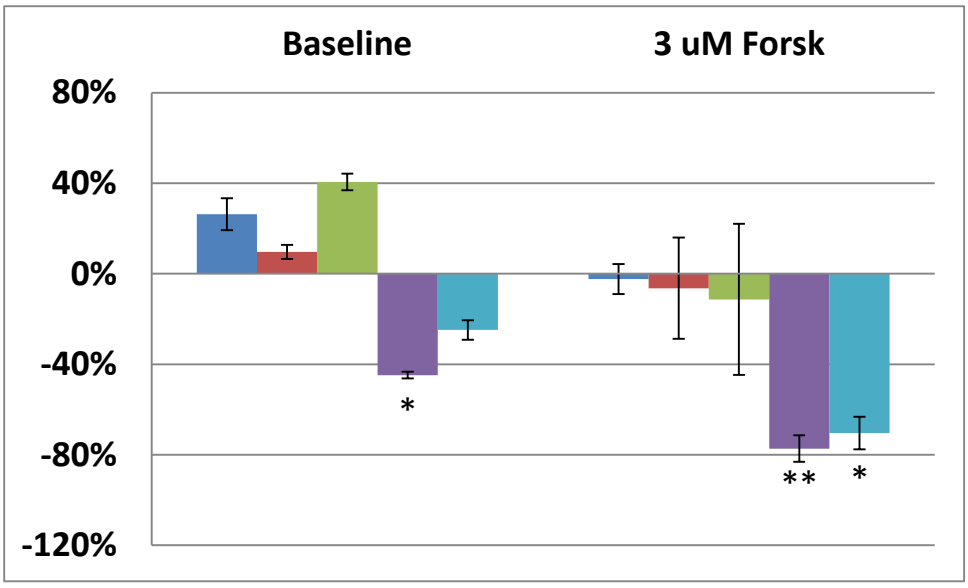

Figure 4.4. "Non-Responder" GPR85 results with single outlier removed. The percent change of cAMP-dependent signaling in CHO-K1 cells transfected with CRE-Luciferase reporter vector and orphan G protein coupled receptors 85 (GPR85). Activity was measured as relative light units (RLU) and normalized between experiments by dividing by the average of the 3 µM Forskolin stimulated control within each plate. This value was then divided by the control for each condition to obtain the fractional change, with a value of zero indicating no change from control levels. Criteria for constitutive activity in this study were 200% stimulation or -40% inhibition. Changes in basal cAMP dependent expression are indicated on the left under "Baseline". Changes in expression in the presence of 3 µM Forskolin (6 hour exposure) is indicated on the right under "3 uM Forsk". Significant divergence from control expression is marked as calculated via Students T-test (n=4, * = $p < .05$, ** = $p < .01$).

4.36. GPR101

First mapped in 2001 [92], GPR101 is matched to $G\alpha_s$ via hidden Markov model analysis and elevated CRE dependent luciferase expression in HEK293 cells, although no

test for forskolin inhibition was done [167]. It has also been linked to acromegaly due to single nucleotide polymorphism mutation [168].

This study agrees with the implication of $G\alpha_s$ signaling, although it did not meet criteria for constitutive signaling, and is the first to show inhibition of forskolin stimulated expression (-68%).

4.37. GPR119

First identified in 2002 [169], GPR119 has been highly studied as a target for metabolic disorders including type 2 diabetes. Its remaining classification as an orphan is due mainly to the ongoing search and disagreement as to its primary native agonist. For example, Lysophosphatidylcholine (LPC) acts as a strong enhancer of rat pancreatic insulin secretion in response to high concentrations of glucose via the simulation of cAMP. LPC also increases cAMP in mouse pancreatic cell lines in a dose dependent fashion. Exposure to siRNA specific to GPR119 blocked these effects [170]. Oleoylethanolamide and N-oleoyldopamine are potent agonists of GPR119, inducing intracellular cAMP accumulation in both pancreatic and gut enteroendocrine cells [171]. These suggested agonists were found to have differential effect on GPR119 pathway signaling preferentially activing cAMP or calcium mobilization [172]. GPR119 and its implications for the treatment of type 2 diabetes and related metabolic disorders was reviewed in 2009 [173], and again 2012 [174]. The search for potential agonists since has concentrated on finding activating molecules that do not cause severe desensitization during in-vivo studies [175]. Recent studies have noted constitutive activity of this receptor that is $G\alpha_s$ dependent [176,177], and is highly dependent on multiple extracellular residues [178].

This work scores GPR119 as a constitutive inhibitor of forskolin stimulated cAMP dependent signaling (-84%), but does not agree with recent findings indicating constitutive $G\alpha_s$ pathway activation. It does not seem common among assumed $G\alpha_s$ constitutive signalers to test if they can inhibit an exogenous cAMP stimulation (forskolin). Given the nature of this orphan in regulation of cAMP dependent insulin release the potential that it may constitutively signal via inhibitory pathways would contribute to its homeostatic mechanism. This could also be explained via differences in cell expression in CHO cells verses cells lines that natively express this orphan.

4.38. GPR132

GPR132, also known as G2A, has been suggested as a receptor for oxidized FFAs- with a potential role in lipid overload and oxidative stress via calcium mobilization [179]. It is also been suggested as a pH-sensor but is missing the Histidine residues of its relatives (GPR4, TDAG8) and did not significantly elevate IP or cAMP [146]. Further examination showed that it is not as sensitive to pH as its relatives, but may signal through $G\alpha_i$ / $G\alpha_q$ / $G\alpha_s$, and $G\alpha_{13}$ to influence Migration, and apoptosis [180]. Lysophosphatidyl-choline (LPC) binding to GPR132 can produce signaling via IP & cAMP, though only the cAMP response is dose dependent [181]. Activation by 9-hydroxyoctadecadienoic acid (9-HODE) in CHO-K1 cells showed Ca mobilization, IP3 increase, and inhibition of cAMP levels, suggesting activation of $G\alpha_i$ signaling pathways [182]. Lysophosphatidyl-serine acts as an agonist for GPR132 causing an increase in cAMP in macrophages and increased their ability to clear recruited neutrophils from areas of inflammation [183].

This work scores GPR132 as a strong inhibitor of baseline (-68%) and forskolin stimulated (-70%) cAMP dependent signaling. This orphan is a highly studied G protein coupled receptor that only remains an orphan due to the disagreement over the primary native agonist coupled to its activation. Most of its agonist-dependent action seems to be related to $G\alpha_q$ / $G\alpha_s$ signaling, the constitutive $G\alpha_i$ implicated within this work may be a new function in maintaining low levels of cAMP until stimulated by agonists.

4.39. GPR150

First discovered in 2005 [184], GPR150 is a possible candidate for tumor bio-marker as it was upregulated in 4 out of 15 different cancer types via methylation of its promotor [185]. Structurally, it is related to gonadotropin releasing hormone receptors, although no agonist has been suggested [39].

This work suggests for the first time that GPR150 is a strong constitutive inhibitor of baseline (-85%) and forskolin stimulated (-78%) cAMP dependent expression. As seen in Figure 4.5, the treatment effect of GPR150 being present in CHO-K1 cells is significant and is similar to results seen in all members found in "Group A" in this work.

4.40. GPR176

GPR176 was first discovered in 1995 [186], and is regulated during smooth muscle cholesterol synthesis but there is no mention of a pathway [45].

This work is the first to show that GPR176 is a strong constitutive inhibitor of baseline (-88%) and forskolin stimulated (-89%) cAMP dependent expression.

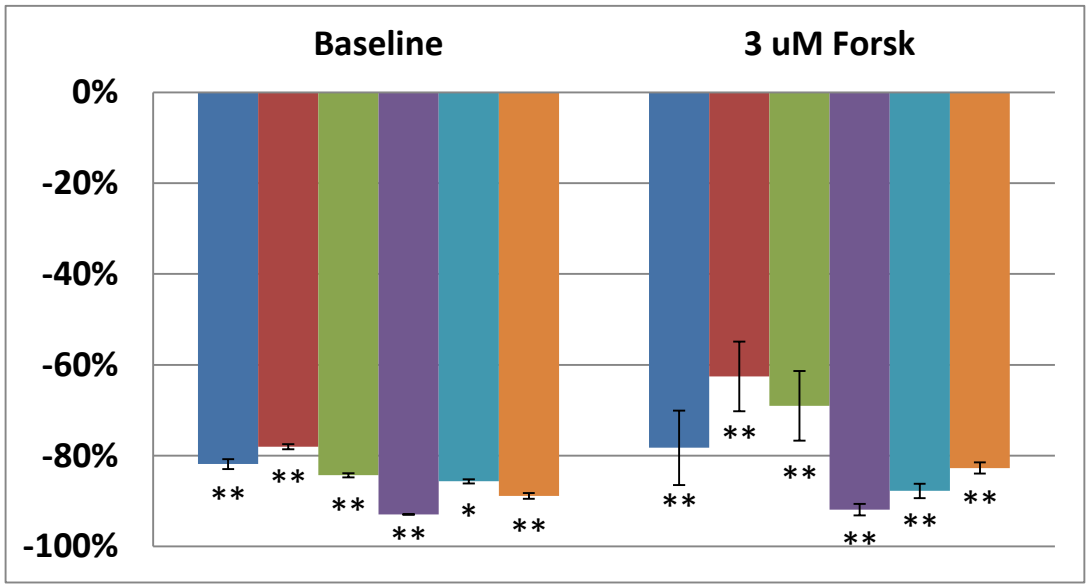

Figure 4.5**.** "Group A" GPR150 results. The percent change of cAMP-dependent signaling in CHO-K1 cells transfected with CRE-Luciferase reporter vector and orphan G protein coupled receptors 150 (GPR150). Activity was measured as relative light units (RLU) and normalized between experiments by dividing by the average of the 3 µM Forskolin stimulated control within each plate. This value was then divided by the control for each condition to obtain the fractional change, with a value of zero indicating no change from control levels. Criteria for constitutive activity in this study were 200% stimulation or -40% inhibition. Changes in basal cAMP dependent expression are indicated on the left under "Baseline". Changes in expression in the presence of 3 µM Forskolin (6 hour exposure) is indicated on the right under "3 uM Forsk". Significant divergence from control expression is marked as calculated via Students T-test (n=4, * = $p < .05$, ** = $p < .01$).

5. CONCLUSIONS

The following conclusions can be drawn from the data and analysis presented in this work:

- Gene expression assays are a viable and meaningful characterization method for receptor signaling and can reveal promiscuity and constitutive activity of G protein coupled receptors.
    - This technique was able to characterize both wild-type and constitutively active versions of all five members of the Muscarinic GPCR family in agreement with canonical understanding.
- This technique allowed for the experimental examination of 40 different orphan class-A G protein coupled receptors to screen for the prevalence of cAMP dependent constitutive signaling, revealing such activity in 75% of receptors studied.
- Constitutive inhibition of cAMP dependent signaling was much more common than stimulation
- Novel findings with respect to potential signaling pathways was found in 23 orphans, and otherwise agrees with previous findings where signaling pathway has been examined.
- Results of this work can be attributed to constitutive signaling and not simple over expression of receptors.
    - “Non-Responders” revealed patterns that suggest either a lack of constitutive signaling or an un-resolved triggering condition (ie. agonists dependent).

- Constitutive signaling is an important physiological property a majority of the remaining orphan class-A GPCRs.
    - The search for inverse agonists may be the most effective approach to understanding their physiological roles as well as selecting targets for pharmacological intervention

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VITA

Adam Lee Martin was born in Blue Earth Minnesota in 1978. Spending his first six years growing up on the family farm, he then moved with his immediate family to Salina, KS. He then was accepted into the Wildlife Biology program at the Kansas State University in Manhattan, KS. During his stay there, he was involved undergraduate research on Collard Lizards with Dr. Eva Horne. After graduating from KSU with a B.S. in Natural History in 2002, he was then accepted into the M.S. program in Applied and Environmental Biology at UMR. During this time he participated in the Missouri Herpetological Association meetings and the Society of Integrative and Comparative Biology, presenting his research at both. After graduating with his Master's in 2004, he was hired as staff within the Department of Biological Sciences at Missouri University of Science & Technology. He spent the next 10 years running the Neurobiology lab for Dr. Aronstam, and was slowly moved into teaching responsibilities along with continuing to manage the lab. In the spring of 2010 he began work towards a PhD in Chemistry under Dr. Ercal and Dr. Aronstam at Missouri S&T. He attended national meetings including SFRBM, ASCB, and GPCR symposium, presenting his doctoral work completed thus far at the latter two. He published papers on NACA's impact on doxorubicin toxicity in 2009, cell-penetrating peptides for protein transduction in 2012, and two papers on the effects of honokiol and oxidative stress on store operated calcium release in CHO cells, along with 12 abstracts during that time. Completing his PhD in the spring of 2015, he plans to continue his career of teaching and research.