FUNCTIONAL IDENTIFICATION AND INITIAL CHARACTERIZATION OF A FISH CO-RECEPTOR INVOLVED IN AVERSIVE SIGNALING

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FUNCTIONAL IDENTIFICATION AND INITIAL CHARACTERIZATION OF A FISH CO-RECEPTOR INVOLVED IN AVERSIVE SIGNALING

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There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.

-J. R. R. Tolkien

To my friends and family, who never let me take myself too seriously

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LIST OF SYMBOLS AND ABBREVIATIONS

AMY	Amylin
AMY1R	Amylin receptor
AT_1	Angiotensin-1
ATP	Adenosine-5'-triphosphate
BLAST	Basic local alignment search tool
BLAT	Basic local alignment tool
cAMP	Cyclic adenosine monophosphate
CaSR	Calcium sensing receptor
CCR	CC chemokine receptor
cDNA	Complementary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
CL-R	Calcitonin receptor-like receptor
cRNA	Complementary ribonucleic acid
CT-R	Calcitonin receptor
CXCR	Chemokine receptor
DAPI	4',6-diamidino-2-phenylindole
DEET	N,N-Diethyl-meta-toluamide
DMSO	Dimethyl sulfoxide
DMSP	Dimethylsulfoniopropionate
DNA	Deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ENaC	Amiloride sensitive sodium channel
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases

EST	Expressed sequence tag
FORM	Formoside
GABA	γ-Aminobutyric acid
GAP	GTPase Activating Protein
GC	Gas chromatography
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HCN	Hyperpolarization-activated cyclic nucleotide gated
HEK293	Human embryonic 293 cells
HPLC	High performance liquid chromatography
IBMX	3-Isobutyl-1-methylxanthine
ISO	Isoproterenol
MEK	MAPK/ERK kinase
mGluR	Metabotrophic glutamate receptor
MOB	Main olfactory bulb
MOE	Main olfactory epithelium
mOR-EG	Mouse eugenol receptor
mRNA	Messenger ribonucleic acid
NMR	Nuclear magnetic resonance
OCT	Octanal
OPR	Opioid receptor
OR	Olfactory receptor
OR-I7	Rat aldehyde receptor
OSN	Olfactory sensory neuron

P2Y1	Purinergic receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKA	Protein kinase A
PMSF	phenylmethylsulphonyl fluoride
PTH2R	Parathyroid hormone 2 receptor
RAMP	Receptor activity modifying protein
RGS	Regulator of G Protein Signaling
RL-TGR	RAMP-like triterpene glycoside receptor
RT-PCR	Reverse transcriptase-polymerase chain reaction
SSTR	Somatostatin receptor 3
T1R1/3	Umami taste receptor
T1R2/3	Sweet taste receptor
T2R	Bitter taste receptor
TAARs	Trace amine-associated receptor
TBS	Tris buffered saline
TEVC	Two-electrode voltage clamp
TRC	Taste receptor cell
TRHR	Thyrotropin-releasing hormone receptor
TRP	Transient receptor potential
TRPV1	Transient receptor potential V1 channel
VPAC-1	Vasoactive intestinal peptide receptor 1
αAR	α-Adrenergic receptor
β_1AR	Beta-1 adrenergic receptor
β_2AR	Beta-2 adrenergic receptor

β_3AR	Beta-3 adrenergic receptor

SUMMARY

Chemoreception plays an important role in predator-prey interactions and feeding dynamics. While the chemoreception of attractant or pleasant tasting compounds has been well studied, aversive chemoreceptive signaling has been difficult to investigate behaviorally in an ecological context because these interactions are species- and context-specific and deterrent compounds vary among prey. Therefore, little is known about the molecular mechanism(s) used in detection of aversive compounds. Using the coral reef system, this thesis explores on a molecular level the deterrent mechanism underlying detection by fish predators of an aversive compound, in order to gain a greater understanding of predator-prey interactions in this community. Like other organisms that are sessile or slow-moving, marine sponges have special mechanisms for defense from predation, commonly containing aversive-tasting compounds that defend these organisms from predation. To this end, we sought to identify and characterize a fish chemoreceptor that detects one or more of these compounds.

A behavioral assay demonstrated that many sponge compounds that are known to be deterrent to coral reef predator fish are also deterrent to zebrafish, a freshwater fish whose genome is well-characterized. Two of these groups of deterrent triterpene glycosides, formoside and a mixture of ectyoplasides A and B, caused electrophysiological changes in *Xenopus* oocytes expressing an entire zebrafish cDNA library, β_2AR, and the ion channel CFTR. Utilizing this electrophysiological bioassay, we fractionated the zebrafish cDNA library and isolated a single cDNA clone encoding RL-TGR, a novel co-receptor involved in the signaling of triterpene glycosides. This co-receptor appears to be structurally and functionally related to receptor activity-modifying

proteins (RAMPs), a family of co-receptors that physically associate with and modify the activity of G protein-coupled receptors (GPCRs). Structurally, this protein is predicted to have a single-pass transmembrane domain, a short intracellular domain, and a long extracellular domain. Expression in *Xenopus* oocytes showed that it responds specifically to triterpene glycosides and no other compound tested in a receptor-mediated manner. Additionally, RL-TGR requires co-expression of a GPCR to enable signaling in oocytes, and both of these receptors may be components of a larger signaling complex, as suggested by immunoblotting evidence. Immunoblotting from expressing *Xenopus* oocyte membranes demonstrated that this protein is membrane associated. A 40 bp portion of the gene is conserved across multiple fish species, but is not found in any other organism with a published genome, suggesting that the expression of this receptor is limited to fish species. Therefore, this fish gene may have coevolved with organisms that produce triterpene glycoside defensive compounds, which include sponges, echinoderms, and vascular plants.

This work suggests that aversive compounds may be detected by RL-TGR and related proteins in fish. The use of a GPCR and RAMP-like co-receptor complex as a detector of deterrent compounds is a clever mechanism in which to perceive potentially harmful compounds. Instead of necessitating expression of a specific *bona fide* receptor (with the ability to both bind ligand and transduce signals) for each possible compound an organism might need to detect in its lifetime, an organism would only require expression of a limited number of GPCRs and a suite of co-receptors, which can combine in numerous combinations to specifically and efficiently detect a vast number of deterrent compounds, protecting these organism from potentially harmful compounds.

This interdisciplinary work crosses the boundaries of behavioral neuroscience, chemical ecology, and molecular biology, and unites fields that rarely overlap. The discovery of RL-TGR is significant not only because it defines a new chemoreceptor-ligand pair in a field where few of these interactions are known, but also because the gene encoding RL-TGR is the first identified that encodes a co-receptor which responds to a chemical defense. This finding may lead the way for the identification of many other receptors that mediate chemical defense signaling in both marine and terrestrial environments, as this protein has the potential to represent the first of an entire family of co-receptors that respond to aversive compounds. The further study of RL-TGR and any related co-receptors will deepen our understanding of the molecular mechanisms of chemical defense compounds and their effects on predator-prey interactions.

CHAPTER 1

INTRODUCTION

Organisms communicate with each other for a variety of reasons, including but not limited to, finding a mate, alarming a conspecific to the presence of a predator, establishing social dominance, and signaling conspecifics to a food source; all of these forms of communication are mediated by signals or cues (Bradbury and Vehrencamp, 1998). Many of these cues are chemical in nature and are produced by a signaler and detected by another organism (Dusenbery, 1992). Since Buck and Axel published their Nobel Prize-winning work on the elucidation of the family of proteins involved in the detection of odorants (Buck and Axel, 1991), the mechanism of how higher organisms chemically sense their environment has been an intensely researched topic (Mombaerts, 1999a; Buck, 2004; Chandrashekar *et al.*, 2006; Lemon and Katz, 2007). However, little is known about the mechanisms regarding how marine predators detect aversive compounds present in potential prey organisms. In this chapter, the molecular mechanisms underlying taste and olfactory sensory systems and their role in marine chemical ecology will be discussed.

1.1 Identification and Mechanism of Chemical Defense Compounds

Many sessile, soft-bodied, and slow-moving marine organisms contain secondary metabolites collectively known as chemical defense compounds, which protect these organisms from an array of detrimental conditions in their environment (Paul *et al.*,

2006). These chemically defended organisms have been subject to intense investigations by groups interested in marine natural products and their chemical ecology.

These studies are typically initiated by isolating and identifying deterrent compounds with a range of chemistry techniques, including fractionation of a crude extract in combination with a bioassay. A common bioassay for the isolation of marine and aquatic defensive compounds is a palatability assay, a behavioral experiment whereby a food pellet is laced with crude extracts or isolated compounds from a potential prey organism and fed to a generalist predator (Hay *et al.*, 1998). Acceptance or rejection is observed, and the deterrent compound is pursued by further separation and testing of the biologically active fractions. To generate the intial crude extract, the organism of interest is exhaustively extracted in various solvents and subjected to liquid-liquid partitioning. Polarity and size are commonly utilized as criteria for fractionation using methods such as high performance liquid chromatography (HPLC), gas chromatography (GC), and gel chromotography. After isolation of the defensive compound, techniques such as nuclear magnetic resonance (NMR) spectroscopy are useful for structural identification.

Using the above chemical techniques, the chemistry and ecology of these compounds has been well studied (Pawlik and Fenical, 1992; Hay, 1996; Pawlik *et al.*, 2002; Lane and Kubanek, 2006; Long and Hay, 2006; Paul *et al.*, 2006); however, far less is known about the sensory mechanism of how a predator determines which prey is palatable and safe enough to ingest. While the detection and processing of palatable compounds, attractants, and feeding stimulants have been well examined, especially in fish (Caprio *et al.*, 1993; Hara, 1994; Sorensen *et al.*, 1998; Derby and Sorensen, 2008),

the neurobiology that mediates the deterrence of these compounds has remained elusive and understudied (Derby and Sorensen, 2008), with few exceptions (Jordt and Julius, 2002; Sheybani *et al.*, 2009).

Defensive compounds could act on potential predators in a number of different ways. Release of the defense ink and opaline by *Aplysia* (sea hare) causes phagomimicry, a defensive mechanism whereby a predator is fooled into treating this chemical secretion as food, allowing the prey to escape unharmed (Kicklighter *et al.*, 2005; Shabani *et al.*, 2007). Several species of ascidians produce inorganic acids that cause them to be unpalatable to potential predators and damage cells of the organisms that ingest them (Pisut and Pawlik, 2002; Stoecker, 1980). Alternatively, phlorotannins, found in marine algae, and tannins, found in terrestrial plants, form indigestible complexes with plant nutrients or inactivate digestive enzymes by binding to them (Boettcher and Targett, 1993; Mole and Waterman, 1987; Targett and Arnold, 2001). Some deterrent compounds are hypothesized to be toxic (Lindquist and Hay, 1995), and potential predators have unknown molecular detection methods which prevent them from ingesting prey bearing these compounds. Other deterrent compounds have been shown to have other, non-ecologically relevant activity, such as cytotoxicity to cancer cell lines (Konig *et al.*, 1998). A study of the cellular effects of chemical deterrents from sea sponges suggested that 4,5-dibromopyrrole-2-carboxylic acid, a deterrent compound found in *Agelas* marine sponges, may alter calcium homeostasis of chemoreceptive cells (Bickmeyer *et al.*, 2005). However, this study investigated calcium responses in rat adrenal cells and *Aplysia* neurons, which are only distantly related to the natural predators

of sponges; therefore, this physiological response may not occur in fish chemoreceptive cells.

The noxious compound capsaicin, another defensive compound, protects chili peppers from predation (Jordt and Julius, 2002) and their seeds from pathenogenic infection (Tewksbury and Nabhan, 2001). Capsaicin, like other pungent compounds such as those found in onions, garlic, and mustard, activates the TRPV1 ion channel (Caterina *et al.*, 1997; Salazar *et al.*, 2008). This channel is a member of the transient receptor potential (TRP) family of cation channels that mediate responses to many noxious compounds (Ramsey *et al.*, 2006). The TRPV1 channel also responds to high temperatures (>43^{o}C) and protons (Caterina *et al.*, 1997). Interestingly, although the avian ortholog does respond to heat and protons, it does not respond to capsaicin (Jordt and Julius, 2002), allowing birds to readily consume peppers and disperse their seeds across a wide geographic area. By testing the electrophysiological response of chimeric proteins in a heterologous system, Jordt and Julius determined that the directed deterrence of chili peppers is conferred by a small segment of the capsaicin channel (Jordt and Julius, 2002). Although there are many other known defensive compounds, only a select few genes have been identified that encode a receptor that responds to a chemical defense compound (Caterina *et al.*, 1997).

The above physiological effects aside, it is likely that deterrence is a response to the odor or taste of the molecules; that is, a predator's chemoreceptors probably respond to chemical deterrent compounds from prey, as chemoreceptors have the ability to respond to numerous compounds (Mombaerts, 2004a). A recent study by Sheybani and colleagues reported electrophysiological evidence that both the gustatory and olfactory

senses are involved in the detection of *Aplysia* ink and opaline (Sheybani *et al.*, 2009). In addition to the phagomimicry effect previously mentioned, ink was also shown to be deterrent to sea catfish, but the specific deterrent components were not identified (Sheybani *et al.*, 2009). A candidate deterrent component is escapin, an L-amino acid oxidase, which reacts with other ink components to produce a complex mixture of compounds; these compounds were tested on sea catfish. Using electrophysiological recordings from the olfactory epithelium and facial barbels of sea catfish, they showed that olfactory and gustatory systems were moderately stimulated by the reaction products of escapin, which are likely found in ink (Sheybani *et al.*, 2009). While this group has not yet behaviorally identified which specific compounds mediate deterrence in ink, the results of this study strongly suggest involvement of these sensory systems in the detection of chemical defense compounds; however to date, only a limited number of genes encoding a chemoreceptor that responds directly to a chemical deterrent have been identified.

1.2 Chemoreception in Vertebrates

1.2.1 Olfaction

The specificity of odorant detection initially lies at the level of individual odorant receptors (ORs), proteins embedded in the cell surface membrane that interact with odorants (Mombaerts, 1999a). These odorants are cues released from a signaler that diffuse through a fluid medium to reach the detecting organism (Dusenbery, 1992). Activation of ORs induces a signaling cascade that culminates in a nerve impulse which is transmitted to the brain for interpretation. Vertebrate OR genes are classified into two

categories: class I and class II genes. Freitag and coworkers (Freitag *et al.*, 1995) originally proposed the designation of these classes on the basis of the OR genes of the African clawed frog *Xenopus laevis*, which has two nasal cavities with distinct anatomies. Its lateral nasal diverticulum detects water-soluble odorants with receptors encoded by class I genes, whereas the medial nasal diverticulum detects odorants from the air with receptors encoded by class II genes. Since amphibian class I receptors have amino acid similarity to fish OR genes, and class II genes were found only in the medial diverticulum, they proposed that class I genes are "fish-like" and class II genes are "mammalian-like" (Freitag *et al.*, 1995). This group also hypothesized that fish OR genes are only members of class I, mammal OR genes are from class II, and genes from amphibians are members from both classes; this further suggested that class I genes encode receptors that detect only water-soluble odorants and class II genes encode receptors that detect airborne odorants (Freitag *et al.*, 1998). Interestingly, phylogenetic analysis of mammalian OR genes showed that some non-fish genes were members of class I (Glusman *et al.*, 2000). Although these were assumed to be pseudogenes, Glusman and coworkers (Glusman *et al.*, 2001) and Zhang and Firestein (Zhang and Firestein, 2002) found that some mammalian class I ORs are not pseudogenes. Thus, the division among these classes is not as clear as once thought (Niimura and Nei, 2006).

Unlike the immune system, which employs *in situ* recombination to produce a vast array of receptors that recognize ligands (Alt *et al.*, 1992), the olfactory system has individual genes that encode each expressed OR within the genome (Mombaerts, 2004a). Genes for all ORs are monoallelic (Chess *et al.*, 1994); hence, an amazing number of OR genes within the genome allow for the detection of thousands of odorants. According to

the one receptor-one neuron hypothesis, a widely accepted but still controversial hypothesis (Mombaerts, 2004b), only one olfactory receptor type is expressed per olfactory sensory neuron (OSN). The mechanism of how only a single type of receptor is expressed in any given OSN is still enigmatic and is one of the major questions remaining in olfactory research. It has been hypothesized that transcriptional regulation affects OR expression in OSNs (Lomvardas *et al.*, 2006). Consistent with this notion, a trans-acting H element was hypothesized by Axel's group as a regulator of genome-wide OR expression (Lomvardas *et al.*, 2006). However, using targeted gene deletion in mice, Mombaert's group refuted this element as a global regulator (Fuss *et al.*, 2007). Other groups have identified trans-acting factors involved in expression of many ORs; these too are not universal regulators of OR expression (McIntyre *et al.*, 2008). Which factors and elements are globally involved in OR regulation and whether this regulation is a feedback mechanism remain to be determined. Current olfactory coding models in vertebrates account for only a single type of olfactory receptor expressed per chemoreceptive cell (Ressler *et al.*, 1994; Mueller *et al.*, 2005; Chandrashekar *et al.*, 2006). These models contrast with the recent examples of chemoreceptors that are co-expressed by invertebrate OSNs, such as *Drosophila* Or83b (Vosshall *et al.*, 1999; Larsson *et al.*, 2004), moth BmOr-2 (Krieger *et al.*, 2003; Nakagawa *et al.*, 2005), and mosquito AgOR7 (Pitts *et al.*, 2004).

Most vertebrate odorant receptors can respond to multiple odorants that are structurally similar, and an individual odorant can activate multiple receptors (Malnic *et al.*, 1999). This broad tuning ensures that an organism is responsive to many pertinent compounds but is not very specific. To compensate, an organism has some receptors that

are more narrowly tuned. These receptors tend to be responsive to highly important odors, such as sex pheromones (Friedrich and Korsching, 1998).

Although recent research suggests that *Drosophila* olfactory receptors are ligand-gated ion channels (Sato *et al.*, 2008; Wicher *et al.*, 2008), all vertebrate olfactory receptors identified to date are canonical G protein-coupled receptors (GPCRs). Odorant receptors, originally identified by Buck and Axel (1991), comprise the largest family of GPCRs in mammals. This highly diverse family is capable of recognizing thousands of odorants (Buck, 1996). As the name suggests, GPCRs are seven-transmembrane proteins that couple to heterotrimeric G proteins, three subunit proteins that bind and hydrolyze GTP (Kristiansen, 2004). In addition to the seven membrane-spanning regions, GPCRs possess an extracellular ligand-binding domain and an intracellular G protein signaling domain (Kristiansen, 2004). Activation of an olfactory receptor by its cognate odorant causes a G protein signaling cascade that culminates in the electrical activation of the olfactory receptor cell (Mombaerts, 1999b) (Fig. 1). More specifically, the olfactory receptor-specific G protein $G_{\alpha olf}$ activates adenylyl cyclase, producing the second messenger cAMP and subsequently activating a cyclic-nucleotide gated Na^+/Ca^+ channel within the plasma membrane. Activation of these channels allows an influx of calcium into the cell, consequently activating calcium-activated chloride channels at the cell surface and leading to depolarization of the OSN. Although the cAMP-dependent pathway is the canonical olfactory signaling cascade, other signal transduction pathways also have been linked to olfactory signaling. Both IP_3-activated and MEK/ERK pathways have been shown to be upregulated in vertebrate OSNs in response to odorants (Bruch, 1996; Moon *et al.*, 2005). Additionally, there are a number of modulators of

olfactory and G protein signaling, such as RGSs, GAPs, and GTPases (Siderovski and Willard, 2005).

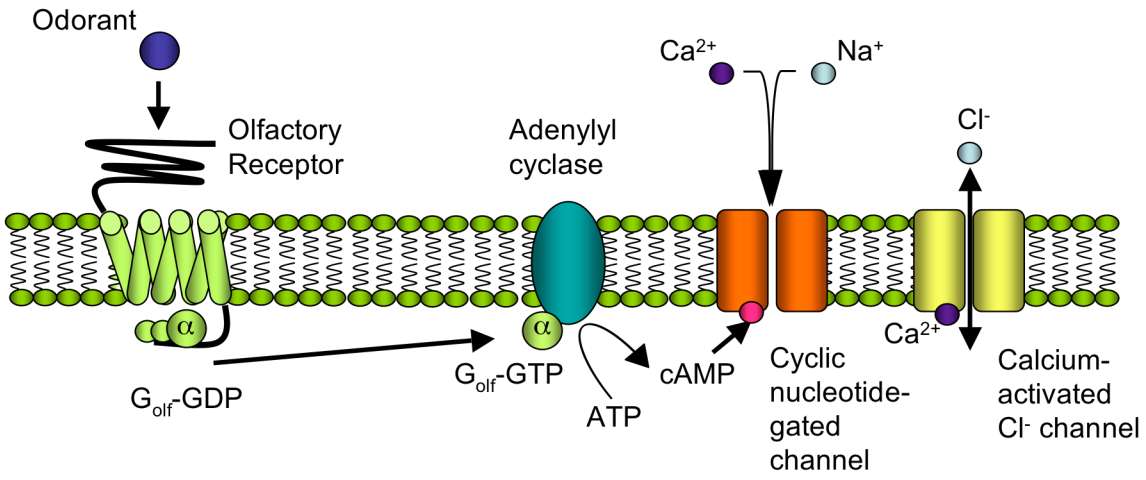


Figure 1. Olfactory receptor signaling schematic. A chemical signal is converted to an electrical signal *via* an odorant that binds to the N-terminal tail of its cognate olfactory receptor, causing the G alpha subunit of the heterotrimeric G protein G_{olf} to have a higher affinity to GTP rather than GDP. The GTP-bound form of $G_{\alpha olf}$ interacts with adenylyl cyclase, which converts ATP to cAMP. cAMP binds to cyclic nucleotide-gated channels in the plasma membrane of OSNs, allowing a open conformational change in the these channels. Calcium and sodium ions enter OSNs *via* these open channels. This localized increase in calcium ions opens calcium-activated chloride channels in the OSN plasma membrane, depolarizing the cell and transmitting a signal to the brain.

Olfactory sensory neurons (OSNs) that express a single type of OR are spatially located in one of four partially overlapping zones in the main olfactory epithelium (MOE) in a seemingly random fashion in rodents (Ressler *et al.*, 1993; Vassar *et al.*, 1993; Buck, 1996; Miyamichi *et al.*, 2005) and project their axons to a specific neuropil called a glomerulus within a corresponding zone in the main olfactory bulb (MOB). Dendrites of mitral cells connect to OSNs in glomeruli and have axon projections to the olfactory sensory cortex (Figure 2A). All of the axons of OSNs that express the same type of OR converge onto the same glomerulus, typically one per bulb (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996).

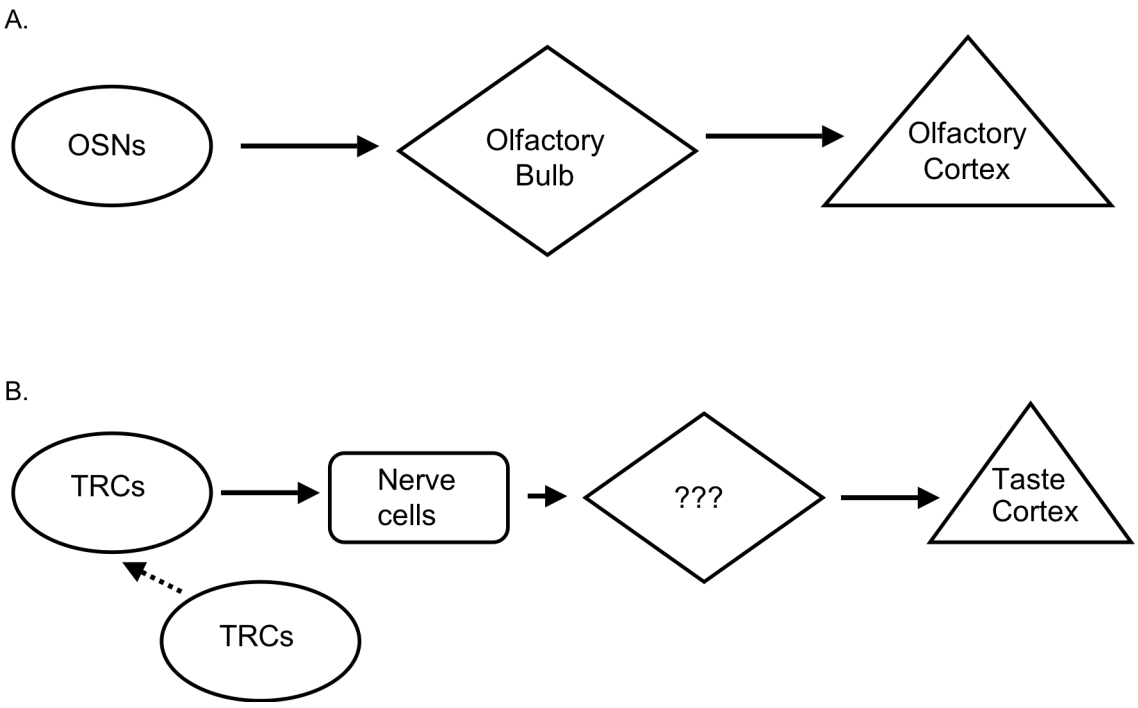


Figure 2. Olfactory and gustatory coding schematic. (A) Olfactory sensory neurons project their axons to the olfactory bulb, where they coalesce with axons of OSNs expressing the same type of olfactory receptors and converge onto discrete glomeruli within the bulb. Mitral cells connect these the OSNs to the olfactory cortex from the olfactory bulb. **(B)** Taste receptor cells (TRCs) interact with nerve cells that relay electrical impulses to the brain. Some TRCs do not directly interact with nerve cells and most likely relay their information to other TRCs through the release of neurotransmitters. It is not clear if there is another relay point analogous to the olfactory bulb that codes information from these cells prior interpretation in the taste cortex.

The mechanism by which axons target the olfactory bulb and converge with axons of OSNs that express the same ORs is not well understood. ORs are expressed in axons, as well as in dendrites, and have been shown to be involved in the targeting process, as mutations in the OR or expression levels have axonal sorting consequences (Mombaerts *et al.*, 1996; Feinstein and Mombaerts, 2004); however, the functional roles that ORs play in axon guidance remain to be determined. Mombaerts and coworkers proposed a contextual model, whereby homophilic interactions guide axons to coalesce into a glomerulus (Feinstein and Mombaerts, 2004). Sakano and Yoshiharo's groups suggested that axonal guidance is regulated by more conventional guidance cues whose expression is regulated by ORs and cAMP/neuronal activity and target axons to a specific spatial location on the bulb (Serizawa *et al.*, 2006; Imai and Sakano, 2007; Kaneko-Goto *et al.*, 2008). Which model holds true remains to be seen, but both models illustrate the elegant design of odor processing in the olfactory system.

1.2.2 Gustation

The primary role of the gustatory system is to regulate the intake of food and nutrition *via* taste perception. In combination with other systems, such as the olfactory and visual systems, food to be ingested is subjected to a quality control test to prevent organisms from consuming harmful compounds while regulating the intake of nutrients. This system is similar in form and organization to the olfactory system, though there are some differences. Like olfaction, gustation is initiated by the interaction of ligand, a tastant, with a corresponding membrane protein, a taste receptor (Lindemann, 2001). There are 5 types of known gustatory receptors (salt, sweet, sour, bitter, and umami) in

the mammalian system, which are designated by the class of molecule that they detect (Fig. 3). Unlike olfactory receptors, which consist of only GPCRs, these five types of receptors can be classified into two types of proteins, GPCRs or ligand-gated ion channels (Scott, 2005).

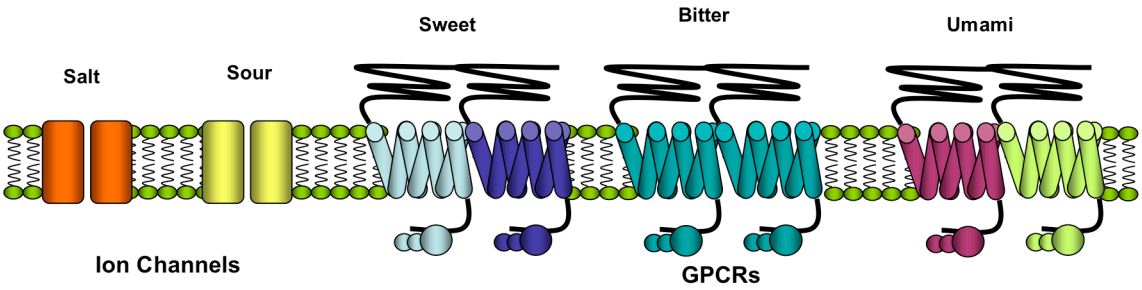


Figure 3. Gustatory receptor types. Gustatory receptors are classified into five categories based on their taste perception quality: Salt, Sour, Sweet, Bitter, and Umami. Salt and Sour receptors are ligand-gated ion channels, which directly change the membrane potential of TRCs. Sweet, Bitter, and Umami receptors are GPCRs, whose activation ultimately leads to the opening of ion channels in the plasma membrane of TRCs.

Ion channels are directly gated by salt and sour tastants, opening pores in the taste receptor cell (TRC) membrane that allow ions to enter and exit the cell in response to these tastants. Salt taste detects sodium and other minerals (Heck *et al.*, 1984). In rodents, this taste quality is mainly mediated by the amiloride-sensitive epithelial sodium channel (ENaC), however the ion channel involved in human salt taste perception has not clearly been identified (Heck *et al.*, 1984; Avenet and Lindemann, 1988). Sour taste perceives spoiled food and acids. A variety of channels serve as sour taste receptors, such as ENaC, the hyperpolarization-activated, cyclic nucleotide-gated channel (HCN), and MDEG1, a member of the ENaC/Deg family of Na^+ channels (Cummings and Kinnamon, 1992; Waldmann *et al.*, 1997; Ugawa *et al.*, 1998; Stevens *et al.*, 2001; Lin *et al.*, 2004; Lyall *et al.*, 2004; Richter *et al.*, 2004).

Sweet, bitter, and umami tastants interact with GPCRs, causing G protein signaling cascades that begin with the activation of the taste specific G protein gustducin and culminate in the opening of ion channels in the TRC membrane. All of these receptors are known to function in the plasma membrane as homo- and heterodimers (Nelson *et al.*, 2001; Nelson *et al.*, 2002; Mueller *et al.*, 2005; Chandrashekar *et al.*, 2006). The sweet sensation detects pleasant-tasting carbohydrate and non-carbohydrate compounds. By expressing T1R2 and T1R3 individually and together in *Xenopus* oocytes, Zuker and coworkers showed that sweet detection is mediated by heterodimers of these GPCRs (Nelson *et al.*, 2001). One of these GPCRs, T1R3, also is involved in another heterodimer pair in the detection of umami (savory) compounds. It heterodimerizes with T1R1 to sense this class of compounds, mainly L-glutamate, an amino acid found in protein-rich foods (Nelson *et al.*, 2002). Bitter compounds are

aversive at high concentrations and therefore prevent ingestion of harmful compounds. Receptors responsive to bitter compounds consist of homodimers of the T2R receptor (Mueller *et al.*, 2005). While the receptors involved in the detection of these tastants are known, the channels that are subsequently activated in response to bitter, sweet, and umami tastants are not as clear (Sugita, 2006; Liman, 2007). Additionally, some evidence has been put forward that fat may also be directly detected by the gustatory system (Gilbertson *et al.*, 1997; Gilbertson, 1998; Liu *et al.*, 2008).

Taste receptors are expressed on the cilia of taste receptor cells (TRCs), specialized epithelial cells that are organized into taste buds. Taste buds include both sensory and non-sensory cells, and buds can detect multiple taste qualities (Scott, 2005). In contrast with OSNs, TRCs are not neurons and do not have axons that relay signals to the brain. Instead, these cells coalesce with sensory nerve fibers that send the brain signals in response to the activation of TRCs (Fig. 2B). There is evidence that some cells within the taste bud do not make synapses onto nerve fibers, and these cells may communicate with other cells *via* neurotransmitters or gap junctions (Roper, 2006). Although evidence of multiple neurotransmitters and neuromodulators has been found within taste buds, those that actually play a role within the synapse are not well defined (Roper, 2007), but serotonin is a strong candidate (Roper, 2006;2007).

Two possible spatial coding mechanisms (and likely a combination of both) are hypothesized for the organization of the wiring of gustatory and olfactory receptor cells to the brain: labeled-line and across-fiber pattern (Laurent, 1999; Smith and St John, 1999; Scott, 2004). Labeled-line organization is characterized by the responses of a dedicated set of neurons that code for a specific odor or taste quality. Across-fiber

patterns are coded by a pattern of inputs from multiple OSN types. Activation of different patterns leads to the perception of different odor or taste qualities. There is evidence of both types of coding (Mueller *et al.*, 2005; Scott, 2005; Chandrashekar *et al.*, 2006), which should be further studied to better understand how chemosensory information is processed.

1.2.3 Orphan Receptors and the Difficulties in Identifying Cognate Ligand-Receptor Pairs

Most chemoreceptors are orphan receptors, that is, receptors without cognate ligands. The standard for deorphanizing receptors to pair orphan receptors with their ligands, is to utilize high-throughput methods. Typically, compound libraries are screened for ligands that activate orphan receptors or *vice versa*, using a variety of functional assays. These high-throughput functional screening methods have not been useful in deorphanizing chemoreceptors because, unlike most other GPCRs, these receptors have been notoriously difficult to functionally express in heterologous cell types (Bush and Hall, 2008). The current hypothesis is that heterologous systems lack endogenous factors or co-receptors that are required for trafficking to the membrane (Gimelbrant *et al.*, 2001; Hague *et al.*, 2004; Bush and Hall, 2008). This notion is supported by the evidence gathered via trafficking studies; although chemoreceptors are translated into protein within these cell types, most of these receptors do not traffic past the endoplasmic reticulum and are subsequently degraded before reaching the cell surface (Gimelbrant *et al.*, 2001; Lu *et al.*, 2003).

1.2.3.1 Fusion proteins

Even though a number of methods have been utilized to increase functional expression and deorphanize chemoreceptors, most of these receptors remain orphans (Touhara, 2007). A widely employed technique to overcome poor cell surface trafficking is to fuse a trafficking export sequence from another GPCR, such as bovine rhodopsin or guinea pig serotonin receptor, onto the sequence of a chemoreceptor. These sequence tags promote cell surface trafficking of some chemoreceptors and allowed the deorphanization of a few receptors, such as mouse mOR-EG (Katada *et al.*, 2003) and human OR17-4 (Wetzel *et al.*, 1999) but also have distinct disadvantages. Their presence does not promote the cell surface expression of all chemoreceptors, suggesting that there is an additional mechanism for trafficking of chemoreceptors. Furthermore, it is not known how these signal sequences affect the function or pharmacology of the chemoreceptor, such as the abilities to bind ligand, interact with binding partners, or signal through G proteins.

1.2.3.2 Accessory Proteins and Co-receptors

Several non-chemosensory GPCRs have endogenous binding partners that allow for endogenous functional expression (Theroux *et al.*, 1996; Hirasawa *et al.*, 1997; Marshall *et al.*, 1999; Chalothorn *et al.*, 2002; Uberti *et al.*, 2003; Uberti *et al.*, 2005) (Table 1) and are helpful in gaining functional expression of these receptors in heterologous cells. These binding partners may help with folding of the receptor, trafficking of the receptor to the cell surface, or binding of ligand. The $GABA_BR1$ receptor, for example, is responsible for binding its ligand GABA but is incapable of trafficking to the cell surface without heterodimerizing with a related GPCR, $GABA_BR2$

(Marshall *et al.*, 1999). The specific interaction of these two receptors masks an ER retention signal on $GABA_BR1$ that allows for proper trafficking to the cell surface (White *et al.*, 1998). While $GABA_BR1$ is capable of interacting with other GPCRs, it specifically requires $GABA_BR2$ for functional expression (White *et al.*, 1998; Marshall *et al.*, 1999).

Table 1. Selected mammalian receptors and their binding partners.

Receptor	*Binding Partners*
β_1AR	α_{2A}AR
β_2AR	β_1AR β_3AR AT δ-OPR κ-OPR
α_{1D}AR	α_{1B}AR, β_2AR
α_{1A}AR	α_{1B}AR
α_{2A}AR	β_1AR μ-OPR
δ-OPR	κ-OPR μ-OPR
Adenosine A1	Dopamine D1 P2Y1 mGluR1α
Adenosine A2A	Dopamine D2 mGluR5
Angiotensin 1A	Angiotensin 2 Bradykinin B2
Calcium sensing (CaSR)	mGluR1 mGluR5 RAMP1 RAMP3
Calcitonin (CTR)	RAMP1 RAMP2 RAMP3
Calcitonin-like (CLR)	RAMP1 RAMP2 RAMP3
CCR2	CXCR4
CCR5	δ-OPR κ-OPR μ-OPR
Dopamine D1	Dopamine D2
Dopamine D2	SSTR5 Dopamine D3
$GABA_BR1$	$GABA_BR2$
Muscarinic M2	Muscarinic M3
OR-M71	β_2AR
PTH2R	RAMP3
SSTR1	SSTR5
SSTR2A	SSTR3 μ-OPR
Substance P (NK1)	μ-OPR
TRHR1	TRHR2
T1R3	T1R1 T1R2
Vasopressin V1a	Vasopressin V2
VPAC-1	RAMP1 RAMP3

Adrenergic receptors, which respond to epinephrine and norepinephrine, are also capable of heterodimerizing; however, unlike the $GABA_B$ receptors, some do not require heterodimerization for proper functional expression (Salahpour *et al.*, 2004). These Family A (rhodopsin-like) GPCRs are classic examples of GPCR heterodimerization, especially the beta-2-adrenergic receptor (β_2AR). β_2AR does not appear to require heterodimerization for functional expression at the cell surface, as it is capable of responding to ligand when expressed alone in heterologous cell systems (Salahpour *et al.*, 2004). Despite this, β_2AR has been shown to heterodimerize with a number of other GPCRs (Lavoie *et al.*, 2002; Uberti *et al.*, 2005) including olfactory receptors (Hague *et al.*, 2004). For example, β_2AR physically associates with β_1AR and β_3AR, as shown through co-immunoprecipitation and BRET studies (Lavoie *et al.*, 2002; Breit *et al.*, 2004). β_1AR is known to heterodimerize with the α_{2A}AR receptor, causing differences in pharmacology such as heterologous internalization (Xu *et al.*, 2003). Furthermore, the physical association of β_2AR with the κ- and δ-opioid receptors causes differences in signaling and internalization (Uberti *et al.*, 2005). Other adrenergic receptors are known to have co-receptors. For example, α_{1D}AR requires a binding partner to shuttle to the cell surface and does not function when expressed alone in nearly all heterologous cell systems (Theroux *et al.*, 1996; Hirasawa *et al.*, 1997; Chalothorn *et al.*, 2002). β_2AR (Uberti *et al.*, 2005) and α_{1B}AR (Uberti *et al.*, 2003; Hague *et al.*, 2004) heterodimerize with α_{1D}AR, increasing its cell surface expression and function.

Like some of the previous examples of co-receptors and accessory proteins, receptor-activity-modifying proteins (RAMPs) also affect the pharmacology of their GPCR binding partners. RAMPs were discovered when research groups had difficulty

expressing certain GPCRs in heterologous expression systems other than HEK293 cells, suggesting the requirement of a co-factor or accessory protein (Parameswaran and Spielman, 2006). Utilizing expression cloning in *Xenopus* oocytes, human RAMP1 was identified, followed by the identification of RAMP2 and RAMP3 through bioinformatics (McLatchie *et al.*, 1998). Despite their relatively low sequence similarity, RAMPs have common structural characteristics: a single predicted membrane-spanning domain, a short cytoplasmic domain, and a long extracellular domain. Co-expression of RAMPs with the calcitonin receptor (CT-R) and other class B and C GPCRs allows the formation of a complex of these membrane proteins and produces a novel binding site for ligand (Christopoulos *et al.*, 2003; Udawela *et al.*, 2004; Parameswaran and Spielman, 2006). This interaction produces a novel signaling response that is not present in cells that expressing either protein alone (Fig. 4).

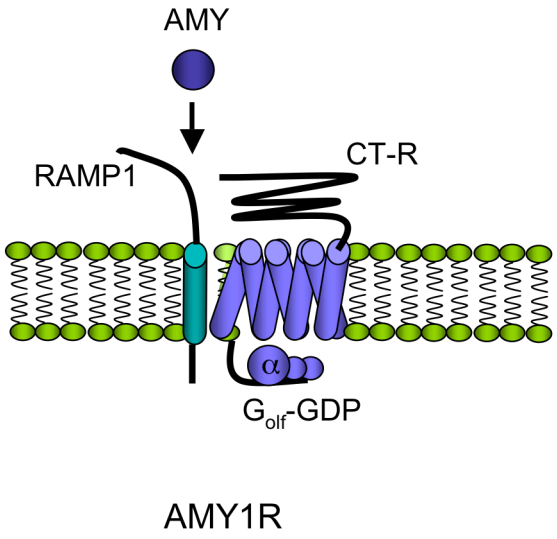


Figure 4. RAMP1 physically interacts with CT-R. This receptor/co-receptor complex, called the amylin 1 receptor (AMY1R), forms a novel binding site for amylin (AMY).

Many other GPCRs physically interact with co-receptors that also cause functional consequences, such as the purinergic receptors (Bush *et al.*, 2007) and the somatostatin receptors (Pfeiffer *et al.*, 2002), and some of these receptor pairs are summarized in Table 1. It has been hypothesized that accessory proteins, related to the co-receptors mentioned above but endogenous to chemosensory cells, are involved in trafficking and/or function of chemoreceptors in sensory cells, given the difficulty in expressing chemoreceptors in heterologous systems (Gimelbrant *et al.*, 2001; Bush and Hall, 2008). In support of this hypothesis, taste receptors have been shown to be another example of GPCRs that must heterodimerize in order to bind ligand (Nelson *et al.*, 2001; Nelson *et al.*, 2002). Specifically, sweet and umami receptors are heterodimers. Heterodimers of T1R2 and T1R3 comprise sweet receptors, which respond to sweet tastants (Nelson *et al.*, 2001). Umami receptors have been identified as the fifth type of taste receptor and consist of a heterodimer of T1R1 and T1R3 that responds to the savory tastant glutamate (Nelson *et al.*, 2002). Although these taste receptors heterodimerize in order to be capable of binding tastants, other vertebrate taste receptors are not known to heterodimerize.

A number of other accessory proteins have been identified recently as involved in chemosensation (Hague *et al.*, 2004; Saito *et al.*, 2004). As mentioned above, β_2AR has been shown to associate with and substantially increase functional expression of one type of olfactory receptor, M71, in heterologous systems (Hague *et al.*, 2004). In HEK293 cells, this association appears to allow these M71 receptors to leave the ER and traffic to the plasma membrane, where they are capable of interacting with their ligand (Hague *et al.*, 2004). Purinergic recepters are also capable of physically associating with M71

receptors and may also have functional consequences (Bush *et al.*, 2007). However, neither of these types of receptors are endogenously expressed at significant levels in olfactory receptors, suggesting that there may be other endogenous binding partners or accessory proteins that serve similar functions in chemosensory cells.

Besides taste receptors and β_2AR, the aforementioned heterodimers are not found in chemoreceptor cells; however, several endogenous proteins have been identified in chemoreceptive cells that appear to promote cell surface expression of chemoreceptors. Matsumani and colleagues identified RTP1 and the related protein RTP2 (Saito *et al.*, 2004). When coexpressed with certain chemoreceptors, these single-pass membrane associated accessory proteins caused increased signaling in response to odorants. Furthermore, a shorter form of RTP, RTP1S, has an even greater effect on signaling and appears to be the more prominently expressed endogenous form (Zhuang and Matsunami, 2007). This group also identified another putative accessory protein, REEP, which appears to increase functional expression (Saito *et al.*, 2004). All of these endogenous accessory proteins do not affect expression of all ORs but can act synergistically with each other and with some export tagged-ORs (Zhuang and Matsunami, 2007).

1.2.4 Other Types of Chemosensation

Chemosensation canonically includes gustation and olfaction in vertebrates; however, other systems serve similar functions, such the vomeronasal system (a subsystem of the olfactory system), the trigeminal system, and lateral-line system. Additionally, genes for trace amine-associated receptors (TAARs) have been identified within the olfactory epithelium of mouse, human, and fish by Linda Buck and coworkers

(Liberles and Buck, 2006). The extent of the involvement and interaction of all these non-canonical forms of chemosensation has yet to be determined.

1.2.5 Chemoreception in Fish

Chemoreception plays a large role in aquatic organisms, such as fish, which utilize their chemosensory systems for migration, predation, and reproduction. (Hara, 1994; Sorensen *et al.*, 1998; Derby and Sorensen, 2008). While the medium used to transport chemical cues is also utilized as a simple distinction between the modalities of olfaction and taste in terrestrial species, these canonical modalities of chemoreception are more difficult to differentiate in aquatic organisms because all chemosensory cues are transported to aquatic organisms *via* water (Derby and Sorensen, 2008). Therefore, the sensory cells that comprise the peripheral olfactory and gustatory nerves define olfaction and gustation in fish (Derby and Sorensen, 2008).

Since chemosensory cues are present in the environment amid many other compounds, fish, like other organisms, must have a means to not only detect and discriminate pertinent from non-relevant compounds, but also to tell pertinent compounds apart. Fish are also able to distinguish complex mixtures (Kohbara and Caprio, 1996; Valentincic and Koce, 2000; Valentincic *et al.*, 2000; Tabor *et al.*, 2004). Our understanding of how detection and discrimination occurs in fish is incomplete; however, the puzzle is beginning to be pieced together (Hara, 1994; Sorensen *et al.*, 1998; Laberge and Hara, 2001; Mombaerts, 2004a; Derby and Sorensen, 2008). The systems of olfaction and gustation are typically thought to allow fish to detect and discriminate chemical stimuli from their environment. Organization of olfactory receptor cells

appears to be random in catfish olfactory epithelium (Chang and Caprio, 1996) and somewhat random in zebrafish (Korsching *et al.*, 1997; Korsching, 2001), which possess overlapping concentric zones of random expression. As in other vertebrates, the chemical stimuli are ligands that interact with receptors, such as odorants or taste molecules. Ngai and colleagues (Alioto and Ngai, 2005) estimated fishes have about 100 OR genes, considerably fewer in number than mammals. Seven major classes of compounds have been identified via electrophysiology as chemosensory stimuli for fish: amino acids, sex steroids, bile acids/salts, aminosterols, amines, nucleotides, and prostaglandins (Hara, 1994; Oike *et al.*, 2007). However, little is known about the correlation of receptor type and ligand class, nor have other types of chemosensory ligands been identified (Laberge and Hara, 2001). Amino acids are the most studied group of fish chemosensory ligand because this class is an obvious indicator of food and nutritional quality. Utilizing electrophysiological and behavioral studies, L-amino acids can be perceived by fish olfactory systems with high sensitivity and specificity (Hara, 1994). However, their gustatory system is not as sensitive. Other systems, such as the trigeminal and lateral-line systems, are also known to be involved in chemosensory signaling in fish, but the extent is yet to be determined.

Genetic analysis of the fish gustatory system has shown that fish possess receptors homologous to those of the mammalian system (Oike *et al.*, 2007). Fish bitter receptors (T2Rs), much like mammalian receptors, respond to denatonium, a compound known to be perceived as bitter to mammals and *Drosophila* and which elicits an avoidance response in fish (Oike *et al.*, 2007). On the other hand, rather than respond to various sweet tastants, fish sweet receptors (T1R2/3s) appear to only be responsive to amino

acids, but activation of these receptors *via* amino acids elicits an attractive response similar to the behavioral response seen in mammals (Oike *et al.*, 2007). Therefore, there are some differences as to the type of tastants to which fish receptors respond, but the type of subsequent behavioral response is conserved. These data suggest that these receptors and cells are conserved as a mechanism by which an organism can distinguish between foods that are nutritious and those that are potentially harmful (Oike *et al.*, 2007). However, the mechanism by which feeding deterrents affect food intake needs further exploration.

1.3 Major Questions Remaining

Although many sessile, soft-bodied, or slow-moving marine organisms contain chemical compounds that defend them from predators (Hay, 1991; Pawlik, 1993; Hay, 1996), the molecular mechanisms by which deterrent compounds are sensed by fish are currently unknown. Many environmental chemicals are detected by chemoreception, but most chemoreceptors are orphan receptors; that is, their ligands are unidentified (Mombaerts, 2004a). While it is not known what kind of chemoreceptors respond to chemical deterrents, these chemicals could activate gustatory receptors, olfactory receptors, or a combination of both. Furthermore, how these receptors molecularly mediate aversive behavior in fish has not been previously characterized. In order to study the impacts of prey chemical signaling on predators, it is important to identify the chemoreceptors responsive to chemical defense molecules and their subsequent signal transduction.

In this thesis, we sought to functionally identify and characterize a chemoreceptor that detected a deterrent compound in order to understand its consequences on predators, using zebrafish as a model organism. To this end, isolated deterrent compounds were obtained from marine sponges and used to functionally screen a zebrafish cDNA expression library in *X. laevis* oocytes *via* electrophysiology and bioassay-guided fractionation.

PART I

RECONSTITUTION OF A CHEMICAL DEFENSE SIGNALING PATHWAY IN A HETEROLOGOUS SYSTEM

CHAPTER 2

INTRODUCTION

Organisms detect and disseminate chemical stimuli to perceive their environments and communicate with other organisms (Dusenbery, 1992). However, the molecular basis for chemoreception is not well understood; although some genes encoding putative receptors are known (Buck and Axel, 1991), their specific ligands remain largely unidentified. Conversely, some ligands have been identified; however, their responsive chemoreceptors have not (Mombaerts, 2004a). Many sessile or slow-moving terrestrial and marine organisms utilize defensive chemicals to protect them from predation, colonization by bacteria, and overgrowth by neighboring organisms (Paul *et al.*, 2006). For example, chili peppers contain capsaicin, a pain-inducing compound that reduces predation by select vertebrates (Caterina *et al.*, 1997). Triterpene glycosides produced by *Erylus formosus* and *Ectyoplasia ferox* protect these Caribbean sponges from predation, microbial attachment, and overgrowth by competing sponges (Kubanek *et al.*, 2002). The decorator crab *Libinia dubia* reduces its predation by decorating its carapace with *Dictyota menstrualis* (Stachowicz and Hay, 1999), a chemically defended brown alga which contains isoprenoid compounds that deter predation and prevent larval settlement on the surface of these plants (Schmitt *et al.*, 1995). Chemical defense compounds, like those utilized by this wide variety of organisms, are secondary metabolites produced either by the organism, a bacterial symbiont, or are sequestered from another species (Moore, 2006).

Defensive compounds could act on predators in a variety of ways. For example, the mediator of the noxious response to chili peppers is the capsaicin receptor, TRPV1, a member of the TRP family of ion channels, which causes the activation of a pain pathway in mammals (Caterina *et al.*, 1997) but not birds (Jordt and Julius, 2002). Some defensive compounds have been shown to be phagomimics that distract predators, who attempt to eat the emitted defensive compounds while the prey escapes (Kicklighter *et al.*, 2005). Several species of ascidians produce inorganic acids that cause them to be unpalatable to potential predators and damage cells of the organisms that ingest them (Stoecker, 1980; Lindquist *et al.*, 1992; Pisut and Pawlik, 2002). Alternatively, phlorotannins, found in marine algae, and tannins, found in terrestrial plants, form indigestible complexes with plant nutrients or inactivate digestive enzymes by binding to them (Mole and Waterman, 1987; Boettcher and Targett, 1993; Targett and Arnold, 2001). Some deterrent compounds are hypothesized to be toxic (Lindquist and Hay, 1995), and potential predators have unknown molecular detection methods to prevent them from ingesting prey bearing these and other unpalatable compounds.

Marine sponges contain a variety of secondary metabolites that are known to be unpalatable to reef predators (Chanas *et al.*, 1997; Assmann *et al.*, 2000; Waddell and Pawlik, 2000; Duque *et al.*, 2001; Kubanek *et al.*, 2001; Pawlik *et al.*, 2002), yet we know very little about how these compounds are perceived by potential predators, other than the fact that predators rapidly reject foods containing these compounds. A study of the cellular effects of chemical deterrents from marine sponges by Bickmeyer *et al.* (2005) suggested that 4,5-dibromopyrrole-2-carboxylic acid, a deterrent compound found in *Agelas* sponges, may alter calcium homeostasis of chemoreceptive cells. However,

this study investigated calcium responses in rat adrenal cells and *Aplysia* (sea hare) neurons, which only are distantly related to natural predators of sponges; therefore, this physiological response may not occur in fish chemoreceptive cells.

It is likely that most cases of deterrence are mediated by a chemosensory response based upon odor or taste; that is, a predator's chemoreceptors most likely respond to deterrent compounds from prey, as chemoreceptors have the ability to respond to numerous chemicals (Mombaerts, 2004a). Chemoreceptors for known odorants or tastants are often G protein-coupled receptors (GPCRs), which may couple to ion channels, such as bitter receptors; in some cases, receptors form ion channels themselves, as in the case of sour receptors (Lindemann, 2001; Mombaerts, 1999). Both bitter and sour taste receptors cause aversive responses in many organisms and help organisms detect unripe fruits, spoiled food, and potentially harmful compounds, and to avoid tissue damage by acids (Lindemann, 2001; Oike et al., 2007). Because predatory fish have been observed to reject foods containing chemical defense compounds within one second of ingestion (Chanas *et al.*, 1997; Assmann *et al.*, 2000; Kubanek *et al.*, 2000; Pawlik *et al.*, 2002), we hypothesized that ion channels (known to cause immediate cellular responses and involved in sour and bitter taste) may be involved, either directly as receptors for these deterrent compounds or *via* coupling to chemosensory receptors. The ligands that interact with chemoreceptors have been identified in very few cases, and relatively little is known about chemoreceptors that respond to chemical deterrents (Caterina *et al.*, 1997). Identifying a gene encoding such a chemoreceptor and investigating its signaling response could be very useful in studying predator-prey interactions on a molecular, behavioral, and evolutionary level.

The long-term goal of this study is to identify a gene encoding a receptor whose ligand acts as a chemical defense in a marine organism by functionally screening a fish cDNA library, in order to investigate the molecular mechanism of an aversive behavioral response. A logical species choice for the library is bluehead wrasse (*Thalassoma bifasciatum*), a common predator on coral reefs (Lindquist *et al.*, 1992) that is one of several reef fishes shown to respond to a variety of chemical defense compounds (Chanas *et al.*, 1997; Assmann *et al.*, 2000; Kubanek *et al.*, 2000; O'neal and Pawlik, 2002; Pawlik *et al.*, 2002; Pisut and Pawlik, 2002; Kicklighter *et al.*, 2003; Jones *et al.*, 2005). Since cDNA libraries from this species are not publicly available, we utilized a library constructed from a different, model fish species, the zebrafish *Danio rerio*. Unlike for *T. bifasciatum* and other generalist reef fishes, the *D. rerio* genome is highly characterized, and high quality libraries are publicly available. Since chemical defense compounds are noxious, and many organisms have protective mechanisms to detect these types of chemicals in order to avoid them, we hypothesized that zebrafish may also be able to detect them. First, we used a behavioral assay to confirm that zebrafish are able to detect sponge chemical defense compounds that also induce aversive responses in reef fishes. We then determined that a deterrent signaling pathway responsive to one of these compounds could be reconstituted by expressing a zebrafish cDNA library in *Xenopus* oocytes.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Isoproterenol, forskolin, IBMX, octanal, sodium alginate, calcium chloride, and ethanol were obtained from Sigma-Aldrich (St. Louis, MO). Sceptrin (a defensive sponge compound) was obtained from A.G. Scientific (San Diego, CA). All other sponge-derived compounds were isolated from sponge tissues by following previously described methods (Chanas *et al.*, 1997; Kubanek *et al.*, 2000; Kubanek *et al.*, 2002).

3.2 Animals

Female *Danio rerio* were obtained from Carolina Biological Supply (Burlington, NC). All fish were housed singly in partitioned 10 gallon aquaria and maintained at 23-27^{o}C in a 12:12 light/dark cycle. *Xenopus laevis* were obtained from Xenopus Express (Dexter, MI) and housed in an aquatic habitat (Aquaneering, Inc.; San Diego, CA). Methods of animal handling are in accordance with the NIH guidelines and the protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology.

3.3 Fish feeding assays

Palatability assays using the zebrafish *D. rerio* were performed as previously reported for marine fishes (Pawlik and Fenical, 1992; Pawlik *et al.*, 1995). Briefly,

isolated sponge compounds, triterpene glycosides or brominated alkaloids, were dissolved in a minimal amount (<0.01%) of methanol and incorporated into a matrix of aqueous sodium alginate (0.06 g/ml) and freeze-dried squid (0.03 g/ml). Concentrations of sponge compounds incorporated into the mixture were chosen based on concentrations known to be deterrent to bluehead wrasse and the amount of compound available to assay. The mixture was packed into a 1 cc syringe, which had an attached 200 μl pipette tip with a slightly enlarged opening, and ejected into a 0.25 M $CaCl_2$ solution to solidify the artificial food. The resulting noodle was rinsed with deionized water, to remove excess $CaCl_2$, and sliced into 3 mm pellets. Control pellets were identical to experimental pellets except that they contained methanol without sponge compound. A minimal amount of food coloring (< 1%) was added to both mixtures to ensure experimental pellets were similar in appearance to control. Using a Pasteur pipet, these pellets were offered to individual zebrafish in a randomized order (n = 7-10 fish), and rejection or acceptance was assessed for each fish. Rejection of a pellet was defined as up to 3 or more unsuccessful attempts by a single fish to ingest the pellet; if the fish swallowed the pellet within 3 attempts it was considered accepted. If a pellet treated with sponge compound was rejected, this was always followed with a control pellet to ensure that rejection was not due to satiation. Statistical analysis was performed using a Fisher's exact test (one-tailed; $p < 0.05$) to determine whether fish responded differently to treated *vs.* control food pellets.

3.4 Molecular biology manipulations

A whole zebrafish *D. rerio* cDNA plasmid library constructed in the pExpress-1 vector and size selected for larger inserts (average size is 2 kb) was obtained from the I.M.A.G.E. Consortium (distributed by Open Biosystems, Huntsville, AL). Dr. David Gadsby (Rockefeller University, NY) kindly provided the construct encoding the human cystic fibrosis transmembrane conductance regulator (CFTR) in the pGEMHE vector, and Dr. Brian Kobilka (Stanford University, CA) kindly provided the construct encoding the human beta 2 adrenergic receptor (β_2AR) in the pSP65 vector. A construct encoding rat aldehyde olfactory receptor OR-I7 was constructed in the pSMYC vector (Wetzel *et al.*, 1999). All cDNA plasmids were isolated from DH5α or DH10B cells with Qiaprep spin kits (Qiagen, Valencia, CA), linearized, and *in vitro* transcribed into cRNA (mMessage mMachine; Ambion, Austin, TX).

3.5 Electrophysiology

We hypothesized that a known chemoreceptor that couples to G_{olf} could activate a G_s signaling cascade, which would result in the opening of heterologously expressed CFTR ion channels in the plasma membrane of oocytes expressing this channel, thus changing current. Both $G_{\alpha olf}$ and $G_{\alpha s}$ lead to activation of adenylyl cyclase and, subsequently, protein kinase A (PKA). CFTR is a PKA-activated chloride channel, and its activation, via the adenylyl cyclase signaling cascade, can be measured using two-electrode voltage clamp (TEVC) (McCarty *et al.*, 1993). *Xenopus laevis* oocytes are a convenient tool for electrophysiological investigations of GPCRs and ion channels. These relatively large cells impale easily with two electrodes so that TEVC can be employed to measure whole cell currents. Furthermore, most of the proteins which

comprise the $G_{\alpha S}$ protein signaling machinery are endogenously expressed within oocytes (Fig. 5), and these cells have been utilized in many other instances to reconstitute GPCR signaling cascades (Lubbert *et al.*, 1987; Abaffy *et al.*, 2006).

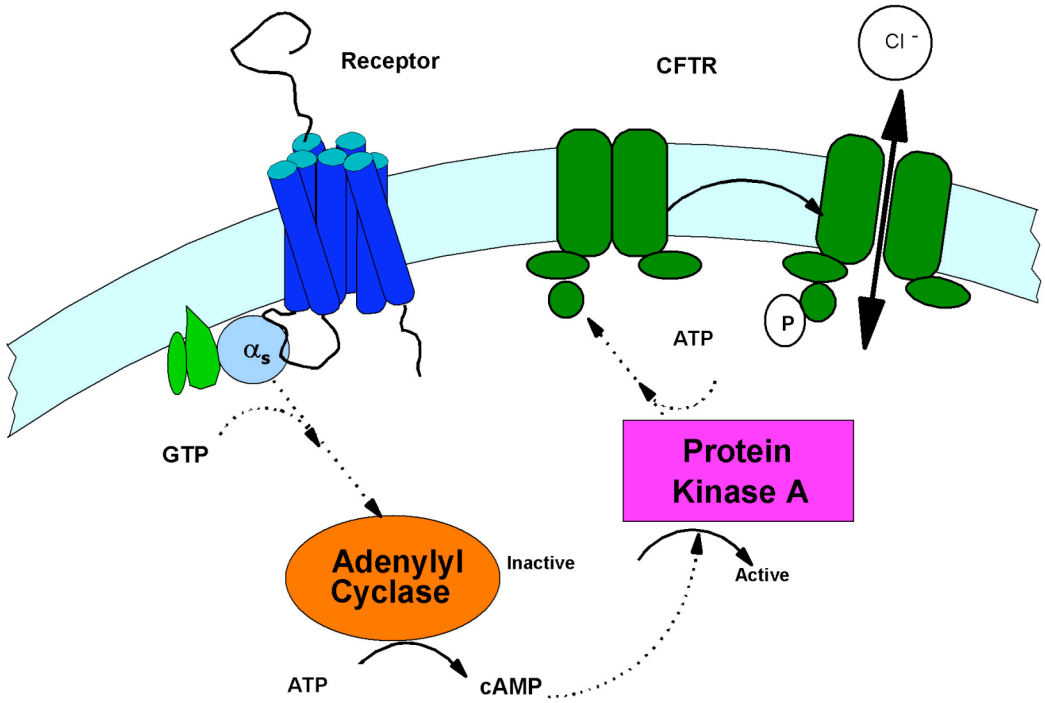


Figure 5. $G_{\alpha s}$ signaling pathway utilized in bioassay. The cyclic AMP dependent response in oocytes co-expressing the zebrafish cDNA library and CFTR is activated by the binding of a ligand to a membrane receptor, causing the receptor to interact with a G protein ($G_{\alpha s}$). Upon dissociation from the heterotrimeric G-protein/ receptor complex, the α subunit activates adenylyl cylase. This action leads to a cAMP signaling cascade, ending in the activation of CFTR. The output of this cascade is measured by electrophysiology and is the basis of the functional assay.

X. laevis oocytes were isolated from adult females and prepared as previously described (Mcdonough *et al.*, 1994; Fuller *et al.*, 2004). Various combinations of library transcript (2.5-10 ng), CFTR transcript (1.25-5 ng), and β_2AR transcript (0.5-2 ng) were microinjected into stage V oocytes. After an incubation of 48-96 hours in L-15 media (Invitrogen; Carlsbad, CA) at 17^{o}C, oocytes were tested *via* TEVC, using a GeneClamp 500 amplifier (Axon Instruments; Sunnyvale, CA). Recording solution was ND96 (96 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl, 5 mM HEPES; pH 7.50) with 1.8 mM $CaCl_2$. Oocytes were treated with deterrent compounds dissolved in ND96 buffer and a minimal amount of solvent (ethanol or DMSO), usually ~0.01% final concentration, via a gravity perfusion system that exchanged the entire recording-chamber in approximately 1 min., and tested for electrophysiological responses *via* TEVC, signaling that the expressed receptor had been activated by a compound which caused a signal cascade that resulted in a change in current. If CFTR were activated by a chemoreceptor-mediated signaling cascade, the electophysiological response would be a slow, broad change in current that slowly returns to baseline. Whole oocyte currents were recorded at V_M= -60 mV. Application of vehicle in ND96 did not cause a change in current.

CHAPTER 4

RESULTS

4.1 Zebrafish reject marine sponge compounds known to be aversive to reef fishes

Zebrafish rejected foods laced with formoside, sceptrin, and ectyoplasides A and B at the same or slightly higher concentrations than those known to deter a common predator on coral reefs, the bluehead wrasse (Table 2). These results suggest that zebrafish possess chemoreceptors that are able to detect at least some marine chemical defenses. However, zebrafish did not have an aversive response to oroidin, even at more than seven times the concentration that was previously found to be aversive to bluehead wrasse (Chanas *et al.*, 1997); therefore, as previously observed, chemosensing can be species-specific (Lindquist and Hay, 1995; Bricelj *et al.*, 2005), and aversive patterns vary based upon chemical structure (Lindel *et al.*, 2000; Lane and Kubanek, 2006).

Table 2. Zebrafish (*Danio rerio*) are deterred by some sponge chemical defenses. Zebrafish rejected formoside, sceptrin, and ectyoplasides A & B, compounds known to deter bluehead wrasse (*Thalassoma bifasciatum*), suggesting that these fish have chemoreceptors capable of responding to these compounds. Oroidin was not rejected by zebrafish, indicating that either this compound does not cause an aversive response by these fish or that they do not have chemoreceptors to detect it. Previous studies have shown that blueheaded wrasse were deterred by formoside (natural concentration: 7.9 mg/ml), sceptrin (natural concentration: 5.3 mg/ml), ectyoplasides A & B (natural concentration: 5.3 mg/ml), and oroidin (natural concentration: 1.4 mg/ml) at and/or below their naturally occurring concentrations (Chanas *et al.*, 1997; Assmann *et al.*, 2000; Kubanek *et al.*, 2000; 2002).

Test Compound	Concentration	Test Pellet	Pellets Eaten	Pellets Rejected	Result
formoside	15.8 mg/mL (15.0 mM)	treatment	0	10	rejected ($p<0.01$)
		control	10	0	
sceptrin	1.0 mg/mL (1.6 mM)	treatment	7	2	accepted ($p=0.24$)
		control	9	0	
	5.0 mg/mL (8.1 mM)	treatment	0	7	rejected ($p<0.01$)
		control	7	0	
oroidin	5.0 mg/mL (28.0 mM)	treatment	10	0	accepted ($p=1.00$)
		control	10	0	
	10.0 mg/mL (56.0 mM)	treatment	9	0	accepted ($p=1.00$)
		control	9	0	
mix of ectyoplasides A & B	5.3 mg/mL (2.8 mM)	treatment	0	9	rejected ($p<0.01$)
		control	9	0	

4.2 Receptor-mediated responses can be reconstituted in *Xenopus* oocytes

In order to characterize chemoreceptors and identify potential signaling pathways, we sought to functionally express them in a heterologous cell expression system, *Xenopus* oocytes, which endogenously contain G protein signaling machinery. Figure 6A shows direct stimulation of CFTR in oocytes by exposure to IBMX, a membrane-permeant inhibitor of phosphodiesterase which leads to sustained activation of PKA and a characteristically slow, broad response that slowly returned to baseline when IBMX was removed from the bathing solution. CFTR can also be activated by exposure to forskolin, a membrane-permeant activator of adenylyl cyclase (Fig. 6B). When the rat aldehyde olfactory receptor, OR-I7, was heterologously expressed in oocytes along with CFTR, CFTR activity increased in response to octanal, an OR-I7 ligand (Fig. 6B), suggesting that this GPCR-mediated signaling pathway can be reconstituted in oocytes.

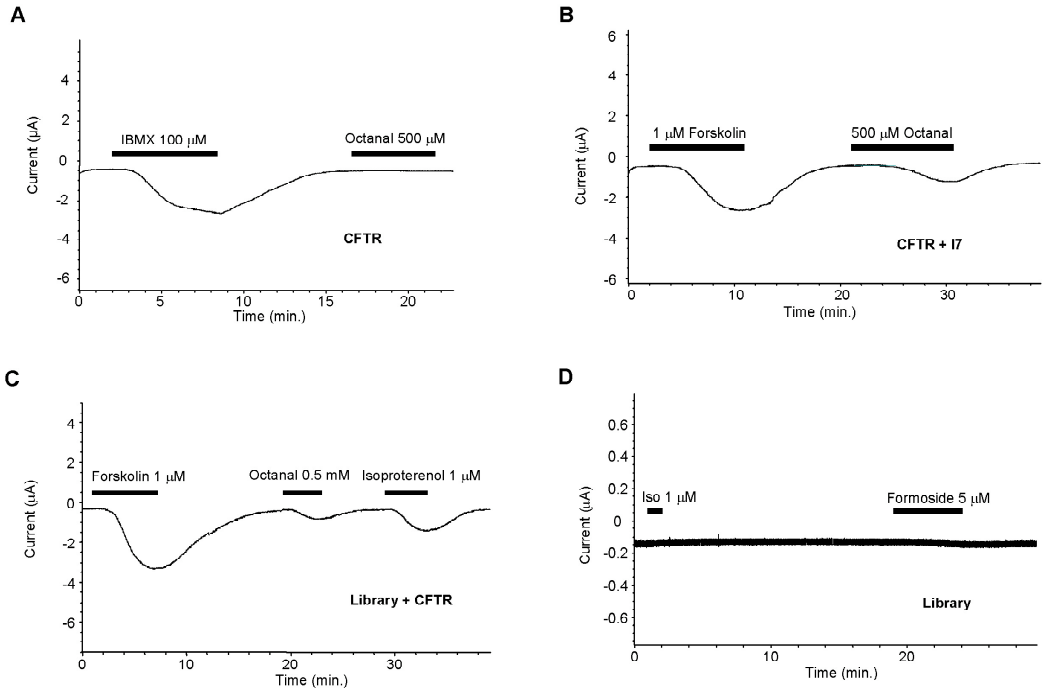


Figure 6. Receptor-mediated responses in oocytes expressing zebrafish cDNA library or OR-I7. (A) Current changed in response to IBMX, an activator of CFTR, but did not change in response to octanal, an agonist for the OR-I7 chemoreceptor, in cells heterologously expressing CFTR alone. **(B)** Both forskolin, an activator for CFTR, and octanal caused increased CFTR activity when applied to oocytes expressing OR-I7 and CFTR. **(C)** Both isoproterenol, an agonist for β2AR, and octanal caused increased CFTR activity when applied to oocytes expressing the library and CFTR but caused no change in oocytes expressing CFTR alone (*data not shown*). **(D)** Oocytes only expressing the library had a very slight change in current in response to formoside. Note the difference in current scale from **(A-C)**.

After determining that signaling by a known chemoreceptor can be reconstituted in oocytes, we sought to determine whether we could reconstitute other receptor-mediated signaling pathways in oocytes expressing a zebrafish cDNA library. When the odorant octanal and the β-adrenergic receptor agonist isoproterenol were applied separately to the oocytes expressing both the library and CFTR, a substantial increase in CFTR activity was observed (Fig. 6C) that did not occur in cells without library (*data not shown*). These data suggest that the zebrafish library included clones encoding a receptor that may be homologous to OR-I7, which would be activated by octanal, and a receptor homologous to the β-adrenergic receptor family, which would be activated by isoproterenol. CFTR served as a read-out in this assay, since the response to isoproterenol was not observed in cells expressing the library alone (Fig. 6D). Figure 6D shows a very slight change in current in response to formoside (note the change in scale) that occurred in oocytes only expressing the library, suggesting that when CFTR is not overexpressed, a G_s pathway is still activated by formoside; expression of CFTR allows enhanced detection of the stimulation of the pathway.

4.3 Responses to chemical defense compounds can be reconstituted in *Xenopus* oocytes

Since receptor-mediated responses to a known odorant could be obtained from library-expressing oocytes, we hypothesized that chemical defense signaling pathways could be reconstituted in these cells as well, allowing the measurement of electrophysiological response to a chemical defense compound. Oocytes co-expressing library, CFTR, and β_2AR were treated with the marine sponge-derived compounds, which we showed in Table 2 to lead to a behavioral response in zebrafish. β_2AR was

included to potentially increase functional expression of chemoreceptors (Hague *et al.*, 2004). Library-expressing oocytes did not have a detectable response to either oroidin (Fig. 7A) or sceptrin (Fig. 7B), when these compounds were applied in the bathing solution.

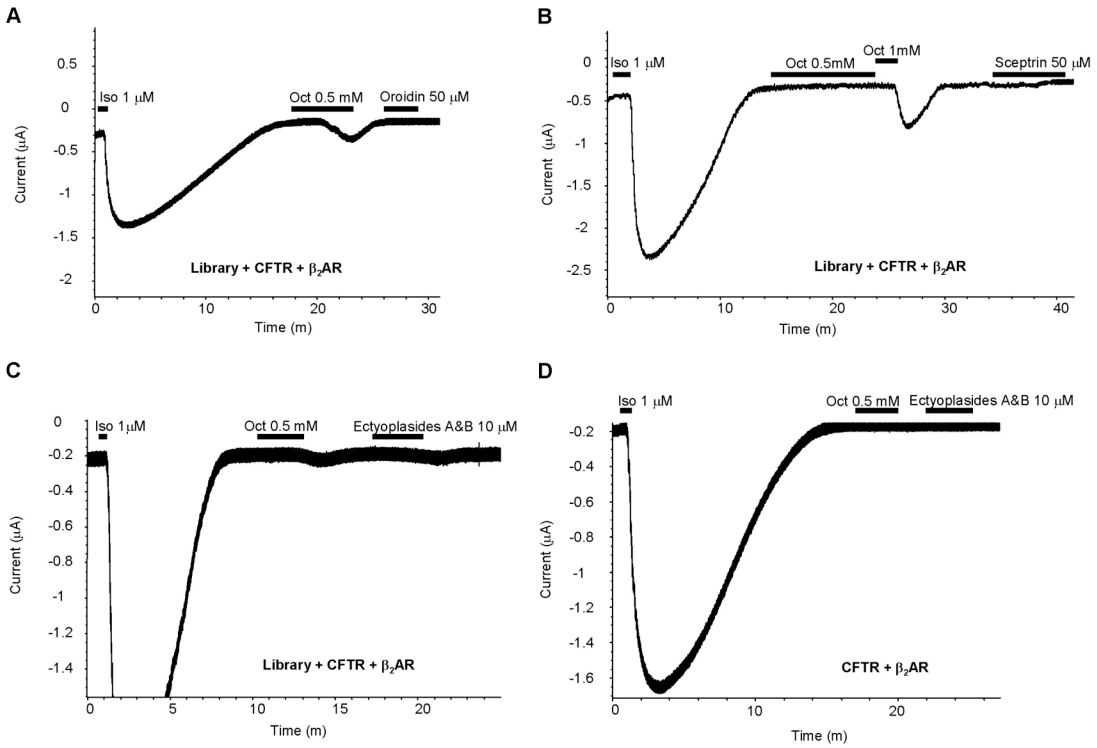


Figure 7. Electrophysiological responses to chemical deterrents. No electrophysiological change was seen in response to application of oroidin **(A)** or sceptrin **(B)**. A mixture of ectyoplasides A & B caused a slight change in current **(C)**, indicating activation of CFTR *via* the G_s signaling pathway, that was not observed in control cells **(D)**.

Exposure to a concentration of 10 μM ectyoplasides A/B (Fig. 7C) and 5 μM formoside (Fig. 8) led to 0.03 +/- 0.01 μA (SEM; range 0-0.1 μA; *n*=11) and 0.2 +/- 0.07 μA response (SEM; range 0.1-0.8 μA; *n*=15), respectively. These concentrations were considerably lower than those utilized in the behavioral assays because higher concentrations of these compounds (at least ten-fold) were cytotoxic to oocytes. The application of formoside or ectyoplasides A/B to oocytes expressing the library led to an electrophysiological response that reflected activation of CFTR (Fig. 7C and 8A), which was not seen in control (Fig. 7D and 8B). The response to formoside was more robust than the response to ectyoplasides A/B, since all cells expressing library, β_2AR, and CFTR responded to formoside but not all cells responded to ectyoplasides A/B. Interestingly, this change in current in response to formoside usually occurred only when the compound was applied after the activation of β_2AR with isoproterenol (Fig. 8A), suggesting that the activation of the $G_{\alpha s}$-mediated pathway may enhance the response to formoside to a detectable level. The response to formoside, unlike ectyoplasides A/B, was very repeatable ($n > 15$) and not seen in oocytes without library (Fig. 8B). Furthermore, multiple presentations of formoside to a library-expressing oocyte did not cause repeatable responses within the same experiment, but with considerable time between presentations (*e.g.*, 3 hours), a second presentation of formoside could lead to a second response of similar magnitude (*data not shown*). These results suggest that the formoside and ectyoplasides A/B signaling pathways were successfully reconstituted in cells expressing the zebrafish library.

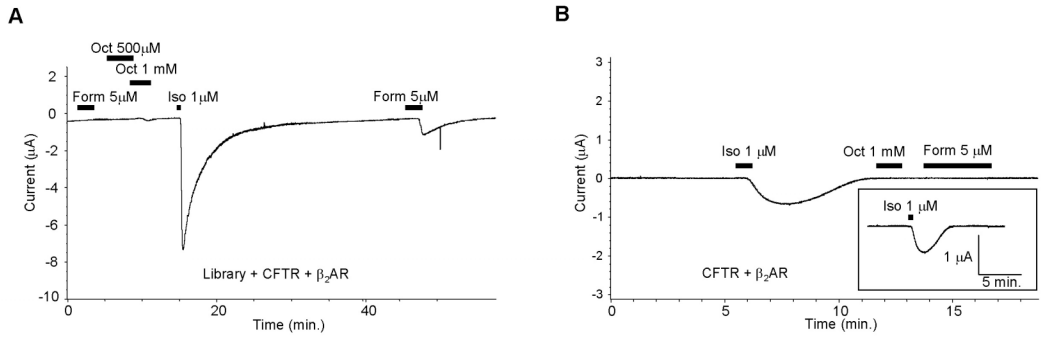


Figure 8. Formoside induces an electrophysiological response in library-expressing oocytes. Formoside caused a response in library-expressing cells after activation of the G_s pathway with isoproterenol **(A)**. No responses to formoside or octanal were seen in oocytes without library **(B)**. Note the difference in time scale between panels **(A)** and **(B)**. Inset shows the response to isoproterenol in **(B)** on the same time scale as **(A)**.

CHAPTER 5

DISCUSSION

5.1 Reconstitution of chemical defense signaling pathways

The molecular detection of chemical defense compounds has rarely been investigated (Bickmeyer et al., 2004) and, therefore, it is generally unproven whether chemical defense compounds are detected in a receptor-mediated manner. We reconstituted the chemical defense signaling pathways for formoside and ectyoplasides A/B, marine sponge compounds, in *Xenopus* oocytes and showed an electrophysiological response to these compounds (Fig. 7C and 8). Interestingly, ectyoplasides A/B and formoside are from the same class of molecules, triterpene glycosides, and the electrophysiological responses to these compounds are also similar. The response to these compounds was observed only in library-expressing cells, indicating that the electrophysiological change occurred due to a receptor-ligand interaction. These putative receptors appear to function as GPCRs that may activate an ion channel in fish endogenously expressing these genes because the response to formoside and ectyoplasides A/B only occurred in oocytes that were expressing the zebrafish cDNA library and was amplified when the ion channel CFTR was co-expressed (Fig. 7D and 8). Activation of an endogenous ion channel *via* these putative receptors may lead to depolarization of the receptor-encoding cell, sending the signal for higher order processing.

Unlike the receptor-mediated response to formoside, it is possible that other sponge compounds cause tissue or cellular damage or a general cellular response, as may be the case with sceptrin, one of the *Agelas*-derived defense compounds utilized in the palatability assays in Table 2. The mechanism of action of sceptrin has been investigated in rat adrenal cells, where it appeared to have an effect on calcium homeostasis (Bickmeyer *et al.*, 2004). Sceptrin may not cause a receptor-mediated response in zebrafish, as no electrophysiological change occurred in response to this compound in our experiments (Fig. 7B). Alternatively, zebrafish sensory cells may exhibit an electrophysiological response to sceptrin that was not measurable in our heterologous expression assay. Oroidin, which zebrafish accepted in the palatability assay (Table 2) but was rejected by coral reef fish (Chanas *et al.*, 1997), also does not appear to cause a receptor-mediated response in this assay (Fig. 7A). These data combined with the behavioral data suggest that zebrafish either: 1) do not possess a chemoreceptor capable of detecting oroidin, or 2) do possess a chemoreceptor capable of detecting oroidin, but its activation causes an acceptance rather than a rejection response. If the second scenario is true, then zebrafish chemoreceptor cells which express this receptor may be wired differently than the same cells in a species which rejects this compound, such as bluehead wrasse.

5.2 Interaction between β_2AR and receptors that detect deterrent compounds

Some library-expressing oocytes did not respond to formoside until after isoproterenol-induced stimulation of the $G_{\alpha s}$ signaling pathway through β_2AR (Fig. 4). There are several possible mechanisms that may explain why activation of β_2AR is

sometimes required in oocytes prior to a response to formoside. β_2AR expression leads to cell surface expression of the mouse M71 olfactory receptor (Hague *et al.*, 2004) and may similarly facilitate cell surface expression of the formoside receptor. G proteins are known to be redistributed in response to the activation of GPCRs (Milligan, 1993; AbdAlla *et al.*, 2000; Cordeaux and Hill, 2002). Therefore, it is possible that $G_{\alpha s}$ proteins are redistributed upon activation of β_2AR, and this action increases the formoside receptor response by providing the receptor with additional G proteins. β_2AR is also known to sequester $G_{\alpha s}$ proteins such that other receptors can not utilize them for signaling (Vasquez and Lewis, 2003), and these G proteins may be made available to the formoside receptor by activating β_2AR with isoproterenol. Alternatively, β_2AR could present G proteins to the formoside receptors, perhaps *via* receptor heterodimerization. Other receptors have been thought to do this, such as the bradykinin receptors, which are hypothesized to present G proteins to the AT_1 angiotensin receptor, thus increasing their signaling ability (AbdAlla *et al.*, 2000; Cordeaux and Hill, 2002). Activation or expression of β_2AR could also recruit other GPCRs, such as formoside receptors, to the plasma membrane, where they become functional. It could also lead to phosphorylation or dephosphorylation of G-protein binding sites, ultimately affecting signaling output. Interestingly, stimulation of the G_S signaling pathway with isoproterenol in the olfactory bulb is known to enhance conditioned olfactory learning in rat pups (Sullivan *et al.*, 1989). Since β-adrenergic receptors are co-expressed along with olfactory receptors in some olfactory sensory cells (Kawai *et al.*, 1999), activation of this pathway in fish peripheral cells may increase formoside signaling in the periphery, to ultimately enhance the rejection process by potential predators of marine sponges.

5.3 Implications of the reconstitution of a defense pathway in frog oocytes

This work demonstrates that a chemical deterrent signaling pathway can be reconstituted in *Xenopus* oocytes and strongly suggests that encoded within this zebrafish cDNA library is a receptor that responds to the chemical defense compound, formoside. A receptor for ectyoplasides A/B also may exist in this library. Using this expression system and electrophysiological assays that direct subdivision of the library clones into smaller and smaller groups, it is possible that the clones encoding these receptors may be isolated from the library and used to study predator detection of chemical defenses. This approach is expected to lead to identification of chemoreceptors used for detection of chemical defense compounds such as formoside.

PART II

FUNCTIONAL IDENTIFICATION AND INITIAL CHARACTERIZATION OF A RAMP-LIKE CO-RECEPTOR THAT IS INVOLVED IN AVERSIVE SIGNALING

CHAPTER 6

INTRODUCTION

Organisms perceive their surroundings using sensory systems, which are designed to detect and integrate these environmental signals. Chemical signals, environmental cues that are detected by chemosensory systems, are commonly utilized as indicators of food, predators, and potential mates (Dusenbery, 1992). Sessile or slow-moving organisms, especially those in marine systems, commonly utilize chemical cues as a means of defense against predation, (Pawlik, 1993; Paul and Ritson-Williams, 2008), but their mechanism(s) of action on potential predators is not well understood, as this topic has been essentially overlooked by the fields of cellular and molecular biology and neurobiology (Derby and Sorensen, 2008; Sheybani *et al.*, 2009).

The cellular effects of a select number of deterrent compounds found in marine sponges have been investigated (Bickmeyer, 2005; Bickmeyer *et al.*, 2005; Bickmeyer *et al.*, 2007). One such study, showed that 4,5-dibromopyrrole-2-carboxylic acid, a deterrent compound found in *Agelas* sponges, alters cellular calcium homeostasis in cultured mammalian cells and *Apysia* neurons (Bickmeyer *et al.*, 2005). Given that this study did not examine responses of chemoreceptive cells from fish or other marine predators, this physiological response may not predict physiological effects in relevant cells.

Chemosensory systems, such as gustatory and olfactory systems, detect and discriminate a variety of compounds (Mombaerts, 2004a). The gustatory system is crucial in food selection, with its primary role regulating food and nutrient intake

(Lindemann, 2001; Scott, 2005; Chandrashekar *et al.*, 2006). Sheybani and coworkers showed that defense compounds from the sea hare *Aplysia californica* were detected electrophysiologically by the olfactory and gustatory systems of sea catfish (Sheybani *et al.*, 2009). Thus, the most probable cellular effect of deterrent compounds is the activation of chemoreceptors, which causes a cascade of events that is subsequently processed as information by the brain and ultimately produces the aversive behavioral response. Despite the growing number of known marine chemical defense (Paul and Ritson-Williams, 2008), no chemoreceptor has been identified that responds to these deterrent compounds. In fact, most chemoreceptors are orphan receptors, *i.e.*, their cognate ligands are unknown (Mombaerts, 2004a).

In the gustatory system, families of taste receptors respond to tastants in taste receptor cells (TRCs). Gustatory receptors are ligand-gated ion channels and G protein coupled receptors (GPCRs) that activate ion channels. Taste receptors specifically interact with tastants according to their taste category, and TRCs, which are specialized epithelial cells, express only one type of taste receptor; these receptors and their cells represent the initial level of taste coding. Although the mammalian gustatory system detects a vast range of ligands, the sensory perception of these tastants can be grouped into relatively few categories based on the type of receptor that detects them: salty, sweet, bitter, sour, and umami. Sweet, salty (at low concentration), and umami tastants induce food acceptance behaviors, whereas bitter, sour, and salty (at high concentration) tastants produce a food rejection response. Genetic analysis of the fish gustatory system has shown that fish possess receptors homologous to those of the mammalian system (Oike *et al.*, 2007). Fish bitter receptors (T2Rs), much like mammalian receptors, respond to

denatonium, a compound known to be perceived as bitter to mammals and which elicits an avoidance response in fish (Oike *et al.*, 2007). On the other hand, rather than respond to various sweet tastants, fish sweet receptors (T1R2/3s) appear to only be responsive to amino acids, but activation of these receptors *via* amino acids elicits an attractive response similar to the behavioral response seen in mammals (Oike *et al.*, 2007). Therefore, there are some differences as to the type of tastants to which fish receptors respond, but the type of subsequent behavioral response is conserved. These data suggest that these receptors and cells are conserved as a mechanism by which an organism can distinguish between foods that are nutritious and those that are potentially harmful (Oike *et al.*, 2007). Many organisms have other mechanisms by which they discriminate harmful substances, such as the nociceptor pathway. For example, capsaicin, a noxious compound that gives chili peppers their spicy sensation, binds to the capsaicin receptor, resulting in the perception of heat and intense pain in mammals (Caterina *et al.*, 1997). Therefore, it is possible that defensive compounds cause pain through nociceptive-mediated signaling, leading to the aversive behavior. Another possibility is that these compounds cause a learned taste aversion (Lindquist and Hay, 1995).

Regardless, relatively little is known about which classes of receptors respond to chemical deterrents (Derby and Sorensen, 2008). In fact, the receptors involved in defensive signaling may be built as ion channels, GPCRs, or accessory co-receptors that work in combination with another receptor to bind ligand. A few co-receptors have been identified in both invertebrates and vertebrates, which facilitate cell surface expression (Dwyer *et al.*, 1998; Gimelbrant *et al.*, 2001; Saito *et al.*, 2004) and, in some cases, affect pharmacology of GPCRs and chemoreceptors (Larsson *et al.*, 2004; Parameswaran and

Spielman, 2006). For example, receptor-activity-modifying proteins (RAMPs) affect the pharmacology of their GPCR binding partners (Udawela *et al.*, 2004). Despite the relatively low sequence similarity within this protein family (McLatchie *et al.*, 1998), RAMPs have three common structural characteristics: a single predicted membrane-spanning domain, a short cytoplasmic domain, and a long extracellular domain (Parameswaran and Spielman, 2006). Co-expression of RAMPs with the calcitonin receptor (McLatchie *et al.*, 1998) or other class B (Christopoulos *et al.*, 2003) and C GPCRs (Bouschet *et al.*, 2005) allows the formation of complexes of these membrane-associated receptors and produces novel binding sites for ligands. This interaction produces novel signaling responses that are not present in cells that express either protein alone (McLatchie *et al.*, 1998).

We previously demonstrated that deterrent compounds from in marine sponges are detectable and unpalatable to zebrafish (Cohen *et al.*, 2008). Furthermore, *Xenopus laevis* oocytes expressing a whole zebrafish cDNA library, the cystic fibrosis transmembrane conductance regulator (CFTR), and the *beta*-2 adrenergic receptor (β_2AR) exhibited a receptor-mediated electrophysiological response to formoside, a chemical deterrent found in the marine sponge *Erylus formosus,* which we have shown to be unpalatable to zebrafish (Cohen *et al.*, 2008). Kinetics of the formoside-mediated response suggest that formoside induces an increase in chloride current *via* activation of the CFTR chloride channel in *Xenopus* oocytes (Cohen *et al.*, 2008). However, this channel is not directly activated by formoside (Cohen *et al.*, 2008), suggesting that the zebrafish library encodes a protein whose activation by formoside subsequently causes a G protein signaling cascade culminating in the opening of CFTR channels.

Given these results, we hypothesized that the zebrafish genome encodes a chemoreceptor underlying the aversive behavioral response to the deterrent compound formoside. We describe here the functional isolation from a zebrafish cDNA library and initial characterization of a chemoreceptor that responds to formoside. Utilizing bioassay-guided fractionation in *Xenopus* oocytes, whereby library clones were functionally tested in smaller and smaller groups, we isolated a cDNA encoding this chemoreceptor, which appears to be related to RAMP proteins and functions as a co-receptor with other GPCRs to respond to formoside.

CHAPTER 7

MATERIALS AND METHODS

7.1 Chemicals

Isoproterenol, octanal, dimethylsulfoxide (DMSO), and ethanol were obtained from Sigma-Aldrich (St. Louis, MO). Cyclohexamide, capsaicin, and sceptrin were obtained from A.G. Scientific (San Diego, CA). Formoside and ectyoplasides A and B were isolated from sponge tissues following previously described methods (Kubanek *et al.,* 2000; 2002).

7.2 Animals

Xenopus laevis frogs were obtained from Xenopus Express (Dexter, MI) and housed in an aquatic habitat (Aquaneering, Inc.; San Diego, CA). Methods of animal handling were in accordance with the NIH guidelines and the protocols were approved by the Institutional Animal Care and Use Committees of the Georgia Institute of Technology and Emory University.

7.3 Plasmid constructs

A whole zebrafish *Danio rerio* cDNA plasmid library constructed in the pExpress-1 vector and size selected for larger inserts (average size is 2 kb) was obtained from the I.M.A.G.E. Consortium (distributed by Open Biosystems, Huntsville, AL). Once isolated by expression cloning (see below), the cDNA encoding RL-TGR, the

functional receptor, was amplified *via* PCR from library clone A9-f4-230 and ligated *via* the *Not I* and *BamH I* sites into the pET-52b(+) vector. This construct encodes a Strep II tag at the amino terminal and a His tag at the carboxy terminal of the insert. A Strep II tagged RL-TGR mammalian expression construct was created by PCR amplifying this insert and ligating into the pcDNA3.1(+) construct *via* the *Hind III and Xho I* sites. Dr. David Gadsby (Rockefeller University, NY) kindly provided the construct encoding the human CFTR in the pGEMHE vector, and Dr. Brian Kobilka (Stanford University, CA) kindly provided the construct encoding the human β_2AR in the pSP65 vector. A construct encoding rat aldehyde olfactory receptor OR-I7 was constructed in the pSMYC vector (Wetzel *et al.*, 1999). All cDNA plasmids were isolated from bacteria, linearized, and *in vitro* transcribed into cRNA (mMessage mMachine; Ambion, Austin, TX) for microinjection into *X. laevis* oocytes.

7.4 Electrophysiology

X. laevis oocytes were isolated from adult females and prepared as previously described (Fuller et al., 2004; McDonough et al., 1994). Various combinations of library transcript (2.5-10 ng), CFTR transcript (1.25-5 ng), β_2AR transcript (0.5-2 ng), and OR-I7 transcript (0.5-2 ng) were microinjected into stage V oocytes. After an incubation of 48-96 hours in L-15 media (Invitrogen; Carlsbad, CA) at 17^{o}C, oocytes were tested via two-electrode voltage clamp (TEVC), using a GeneClamp 500 amplifier (Axon Instruments; Sunnyvale, CA). Recording solution was ND96 (96 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl, 5 mM HEPES; pH 7.50) with added 1.8 mM $CaCl_2$. Oocytes were treated with deterrent compounds dissolved in ND96 buffer and a minimal amount of

solvent (ethanol, DMSO, or water), usually ~0.01% final concentration. Electrophysiological responses were detected *via* TEVC, signaling that the expressed receptor was activated by the compound, inducing a signal cascade that resulted in a change in current. Whole oocyte currents were recorded at V_M= -60 mV. Application of vehicle alone did not cause a change in current (*data not shown*).

7.5 Isolation of chemoreceptor gene

We previously showed that the rat aldehyde receptor (OR-I7), which couples to G_{olf}, is capable of activating a G_s-mediated signaling cascade, resulting in the opening of heterologously expressed CFTR ion channels in the plasma membrane of *Xenopus* oocytes expressing this channel, thus changing current (Cohen *et al.*, 2008). Specifically, activation of $G_{\alpha S}$ leads to activation of adenylyl cyclase and, subsequently, protein kinase A (PKA). CFTR is a PKA-activated chloride channel, and its activation can be measured using TEVC in oocytes. All of the proteins which comprise the $G_{\alpha S}$–mediated signaling machinery are endogenously expressed within oocytes, and these cells have been utilized in many other instances to reconstitute GPCR signaling cascades (Lubbert *et al.*, 1987; Abaffy *et al.*, 2006). Futhermore, we showed that an application of formoside to oocytes co-expressing a whole zebrafish cDNA library, β_2AR, and CFTR causes a CFTR-like electrophysiological response (Cohen *et al.*, 2008). The electrophysiological response for CFTR channels activated by a chemoreceptor-mediated signaling cascade is a slow, broad change in current that slowly returns to baseline (Cohen *et al.*, 2008). Thus, we hypothesized that we could utilize this bioassay to isolate a cDNA encoding a formoside-responsive receptor.

The bioassay-guided fractionation technique employed here makes use of the above electrophysiological bioassay to separate zebrafish cDNA library clones by further fractionating the pools of clones that induced a positive response (Appendix A). Briefly, pools of library clones were linearized with *Pac I* and transcribed into cRNA, as described above. These pools of cRNA were microinjected into *X. laevis* oocytes along with cRNA encoding the CFTR and β_2AR. These oocytes were tested *via* the electrophysiological bioassay, which utilizes TEVC to probe for an increase in CFTR current in response to formoside. The cDNA library clones corresponding to the cRNA-injected oocytes that responded to the application of formoside ("active fractions") were sub-fractionated. These cDNA clones were miniprepped as a pool, *in vitro* transcribed into cRNA, microinjected into oocytes, and tested *via* the bioassay. This iterative process continued until one "active" clone was isolated and sequenced in both the forward and reverse directions with T7 promoter and T7 terminator universal primers, respectively.

7.6 Immunoprecipitation and western blot analysis

7.6.1 Protein extraction

Two to three days post-microinjection, *X. laevis* oocytes were tested for expression using TEVC. Thirty to fifty oocytes from expressing batches were placed in 1 mL ice-cold homogenization buffer (5 mM $MgCl_2$, 5 mM Na_2HPO_4, 1 mM EDTA, 80 mM sucrose, 1 mM PMSF, 20 mM Tris; pH 7.4) with added protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO) and homogenized on ice *via* several passages through a syringe and 25-gauge needle. This mixture was centrifuged at 4^{o}C for 30 min. at 13,000 rpm. The yolk was carefully removed with a sterile cotton-tipped applicator. The

supernatant was stored at -20°C, and 100 µl solubilization buffer (10% glycerol, 5 mM EDTA, 1% Na deoxycholate, 1 mM PMSF, 20 mM Tris; pH 6.8) with added protease inhibitor cocktail was used to resuspend the membrane pellet. The pellet was solubilized at 37°C for 1 hr. and centrifuged at 4°C for 1 hr. The supernatant was removed to a new tube, diluted in 700 µl sucrose buffer (100 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, 10% sucrose, 1 mM PMSF, 20 mM Tris-Cl; pH 6.8) with added protease inhibitor cocktail, and stored at -20°C until use.

7.6.2 Immunoprecipitation

Extracted protein was immunoprecipitated using mouse anti-His antibody (Invitrogen; Carlsbad, CA) conjugated to protein A agarose beads (Roche; Indianapolis, IN). The beads were pre-cleared and conjugated to the antibody by following the manufacturer's protocol. However, wash steps utilized less stringent buffers (buffer 1: 150 mM NaCl, 0.1% NP40, 0.05% Na deoxycholate, 1 mM $CaCl_2$, 50 mM Tris-Cl, pH 7.5; buffer 2: 0.1% NP40, 0.05% Na deoxycholate, 1 mM $CaCl_2$, 50 mM Tris-Cl, pH 7.5), so as to not disrupt pertinent protein-protein interactions.

7.6.3 Western blot

The antibody-protein complexes were resuspended in sample buffer containing 5% betamercaptoethanol, boiled, and loaded into the wells of a 4-20% Tris-Cl pre-cast gel (Bio-Rad). Standard electrophoresis and blotting procedures were utilized to transfer proteins onto a nitrocellulose membrane (Bio-Rad; Hurcules, CA) (Sambrook *et al.*, 2001). After the membrane was blocked with 3% nonfat milk in TBS-tween20 (TBS-T),

it was incubated with mouse anti-Strep tag II (Novagen; Darmstadt, Germany) antibody at 4°C overnight. Three 10 min. washes in TBS-T were performed, after which the membrane was incubated with goat anti-mouse-HRP antibody (Pierce; Rockford, IL) for 30 min. at room temperature and washed again. SuperSignal West Pico chemiluminescent substrate (Pierce; Rockford, IL) was utilized for detection on autoradiography film.

7.7 Cell culture and transient transfection

HEK293 cells were maintained at 37°C with 5% CO_2 in Dulbecco's Modified Eagle's Medium supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin and 10% fetal bovine serum. One day prior to transfection, cells were seeded into 100 mm dishes containing sterile glass coverslips at a density of approximately $1x10^6$ cells/dish. Two to three hours prior to transfection, the media was replaced with fresh media and incubated at 37°C. Cells were transiently transfected using calcium phosphate. A total of 10 μg DNA was dissolved in 200 μl of 0.25 M $CaCl_2$, added dropwise to 200 μl of 2X HEPES buffered saline (140 mM NaCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 50 mM HEPES; pH 7.1), and incubated at room temperature for 20 minutes. This solution was added dropwise to the cells. After an overnight incubation, the media was changed and the cells were incubated for an additional 18-24 hours. Estimated transfection efficiencies of 70-90% were routinely obtained using this protocol.

7.8 Indirect immunofluorescence

Cells were fixed in 5% paraformaldehyde in phosphate buffered saline (PBS) for 10 min. and rinsed with 10% fetal bovine serum and 0.02% azide in PBS (PBS/serum). Fixed cells were incubated with primary antibodies diluted in PBS/serum for 45 min. and washed with PBS/serum (3 x 5 min.). The cells were then incubated with fluorescently labeled secondary antibodies in PBS/serum for 45 min., washed with PBS/serum (3 x 5 min.) and once with PBS, and mounted with 4',6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories; Burlingame, CA) onto glass slides. Images were acquired using a Zeiss AxioObserver A1 microscope with an attached Zeiss AxioCamHRC camera and were analyzed using the Zeiss AxioVision software.

7.9 Bioinformatics

Nucleotide and protein sequences were assessed *via* NCBI BLAST (Blastn, Blastp, EST Blast), Zebrafish BLAT (UCSC Bioinformatics Server), and Multiz Alignment (UCSC Bioinformatics Server). The TMHMM Server v. 2.0 (CBS prediction server) was utilized to predict the transmembrane helix of RL-TGR. The helical net diagram was created utilizing TOPO2 Transmembrane protein display software (http://www.sacs.ucsf.edu/TOPO2/).

CHAPTER 8

RESULTS

8.1 Fractions of a zebrafish cDNA library cause an electrophysiological response to formoside

As previously shown (Cohen *et al.*, 2008), oocytes that co-express a whole zebrafish cDNA expression library along with CFTR and β_2AR exhibited an electrophysiological response to the application of formoside. After dividing this cDNA library into fractions, each fraction was transcribed into cRNA, microinjected into oocytes, and tested *via* TEVC (see Appendix A). Oocytes expressing fraction A responded to a 5 μM application of formoside in a manner similar to that of the entire library (Fig. 9A). Furthermore, oocytes expressing fraction A9, a sub-fraction of library fraction A, and subsequent sub-fractions (Fig. 9B), also responded to formoside when co-expressed with CFTR and β_2AR.

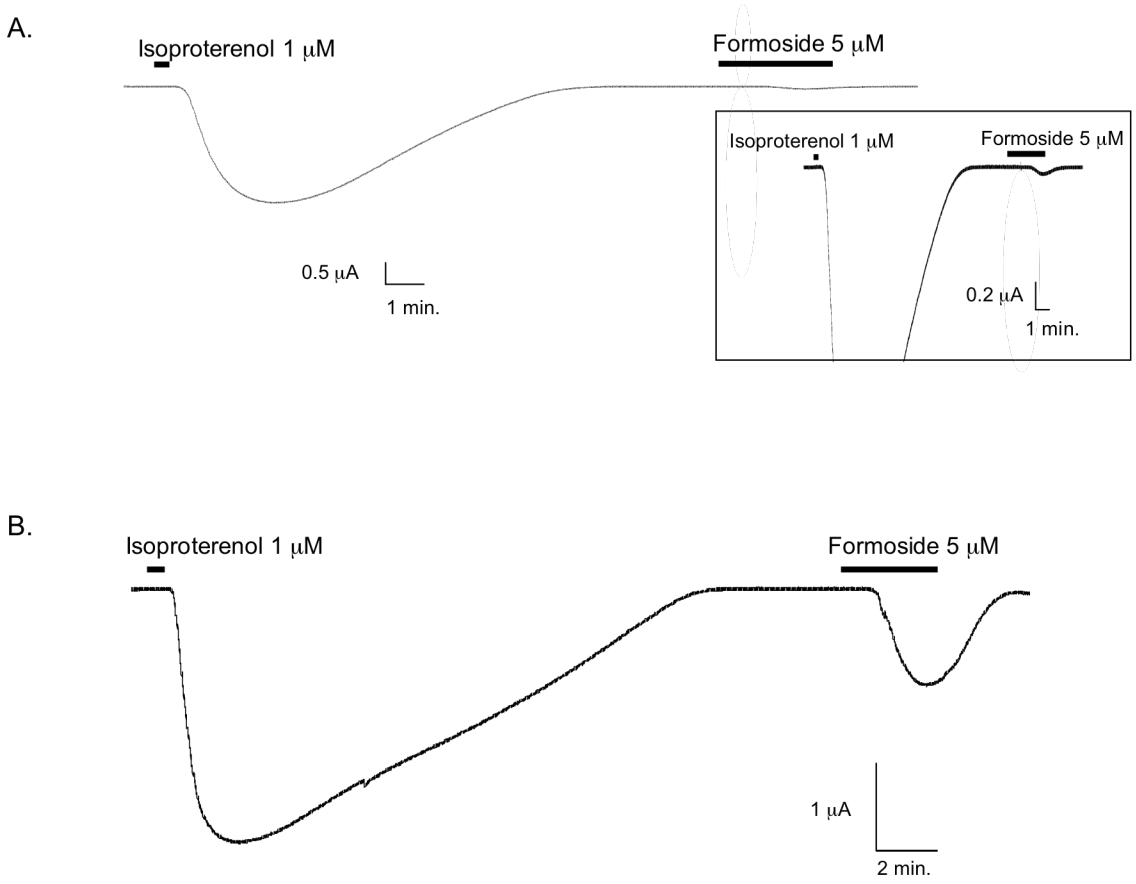
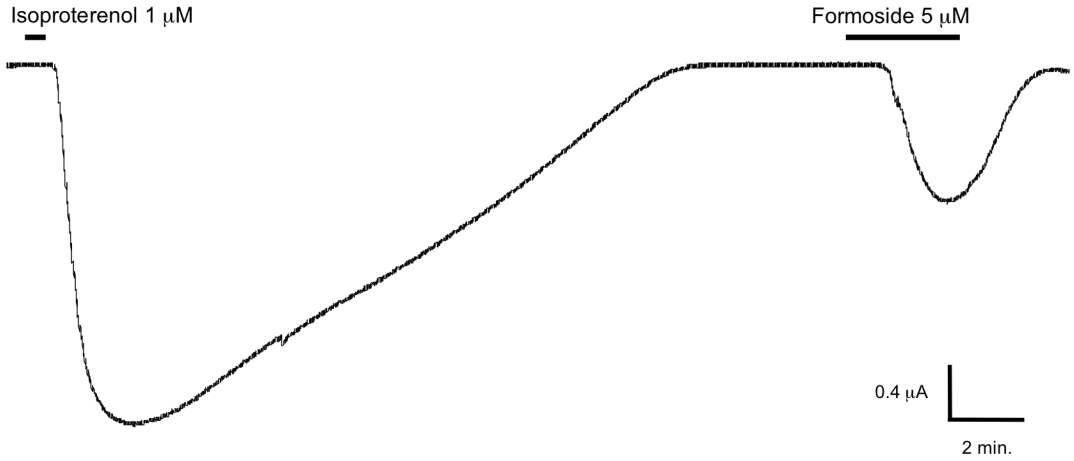


Figure 9. Electrophysiological responses to formoside from *X. laevis* oocytes expressing zebrafish cDNA library fractions. Representative traces are shown from oocytes co-expressing CFTR, β_2AR and **(A)** fraction A (*n*=10; range =0.05-0.2 μA) or **(B)** fraction A9-f4 (*n*=5; range =0.1-6.7 μA). Cells were tested for expression of CFTR and β_2AR with an application of 1 μM isoproterenol. When 5 μM formoside was applied to these cells, there was a broad, slow increase in current that slowly returned to baseline current levels, suggesting activation of CFTR. Inset in **(A)** shows an expanded view of the same trace.

8.2 A 291 base pair segment of clone A9-f4-230 is responsible for the electrophysiological response to formoside

Using bioassay-guided fractionation to isolate a single clone that enabled the functional response in oocytes, clone A9-f4-230 was identified. The response of oocytes expressing the protein encoded by this clone was similar to the formoside-mediated response of oocytes expressing the entire zebrafish cDNA library (Fig. 10A). Sequencing of the 1199 bp insert of the full-length clone followed by BLAT/BLAST analysis revealed that this gene is located on zebrafish chromosome 12:3886126-3887324. Furthermore, the cDNA is 100% identical to the zebrafish genomic DNA, suggesting that the gene is intronless. A 40 bp segment within the presumed open-reading frame appears to be conserved in fish because it aligned to chromosomal DNA from five fish species: zebrafish, stickleback, medaka, tetraodon, and *Fugu rubripes* (Fig. 10B). The sequence is <100% conserved because tetroadon and stickleback each have a single base insertion. This same 40 bp segment is only found in fish species and not found in any mammalian, reptilian, or amphibian genomes available through NCBI and UCSC, suggesting that it is a novel fish gene.

A.



B.

^{N}-Met Tyr Leu Asp Ile Ile Asp Met Val Glu Thr Cys Leu Phe Ser Ser Phe Ala Ile Phe Ser Lys Ile Phe Pro Gly Leu Leu Leu Ile Phe Ser Pro His His Ala Phe Cys Ile Trp Gly Asn Met Arg Ala Leu Leu Lys Tyr Thr Trp Thr His Phe Phe Val Lys Tyr Glu Asp Ala Leu Lys Val Thr Val Leu Lys Phe Val Lys His Leu Glu Leu Ile Phe Ile Pro Glu Phe Gln Leu Cys Leu Tyr Glu Leu Cys Phe Ser Ile Thr Asp Cys Leu Lys-^{C}

Figure 10. Clone A9-f4-230 encodes a zebrafish protein that is activated by formoside. (A) Electrophysiological response to formoside of an oocyte expressing CFTR, β_2AR, and isolated full-length clone A9-f4-230 (n> 30; range =0.1-3.7 μA). The representative response to formoside shown here suggests that this clone encodes a receptor responsive to this compound. **(B)** Predicted peptide sequence of RL-TGR. Red residues indicate a possible PDZ binding domain. Boxes denote extracellular cysteines that may be involved in protein-protein interactions. Blue residues are conserved across five fish species. The predicted transmembrane region is denoted with an underline.

We hypothesized that the formoside-responsive receptor was encoded by a 291 base pair segment (Fig. 10B), which encompassed the longest open reading frame within this clone, although a strong Kozak sequence was not apparent. To test this hypothesis, we subcloned this open reading frame into pET-52b(+), which would generate protein as a doubly-tagged fusion peptide, with Strep tag II at the N-terminus and His tag at the C-terminus, thus confirming reading frame (Fig. 11A). Oocytes injected with a cRNA transcript from this clone, CFTR, and β_2AR responded to formoside, confirming both the open-reading frame and the translation frame (Fig 11B). A BLAST analysis of the predicted peptide sequence found no homologs, indicating that clone A9-f4-230 encodes a novel protein. The molecular weight of the native protein is predicted to be approximately 10 kDa and that of the tagged receptor approximately 17 kDa. To determine if formoside-responsive oocytes were expressing the tagged receptor, we immunoprecipitated His-tagged protein from responsive oocytes that were injected with transcript for the doubly tagged protein, CFTR, and β_2AR. Although the expected band size for the tagged protein was 17 kDa, immunoblotting for Strep tag II showed bands at ~34, ~60, and ~111 kDa (Fig. 11C). The band at ~60 kDa detected in all lanes is from a non-specific IgG conglomerate artifact. The lower weight band at ~34 kDa likely represented homodimers of the 17 kDa receptor, since it was approximately double the predicted size of the tagged receptor. The higher weight band at ~111 kDa possibily represented a complex of proteins, including this tagged receptor. Since these oocytes were co-expressing β_2AR, which has a molecular weight of ~47 kDa, the ~111 kDa complex could have been comprised of a β_2AR homodimer associated with the tagged

protein. These two bands were detected in the membrane fraction, suggesting that the formoside receptor is membrane-associated. Additionally, these bands were not present in the membrane fractions from uninjected oocytes or oocytes expressing only β_2AR and CFTR, indicating that they are specific to formoside-responsive oocytes. These results also indicate that oocytes injected with transcript from the doubly-tagged protein, CFTR, and β_2AR produced full-length tagged receptor, given that these specific bands were detected only after immunoprecipitating for one tag and immunoblotting for the other tag. Moreover, since these same oocytes also were responsive to formoside, whereas cells co-expressing CFTR and β_2AR but not the tagged receptor had no response, expression of this receptor either as a monomer or as a component of a protein complex is required to respond to formoside. Immunofluorescence in mammalian cells that were overexpressing tagged RL-TGR further confirms that this cDNA encodes a protein (Fig 11D).

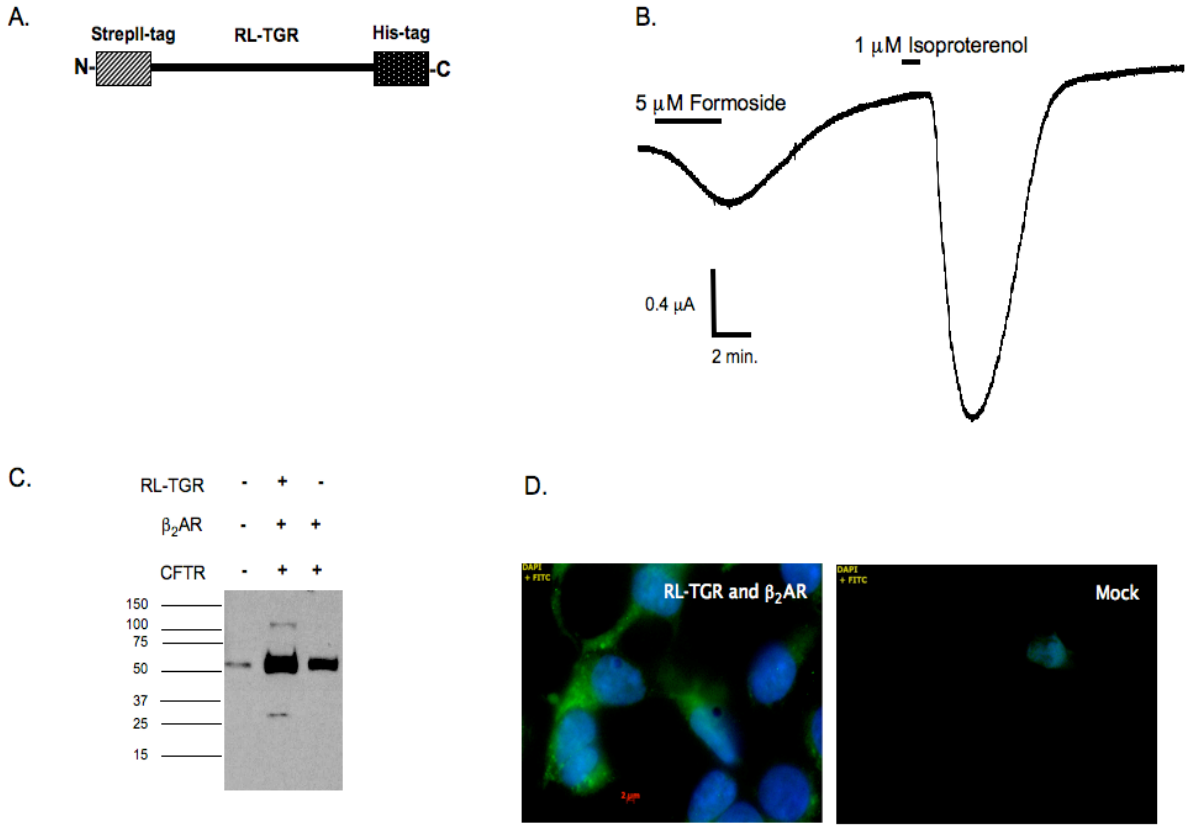


Figure 11. RL-TGR is responsible for the formoside-induced response. (A) Diagram of StrepII/His-tagged RL-TGR fusion protein. The StrepII tag is on the N-terminus and the His tag is located on the C-terminus of a 291 base pair segment of the full-length clone that encodes RL-TGR. **(B)** Electrophysiological response upon the application of formoside to oocytes expressing CFTR, β_2AR, and StrepII/His-tagged RL-TGR. These cells (n=19; range =0.05-2.2 μA) recapitulated the responses seen in Figures 9 and 10A, strongly suggesting that this tagged open reading frame correctly encodes a formoside-responsive receptor and that the tags do not interfere with the function of this receptor. **(C)** Western blot showing StrepII/His-tagged RL-TGR heterologously expressed in formoside-responsive *X. laevis* oocytes that were also expressing CFTR and β_2AR. Protein from oocytes was extracted, immunoprecipitated with anti-His antibody, and immunoblotted with anti-StrepII. A low molecular weight band was detected at ~34 kDa, suggesting the presence of homodimers of RL-TGR. Additionally, a high molecular weight complex was detected at ~111 kDa, suggesting that RL-TGR physically interacts with another protein, which may be β_2AR. The band at ~60 kDa is a non-specific artifact from IgG conglomerates. **(D)** Indirect immunofluorescence of heterologously expressed StrepII-RL-TGR. HEK293 cells that were transiently transfected with plasmids encoding StrepII-RL-TGR (green) and β_2AR were probed for expression of RL-TGR with a Strep

tag II specific antibody. Nuclei were stained with DAPI (blue). Because the StrepII tag is predicted to be internal, the cells were permeabilized prior to incubation with antibody. These cells show staining throughout the cell but very little perinuclear staining, in contrast to the mock transfected cells, which show mostly perinuclear staining, indicating that the antibody is specifically detecting expression of tagged RL-TGR.

8.3 RL-TGR has predicted structural similarity to RAMP proteins

A transmembrane prediction program predicted a single-pass transmembrane domain (Fig. 12A and 12B), similar to RAMP proteins, which act as accessory proteins to many GPCRs (Morfis *et al.*, 2003; Parameswaran and Spielman, 2006). Given this similarity, we have named this protein RAMP-like Triterpene Glycoside Receptor (RL-TGR). The topology of RL-TGR is similar to RAMPs: the short amino-terminal is predicted to be intracellular, and the long carboxy-terminal is predicted to be extracellular; however, the carboxy- and amino-terminals oppositely oriented from RAMPs described so far (Fig. 12A and B). All known RAMPs require physical interaction with a true receptor, and consistent with that, RL-TGR has features that suggest it is involved in protein-protein interaction. A PDZ binding domain, found in some RAMPs (Bomberger *et al.*, 2005a; 2005b), is predicted to be located in the cytoplasmic tail and likely helps anchor it into a plasma membrane complex (Fig. 12B). The extracellular domain of RL-TGR has four cysteines, which also may be involved in protein-protein interactions.

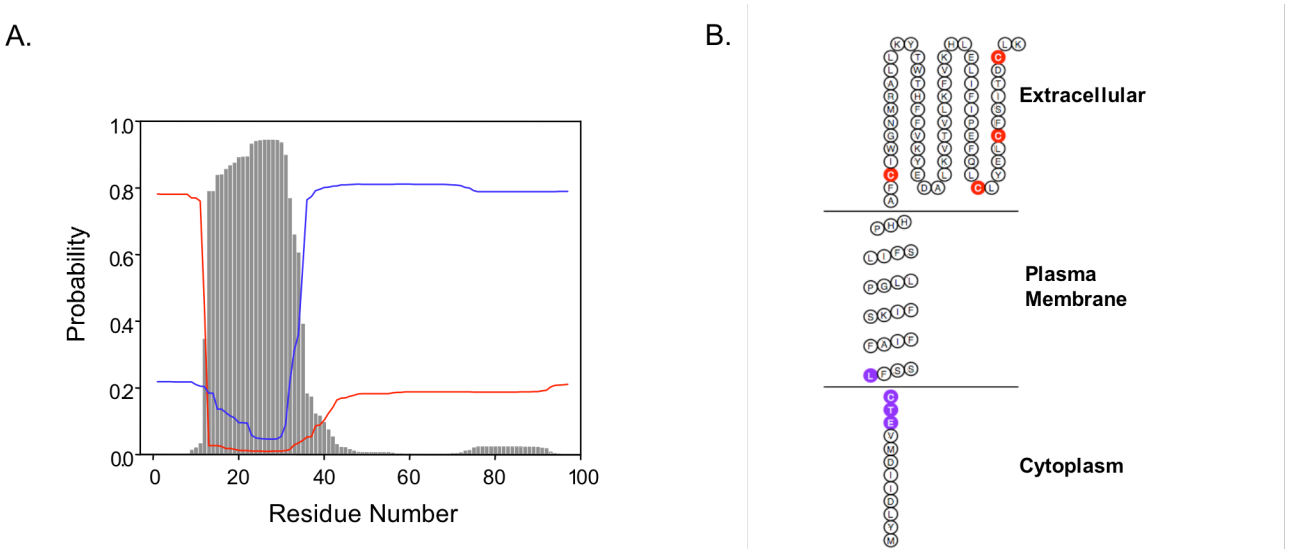


Figure 12. RL-TGR is predicted to be a single-pass membrane associated receptor. **(A)** Transmembrane prediction plot of RL-TGR. The red plot indicates the residues' probability of being intracellular, and the blue plot shows the residues' probability of being extracellular. The grey bars, indicating the residues' probability of being transmembrane, predicted one transmembrane helix consisting of amino acids 13-35. A short intracellular region (amino acids 1-12) is predicted on the N-terminus, and a long extracellular region (amino acids 36-97) is predicted on the C terminus. **(B)** Annotated protein structure schematic of RL-TGR. The predicted transmembrane helix is represented as a helical net. Cysteine residues in the extracellular region that may be involved in protein-protein interactions are highlighted in red. Purple residues indicate a possible PDZ binding domain.

8.4 RL-TGR responds to other triterpene glycosides

We hypothesized that RL-TGR may be a generalized aversive receptor. When we tested the specificity of this receptor by assaying other aversive compounds, oocytes expressing the full-length clone, β_2AR, and CFTR responded to a mixture of ectyoplasides A and B, defensive triterpene glycoside compounds found in marine sponges (Fig. 13A). These cells did not respond to the odorant octanal (Fig. 13B), cyclohexamide (Fig. 13B), which is perceived as bitter by humans, the pain-causing compound capsaicin, or the marine chemical defense compound sceptrin (*n*=5; *data not shown*). Since only triterpene glycoside compounds caused a CFTR-like electrophysiological response in these cells, it is possible that this receptor may be specific for triterpene glycosides or related compounds.

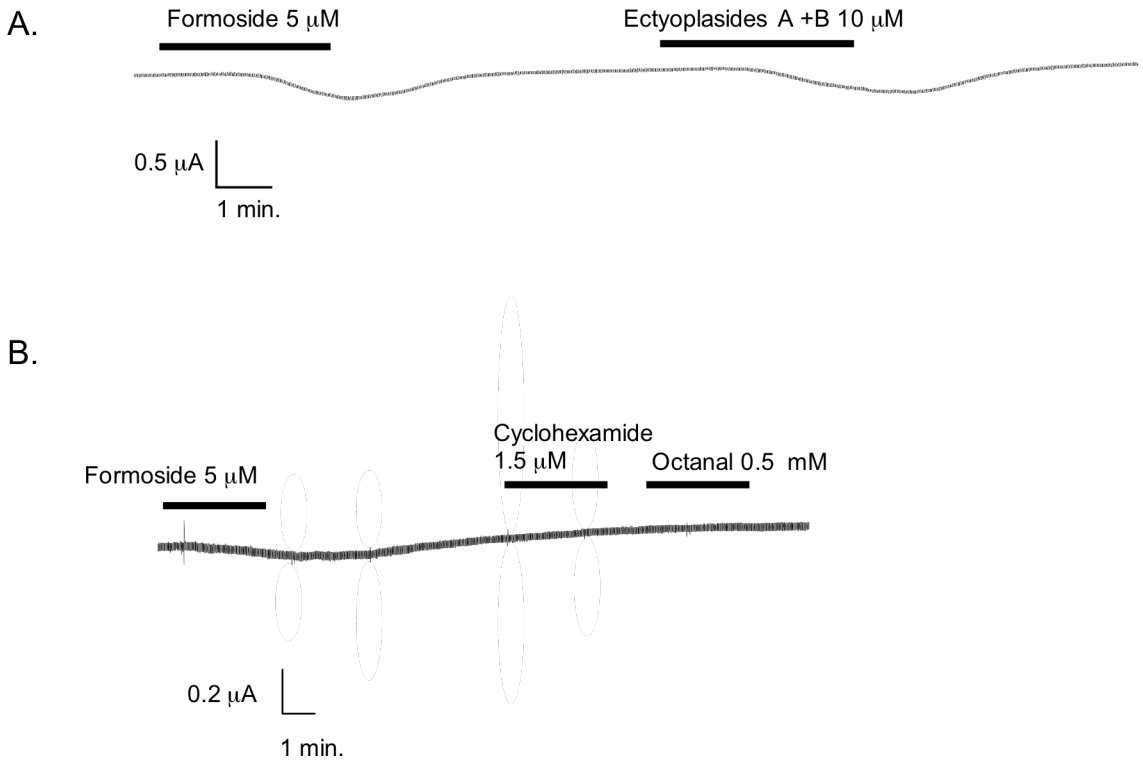


Figure 13. RL-TGR responds specifically to triterpene glycoside compounds. Electrophysiological responses of oocytes expressing CFTR, β_2AR, and the full-length clone to various compounds are shown. **(A)** Application of a mixture of ectyoplasides A and B, triterpene glycoside compounds known to be unpalatable to marine (Kubanek *et al.*, 2001) and freshwater (Cohen *et al.*, 2008) fish, caused an electrophysiological response comparable to a formoside-induced response (*n*=5; range =0.2-0.3 μA). **(B)** No responses were seen to applications of the bitter compound cyclohexamide or to octanal (*n*=5).

8.5 RL-TGR requires co-expression of a GPCR to respond to formoside

Oocytes expressing the full-length clone and β_2AR but not CFTR did not respond to formoside (Fig. 14A). Therefore, CFTR was needed in this system to obtain a detectable response to formoside, suggesting that the receptor itself did not directly cause the change in current and is not a ligand-gated ion channel. In cells expressing the full-length clone, β_2AR, and CFTR, current-voltage plots showed that reversal potentials of isoproterenol-activated CFTR current (-29.1 +/- 3.3 μA; SEM; *n*= 5) and formoside-activated current (-28.6 +/- 5.3 μA; SEM; *n*=7) were the same (Fig. 14B), indicating that both responses are reflective of increased CFTR chloride current. RL-TGR responded to multiple applications of formoside with diminishing amplitude, not unlike β_2AR-mediated responses to isoproterenol (Fig. 14C). Therefore, it is likely that formoside activates a receptor-mediated signaling cascade, which culminates in the activation of CFTR.

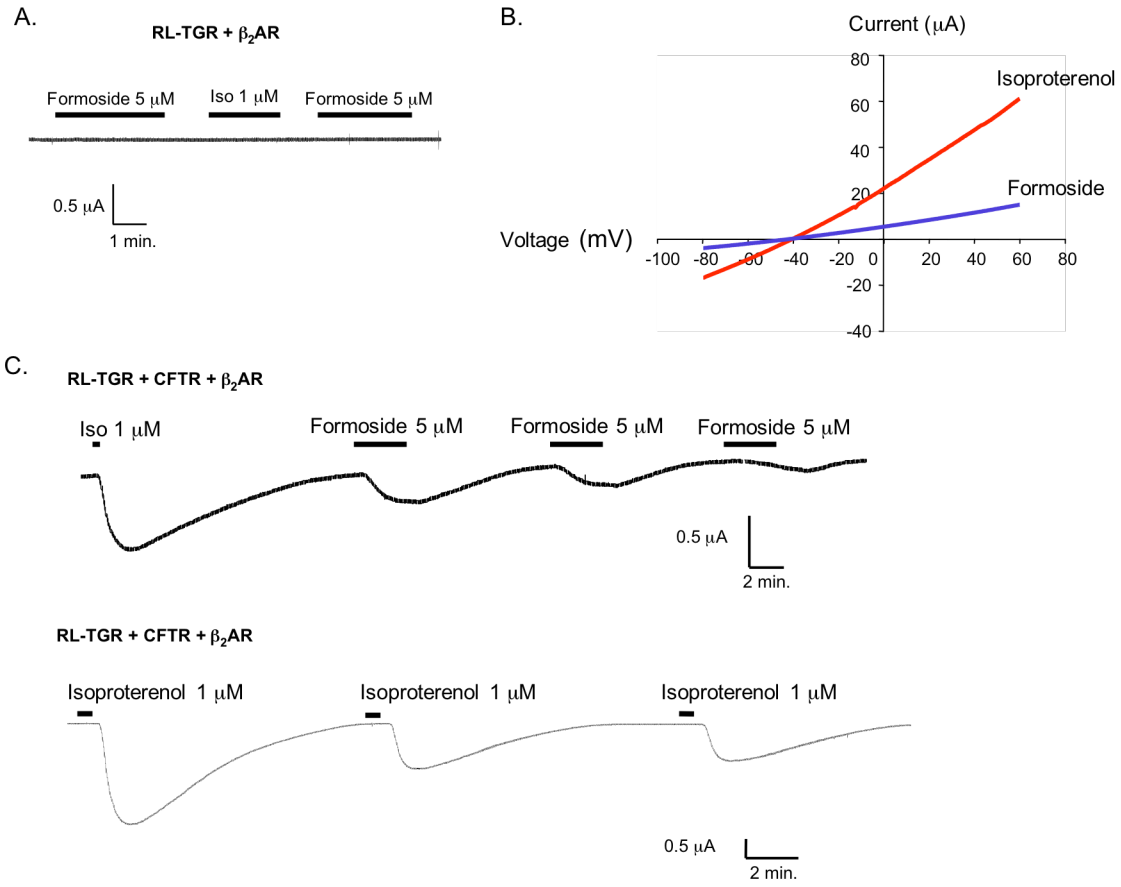


Figure 14. Formoside induces receptor-mediated activation of CFTR. (A) Oocytes expressing the full-length clone and β_2AR but not CFTR did not respond to formoside (*n*=5), suggesting that CFTR is required for a detectable response. **(B)** Current-voltage plot for isoproterenol-induced and formoside-induced responses in oocytes expressing CFTR, β_2AR, and the full-length clone. Both plots crossed the X-axis at the same point, suggesting that chloride is the main charge carrier for both currents. Furthermore, CFTR is the most likely source of chloride current in these cells and the kinetics of the formoside-induced current are the same as those of the isoproterenol-induced current, suggesting that CFTR is ultimately activated by formoside. Since formoside did not activate CFTR when expressed alone in oocytes (Cohen *et al.*, 2008), formoside likely activates CFTR indirectly through a receptor-mediated cascade. **(C)** Multiple applications of formoside to oocytes expressing CFTR, β_2AR, and clone A9-f4-230 caused repeatable electrophysiological responses similar to the receptor-mediated responses to isoproterenol (*n*=5), further suggesting that formoside induces a receptor-mediated response.

We hypothesized that if RL-TGR functions similarly to RAMPs, it would require co-expression of a GPCR to respond to formoside. Indeed, electrophysiological responses to formoside require oocytes to co-express the full-length clone plus CFTR along with a GPCR, as cells not co-expressing a GPCR have minimal response to formoside (Fig. 15). Interestingly, the robust response to formoside occurs in cells co-expressing CFTR, RL-TGR, and one of two different $G_{\alpha S}$-coupled GPCRs: β_2AR or OR-I7 (Fig. 15). Thus, the response to formoside requires both RL-TGR and a GPCR, further supporting our hypothesis that RL-TGR is RAMP-like co-receptor, which forms a signaling complex with a GPCR to detect formoside.

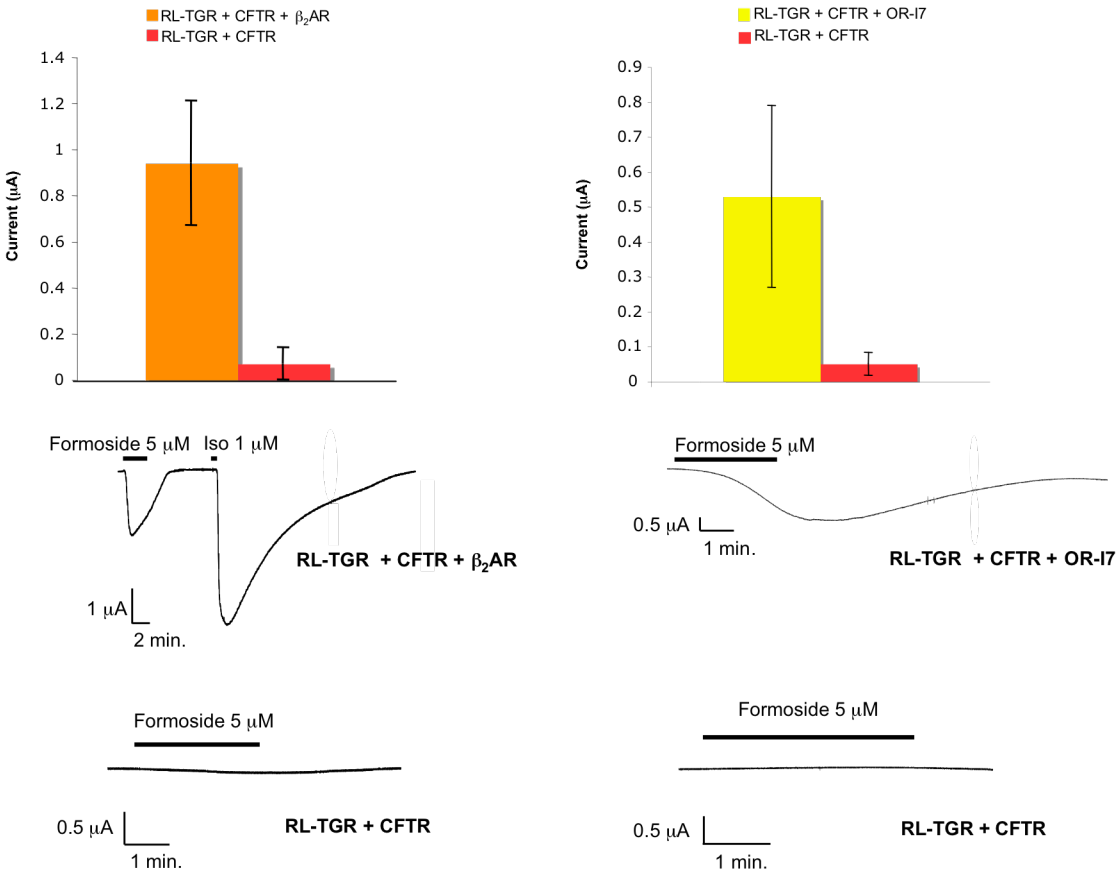


Figure 15. Oocytes require expression of RL-TGR and a GPCR to respond to formoside. (A) Electrophysiological responses of cells co-expressing CFTR and the full-length clone with and without β_2AR (*left panel; n*=5; *p*<0.5) or OR-I7 (*right panel; n*=7; *p*=0.5). Bar charts summarizing data for responses to formoside are shown above and representative traces are depicted below. When formoside was applied, cells that were co-expressing a GPCR were able to robustly respond; cells that were not co-expressing a GPCR were not able to consistently respond. Error bars represent standard error.

CHAPTER 9

DISCUSSION

We showed previously that the formoside-mediated signaling pathway could be reconstituted in *Xenopus* oocytes by heterologously expressing CFTR, β_2AR, and a whole zebrafish cDNA library (Cohen *et al.*, 2008). Here we present the functional identification and initial characterization of RL-TGR, an accessory protein that responds to formoside (Fig. 9 and 10A) and other deterrent triterpene glycosides (Fig. 13). We demonstrate that this protein is membrane-associated, as we are able to detect StrepII/His-tagged RL-TGR from the membrane fraction of oocytes (Fig. 11C) that electrophysiologically respond to formoside. This response appears to be receptor-mediated because multiple applications of formoside elicited multiple responses from these oocytes in a receptor-mediated fashion (Fig. 14C). Further supporting a receptor-mediated mechanism, current-voltage plots showed that the electrophysiological response in oocytes is reflective of activation of CFTR-mediated chloride current (Fig. 14B). The response is not likely attributable to direct activation of CFTR; rather, it is a GPCR-mediated signaling cascade that indirectly activates CFTR, since the response required CFTR but did not occur in oocytes only expressing CFTR (Fig. 14A) and did not occur in cells expressing CFTR but not a $G_{\alpha S}$-coupled GPCR (Fig. 15).

Despite the fact that there was little sequence homology between RL-TGR and members of the RAMP family, this 10 kDa protein did bear remarkable similarity to this class of proteins in both structure (Fig. 12A and B) and function (Fig. 15). Like the

structure of RAMPs described to date (Udawela *et al.*, 2004; Parameswaran and Spielman, 2006), the predicted structure of RL-TGR was a single-pass transmembrane protein with a large extracellular domain and short intracellular domain, suggesting that it could function similarly. The extracellular domain of RL-TGR was large enough to interact with formoside; however, the intracellular domain was not typical of a G protein binding domain. Therefore, the apparent signaling response was not likely directly mediated by this receptor alone, given that the formoside-mediated signaling involved a G protein signaling pathway. Since RAMPs have the ability to modify GPCR receptor activity, we hypothesize that our RAMP-like receptor may be interacting with heterologously expressed β_2AR receptor in oocytes to enable formoside-mediated signaling, explaining the need for expression of both β_2AR and RL-TGR in oocytes to elicit a response to formoside (Fig. 15). Our results suggest that the RL-TGR receptor was also able to modify the activity of another $G_{\alpha S}$-coupled GPCR, OR-I7, to enable formoside-mediated signaling (Fig. 15). These functional data, combined with the structural parallels to known RAMPs, support our hypothesis that RL-TGR is an accessory protein related to the RAMP family. Moreover, the reverse structural topology of RL-TGR, as compared to RAMPs, suggests that these co-receptors may have evolved separately through convergent evolution.

9.1 Signaling mechanism of RL-TGR

RAMP proteins are a family of accessory proteins that affect the localization and pharmacology of GPCRs (Morfis *et al.*, 2003; Parameswaran and Spielman, 2006). They are expressed fairly ubiquitously across tissues, suggesting that they play a widespread

role (Husmann *et al.*, 2000). In a unique mechanism, these single-pass transmembrane receptors act as chaparones that associate with class B and class C GPCR families to bind ligands that these GPCRs cannot bind alone, producing novel signaling responses (Udawela *et al.*, 2004; Parameswaran and Spielman, 2006). Like RAMP proteins (Flahaut *et al.*, 2003), the extracellular tail of RL-TGR has several cysteines that may be involved in protein-protein interactions with the extracellular tail of a GPCR, in addition to a possible PDZ binding domain on the C-terminal tail. This type of binding domain helps to anchor transmembrane proteins to the plasma membrane and participates in the formation of macromolecular signaling complexes *via* interactions with scaffolding proteins (Sheng and Sala, 2001). Although it is uncommon to have a PDZ binding domain that is not at the extreme C-terminus, there are examples of these non-canonical binding domains (Sheng and Sala, 2001), such as the well-studied interaction between neuronal nitric oxide synthase (nNOS) and syntrophin (Brenman *et al.*, 1996).

Since RL-TGR is a small RAMP-like protein, it probably does not have direct signaling capabilities. Instead, the electrophysiological response may occur because the extracellular tail of this accessory protein facilitates binding of triterpene glycosides in cooperation with a GPCR (Fig. 16), in the case of our experimental set-up β_2AR or OR-I7, which causes a conformational change in this GPCR, resulting in the activation of its cognate G protein. Thus, triterpene glycoside-mediated G protein signaling *via* RL-TGR takes advantage of the G protein activation mechanism provided by the associated GPCR.

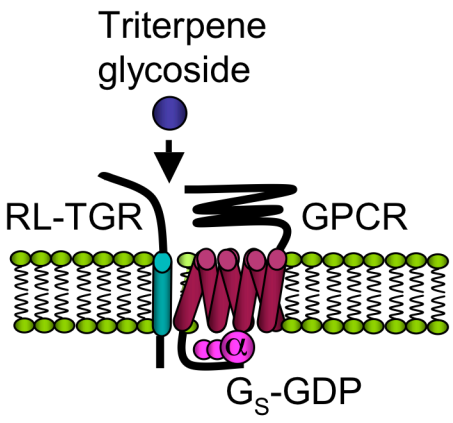


Figure 16. Proposed schematic of co-receptor/GPCR complex. We propose that RL-TGR, like other RAMPs, forms a complex with a GPCR to cooperatively bind ligand. The ligand-bound complex activates a signaling cascade through the GPCR's cognate G protein, resulting in the activation of signaling pathways that regulate ion channels.

Although for the experiments presented here we have utilized β_2AR and OR-I7 as representative $G_{\alpha S}$-coupled GPCRs, it is not likely that these receptors co-express with RL-TGR endogenously in zebrafish and form a receptor complex that responds to triterpene glycososides. In fact, an endogenous GPCR may have a higher affinity for interacting with RL-TGR, allowing for more robust responses to triterpene glycosides than we were able to detect in the experiments presented here. The identity of an endogenous GPCR whose activity is modified by RL-TGR remains unclear and should be investigated in further studies. Furthermore, future studies should investigate whether an RL-TGR/GPCR complex in peripheral chemosensory tissue underlies the aversive behavioral response in zebrafish, as well as in fish that co-occur with marine sponges containing chemical defense compounds.

9.2 Broad implications of this work

The identification of RL-TGR represents the first discovery of a receptor that responds to marine chemical defense compounds, such as triterpene glycosides. This novel accessory protein, which bears functional and structural similarity to RAMPs, may have evolved as a flexible mechanism by which organisms can detect and avoid potentially harmful compounds. Further characterization of this co-receptor will enable a broad range of studies in the mechanism of the detection of these types of defensive compounds by predators.

CHAPTER 10

PERSPECTIVE AND FUTURE DIRECTIONS

10.1 Summary of presented work

Chemoreception plays an important role in predator-prey interactions and feeding dynamics. While attractant or pleasant tasting compounds have been well studied, aversive chemoreceptive signaling has been difficult to investigate behaviorally in an ecological context because these interactions are species- and context-specific, and deterrent compounds vary among prey. Therefore, little is known about the molecular mechanism(s) used in detection of aversive compounds. Using the coral reef system, this thesis explores on a molecular level the deterrent mechanism underlying detection by fish predators of an aversive compound, in order to gain a greater understanding of predator-prey interactions in this community. Like other organisms that are sessile or slow-moving, marine sponges have special mechanisms for defense from predation. They commonly contain aversive compounds that defend these organisms from predation. To this end, we sought to identify and characterize a fish chemoreceptor that detects one or more of these compounds.

A behavioral assay demonstrated that many sponge compounds that are known to be deterrent to coral reef predator fish are also deterrent to zebrafish, a freshwater fish whose genome is well-characterized. Two of these groups of deterrent triterpene glycosides, formoside and a mixture of ectyoplasides A and B, caused electrophysiological changes in *Xenopus* oocytes expressing an entire zebrafish cDNA

library, β_2AR, and the ion channel CFTR. Utilizing this electrophysiological bioassay, we fractionated the zebrafish cDNA library and isolated a single cDNA clone encoding RL-TGR, a novel co-receptor involved in the signaling of triterpene glycosides, defensive compounds that are found in marine sponges and other slow-moving or sessile organisms. This co-receptor appears to be structurally and functionally related to receptor activity-modifying proteins (RAMPs), a family of co-receptors that physically associate with and modify the activity of G protein-coupled receptors (GPCRs). Structurally, this protein is predicted to have a single-pass transmembrane domain, a short intracellular domain, and a long extracellular domain. Expression in *Xenopus* oocytes showed that it responds to triterpene glycosides and no other types of compounds tested in a receptor-mediated manner. Additionally, RL-TGR requires co-expression of a GPCR to enable signaling in oocytes, and both of these receptors may be components of a larger signaling complex, as suggested by immunoblotting evidence. Immunoblotting from *Xenopus* oocyte membranes demonstrated that this protein is membrane associated. A 40 bp portion of the gene is conserved across multiple fish species, but is not found in any other organism with a published genome, suggesting that the expression of this receptor is limited to fish species. Therefore, this fish gene may have coevolved with organisms that produce triterpene glycoside or related defensive compounds.

This work suggests that aversive compounds may be detected by RL-TGR and related proteins in fish. The use of a GPCR and RAMP-like co-receptor complex as a detector of deterrent compounds is a flexible mechanism in which to perceive potentially harmful compounds. Instead of necessitating expression of a specific *bona fide* receptor (with the ability to both bind ligand and transduce signals) for each possible compound

an organism might need to detect in its lifetime, an organism would only require expression of a limited number of GPCRs and a suite of co-receptors, which can combine in numerous combinations to specifically and efficiently detect a vast number of deterrent compounds, protecting these organism from potentially harmful compounds.

10.2 Protective mechanisms and evolutionary implications

Although all of the compounds tested in our palatability assays (Chapter 4) are found in marine sponges, our experiments used the freshwater zebrafish *Danio rerio*. While not ecologically relevant due to the geographic separation of these two organisms, this finding may have evolutionary implications, as the behavioral rejection response of the zebrafish to some marine sponge compounds (Table 2) indicates that this aversion may be evolutionarily conserved in fish, while other chemically-mediated interactions are more species-specific (Lindquist and Hay, 1995; Bricelj *et al.*, 2005). Furthermore, because the response to formoside appears to be receptor-mediated, the receptor(s) involved in the detection of this compound also may be conserved. Conservation of receptors that detect potentially harmful compounds would not be surprising, given that these receptors would afford an evolutionary advantage to organisms that would be predisposed to avoid noxious prey, and a variety of marine and terrestrial organisms produce triterpene glycosides (Zhang *et al.*, 2006; Ukiya *et al.*, 2007), which are known in some organisms to act as defenses (Kubanek *et al.*, 2000). Many organisms exploit such a predisposition, such as the directed-deterrence of chili plants. Chilies contain capscaicin, a compound which deters predation by mammals that possess a nociceptor capable of activating a pain pathway in response this compound (Caterina *et al.*, 1997).

However, the equivalent avian receptor contains a mutation that renders birds insensitive to capsaicin (Jordt and Julius, 2002); birds readily consume chilies and effectively disperse their seeds (Tewksbury and Nabhan, 2001). Therefore, these plants benefit by containing a chemical defense, as do marine sponges.

The potentially widespread occurrence of an aversive response in a predator also would be advantageous for prey species that possess these chemical deterrents, making it more likely that a variety of potential predators would be inclined to avoid these prey as food. For example, as shown by field experiments, formoside (Kubanek *et al.*, 2002) and some other marine chemical defense compounds (Chanas *et al.*, 1997; Vervoort *et al.*, 1998; Wilson *et al.*, 1999) are deterrent to a variety of generalists (*i.e.*, predators that utilize multiple resources). However, some specialists (*i.e.*, predators that specialize on particular prey) have a higher tolerance to defensive compounds and are typically not deterred by defensive compounds of their preferred prey (Hay *et al.*, 1990; Pennings *et al.*, 1996). Our results suggest that marine sponges are broadly defended by deterrent compounds, since several sponge compounds deter feeding by a fish not present in the sponges' natural environment (Table 2). Because our data demonstrate that consumers from two very different habitats have the ability to detect some of the same deterrent compounds, suggesting that neither species has evolved resistance to these chemical defenses, sponge geographic distribution patterns may not be predominantly limited by predation pressure by generalist fishes.

Triterpene glycosides are found in a number of marine and terrestrial organisms as putative defenses (Kubanek *et al.*, 2002; Zhang *et al.*, 2006; Ukiya *et al.*, 2007). Therefore, there is likely a conserved mechanism for detecting these compounds among

predators and herbivores that encounter these potential food items. It is interesting to note that RAMPs seem to be common GPCR regulators in numerous tissue types and are found in many organisms (McLatchie *et al.*, 1998; Husmann *et al.*, 2000; Benitez-Paez, 2006; Parameswaran and Spielman, 2006), suggesting that they have a conserved purpose. Furthermore, a single RAMP has the capability to detect multiple types of ligands with great specificity, depending upon which GPCR it is associated with at any given time (Udawela *et al.*, 2004). Such a mechanism for detecting harmful compounds would be evolutionarily advantageous, as an organism would not need to expend unnecessary resources or evolve specific receptors for every possible compound that it may come in contact with. Using this flexible signaling mechanism, a vast number of specific compounds could be detected with a relatively small number of full-sized GPCRs, which physically combine in limitless permutations to form specific receptors, allowing an organism to easily detect and avoid potentially harmful compounds with as little energy as possible. Furthermore, since RL-TGR seems to be specifically expressed in fish, this co-receptor in fish may have co-evolved with the triterpene glycoside compounds found in their potential prey organisms.

Triterpene glycosides are closely related to steroidal and saponin compounds, which are found in a variety of marine and terrestrial organisms (Kubanek *et al.*, 2000; Kubanek *et al.*, 2001; Zhang *et al.*, 2006; Ukiya *et al.*, 2007; Xu *et al.*, 2007; Feng *et al.*, 2008; Fu *et al.*, 2008; Li *et al.*, 2008; Lin *et al.*, 2008; Nakamura *et al.*, 2008; Peng *et al.*, 2008; Yoshikawa *et al.*, 2008; Zhu *et al.*, 2008). Thus, RL-TGR may be a general detector of these classes of compounds, and potentially, other RAMP-like co-receptors may have evolved as detectors of other chemical defenses. Since RAMP homologues are

expressed in diverse organisms, such as fish and mammals (Benitez-Paez, 2006), and RL-TGR is conserved across freshwater and marine fish species (Part II) , these species may have evolved the same or homologous RAMP-like co-receptors for the detection of chemical defenses. That is, these co-receptors may have evolved through divergent evolution as a protection mechanism for predators, herbivorous/browsing animals, and other vertebrate consumers.

10.3 Future directions

This work opens many doors for future research. First, given that RL-TGR is a novel chemoreceptor-related protein that responds to a marine chemical defense compound, it may also be the first of a new family of RAMP-like co-receptors. Our study used bioinformatics to initially investigate this hypothesis. While a 40 bp portion of the gene encoding RL-TGR was found to be highly similar to the genomic sequence of multiple fish species, BLAST performed against the protein sequence failed to predict any other related protein sequences. This *in silico* investigation was limited by the amount of publicly available bioinformatic data and could indicate that proteins homologous to RL-TGR have yet to be identified in other organisms. To define whether RL-TGR is a member of a larger family of co-receptors, RT-PCR should be performed on zebrafish mRNA with degenerate primers that overlap conserved regions, such as the 40 bp sequence identified in Chapter 8.

Additionally, although the molecular mechanism of RL-TGR was initially investigated by the experiments reported in this dissertation, this mechanism needs further characterization. For example, an important goal is to define the role of β_2AR in

RL-TGR-mediated signaling in *Xenopus* oocytes. Does β_2AR affect function of RL-TGR, similar to how RAMP proteins affect GPCRs? Does it affect trafficking? Furthermore, if RL-TGR is indeed related to the RAMP family of co-receptors, then RL-TGR will be physically associated with another GPCR when expressed together in a cell system. Preliminary evidence from colocalization studies hint that these receptors were physically associated in transiently transfected HEK293 cells, when Strep-tagged RL-TGR and Flag-tagged β_2AR were co-expressed (Fig. 17). Discrete pockets of colocalization of these proteins were seen in the confocal images of these cells.

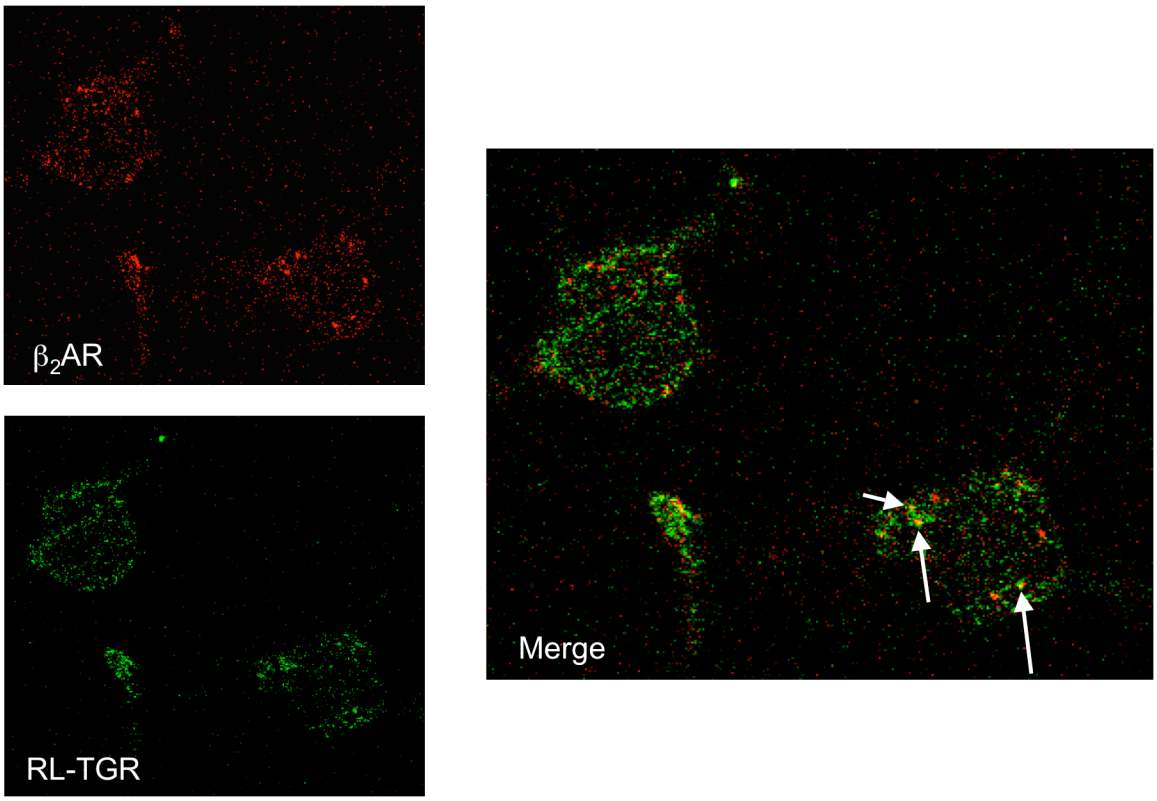


Figure 17. Colocalization of RL-TGR and β_2AR in HEK293 cells. HEK293 cells were seeded on glass coverslips and transiently co-transfected with plasmids encoding StrepII-tagged RL-TGR and Flag-tagged β_2AR. After two days, the cells were permeabilized, fixed, and probed with Flag antibody (red) and Strep-tag II antibody (green). Confocal images show that β_2AR (top panel) and RL-TGR (bottom panel) are co-expressed in discrete punctae in these cells. The merged image (right panel) shows evidence that some RL-TGR and β_2AR are colocalized (yellow; arrows) in these cells.

To confirm these preliminary data, co-immunoprecipitation (Co-IP) studies should be undertaken to determine whether both a GPCR and RL-TGR physically interact in cells expressing both proteins. Unfortunately, our preliminary colocalization experiments do not conclusively show that RL-TGR is expressed at the cell surface because the StrepII tag is internal; use of a plasma membrane marker or an external epitope tag would be beneficial in definitively showing localization. Additionally, pharmacological agents (inhibitors) or mutant G proteins/GPCRs/RL-TGR should be utilized to help define the signaling interaction of these proteins further.

Additionally, since all of the experiments in this dissertation utilized cells that overexpressed both receptors heterologously, this system may not be physiologically relevant. Thus, it is imperative to study the expression and interaction of these receptors in fish tissue, after the development of RL-TGR specific antibody. Moreover, it is important to note that RL-TGR is not expected to associate primarily with β_2AR in tissue, as β_2AR is generally expressed at low levels in chemoreceptive cells (Hague *et al.*, 2004). RL-TGR also appeared to have functional consequences upon interaction with another GPCR, more relevant to olfaction and taste. We hypothesize that an as yet unknown endogenous GPCR (or GPCRs) in native tissue associates with RL-TGR, forming a complex that responds to triterpene glycosides.

Furthermore, if this receptor does in fact underlie the mechanism of the aversive behavioral response in fish, it should be expressed in the taste or olfactory epithelium. Therefore, the tissue in which RL-TGR is expressed needs to be investigated *via* RT-PCR or *in situ* hybridization in zebrafish. It would be interesting to see whether expression of

this receptor is restricted to certain tissue types such as chemoreceptive tissue, suggesting a specific purpose for this receptor, or whether it is widely expressed, suggesting a ubiquitous role. As controversial work from Zucker and colleagues (Mueller *et al.*, 2005) suggests in mammals, the chemoreceptive cell type, not just the chemoreceptor type, which is activated in response to a compound may be an important factor in determining the subsequent behavioral output. Their work in rodents suggests that chemoreceptor cells are wired to the brain to evoke certain behavioral responses, aversive or attractive, in response to receptor activation, no matter the type of chemoreceptor expressed in the cell; sweet cells evoke positive behavioral responses, even when these cells have been engineered to express bitter receptors (Mueller *et al.*, 2005). However, many other groups do not show evidence of labeled-line coding (Sugita, 2006). Therefore, it would be of interest to investigate which coding model our research supports by determining whether a certain chemoreceptive cell type, such as sweet or bitter, expresses RL-TGR and is activated in response to triterpene glycosides. Moreover, is there another type of chemoreceptor expressed in these cells and with what kind of behavioral response is that chemoreceptor associated? All of these factors are important for downstream neuronal processing and would indicate whether the activation of RL-TGR specifically triggers the aversive behavioral response in fishes.

The behavioral response can also be tested by any or several knock-down approaches to determine whether this receptor underlies the aversive behavior; an example includes employing morphilinos in zebrafish. These 25-mer oligonucleotides bind to RNA and prevent expression of specific proteins, a technique that facilitates investigation of behavioral responses in the absence of certain receptors. By making use

of this molecular technique, the electrophysiological and behavioral phenotype of zebrafish that do not express RL-TGR can be characterized to show whether the signaling of this receptor mediates the aversive behavioral response to triterpene glycosides. Moreover, morpholino fish have other experimental advantages. While little is known thus far about the physiological consequences of deterrent compound ingestion on the survival and fitness of predators, these consequences can be easily assessed by studying morpholino zebrafish. Zebrafish lacking RL-TGR, which should lack the rejection response to triterpene glycoside compounds, could be fed triterpene glycoside-laced diets and then examined for the physiological effects. Alternatively, genetically engineered zebrafish can be made for the above studies by utilizing a new technique that employs zinc-finger nucleases to target inactivation of specific genes (Meng *et al.*, 2008), in this case RL-TGR. Moreover, either of these types of mutant fish could be utilized to examine the behavioral consequences of not expressing RL-TGR and could also enable the investigation of the loss of this co-receptor on a cellular level (*i.e.,* does it affect the trafficking or function of an endogenous GPCR?).

As the previously discussed proposed experiments illustrate, zebrafish are extremely advantageous as a molecular tool, and we chose to investigate the mechanisms of deterrent compounds in this model organism for this reason. However, we do not yet know whether RL-TGR, which was functionally identified from a zebrafish cDNA library, is also expressed fish that encounter prey defended by triterpene glycosides. Since previous studies demonstrated that bluehead wrasse, a generalist marine predator, can detect and reject triterpene glycosides and other marine sponge deterrent compounds (Chanas *et al.*, 1997; Assmann *et al.*, 2000; Kubanek *et al.*, 2000; Waddell and Pawlik,

2000; Duque *et al.*, 2001; Kubanek *et al.*, 2002; Pawlik *et al.*, 2002), this species is an ideal choice. PCR or southern blot analysis should reveal whether the RL-TGR gene is present in the bluehead wrasse genome, and RT-PCR and/or *in situ* hybridization should determine whether the gene is expressed.

Several compounds found in marine sponges were shown to be deterrent to zebrafish *via* our behavioral assay. Aside from formoside and a mixture of ectyoplasides A and B, these other compounds were not found to activate the zebrafish cDNA library or RL-TGR expressed in oocytes, nor were some other commercially available compounds that are known to be aversive, but not previously tested behaviorally on zebrafish. It would be interesting to test a broader range of aversive compounds, especially those known to cause a rejection behavioral response in zebrafish. For example, using a different behavioral assay, Oike and coworkers (Oike *et al.*, 2007) demonstrated that zebrafish were able to detect and reject denatonium, a compound also known to taste bitter to mammals. Given this finding, it would be interesting to test this aversive compound on oocytes expressing the whole zebrafish cDNA library or RL-TGR using our electrophysiological assay. Additionally, it is important to note that multiple fractions of the zebrafish cDNA library induced an electrophysiological response to formoside in our tests but were not further tested because of time restraints. Two likely possibilities underlie this observation: 1) there were multiple occurrences of the cDNA encoding RL-TGR in our screening process or 2) there are multiple receptors encoded within this library that are capable of responding to formoside with different affinities. To differentiate between the two hypotheses, PCR should be performed with RL-TGR specific primers on positive fractions to amplify any RL-TGR encoding cDNAs within

these fractions. Additionally, our electrophysiological assay should be employed to further screen the zebrafish library for additional receptors, by subfractionating the fractions that previously gave a positive response to formoside.

Although questioned by some investigators (Syed and Leal, 2008), recent data by Vosshall and colleagues (Ditzen *et al.*, 2008) suggested that the repellent compound DEET masked the detection of food. Given that possible mechanism of DEET, the conclusions drawn from our palatability behavioral assay that tested specific compounds for rejection in our study may be limited because we tested specific aversive compounds incorporated into a food matrix. It would be interesting to test these compounds in a neutral background to examine whether zebrafish responded negatively to deterrent compound or whether the deterrent compound inhibited the taste of food components.

10.4 Final conclusions

This interdisciplinary work crosses the boundaries of behavioral neuroscience, chemical ecology, and molecular biology, and unites fields that rarely overlap. The discovery of RL-TGR is significant not only because it defines a new chemoreceptor-ligand pair in a field where few of these interactions are known, but also because the gene encoding RL-TGR is the first identified that encodes a co-receptor which responds to a chemical defense compound. This finding may lead the way for the identification of many other receptors that mediate chemical defense signaling in both marine and terrestrial environments, as this protein has the potential to represent the first of an entire family of co-receptors that respond to aversive compounds. The further study of RL-

TGR and any related co-receptors will deepen our understanding of the molecular mechanisms of chemical defenses and their effect on predator-prey interactions.

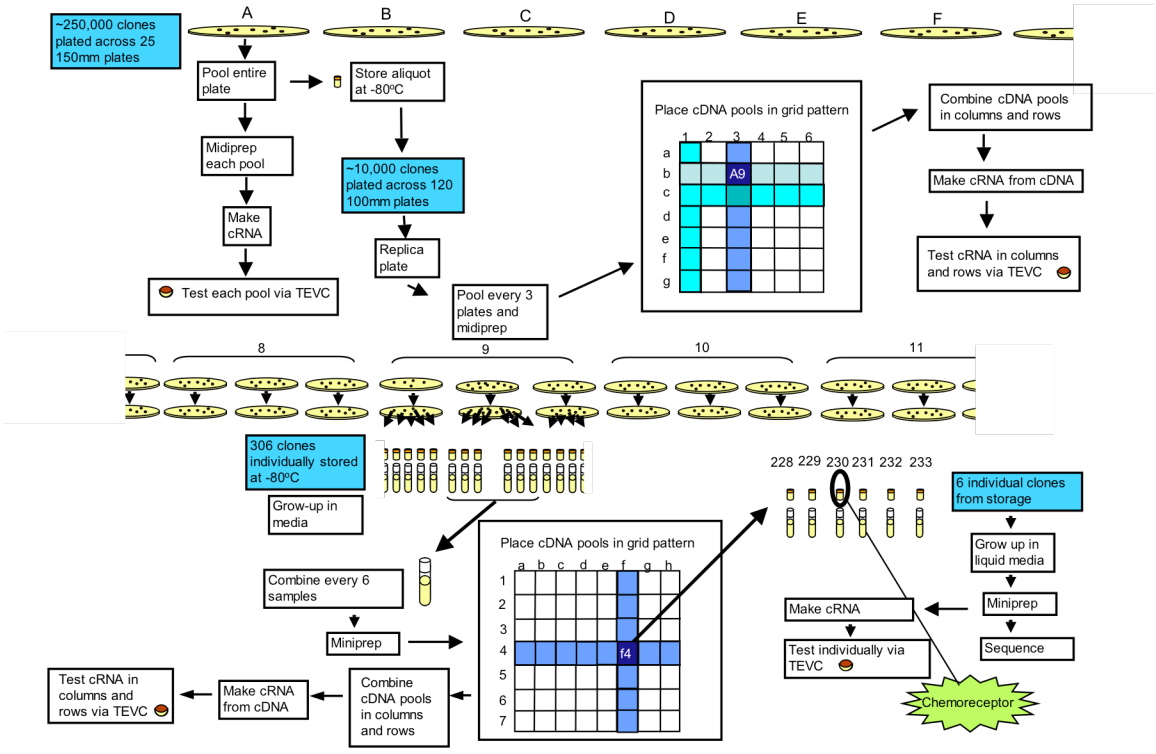


Figure 18. Schematic of bioassay-guided fractionation of a whole zebrafish cDNA library. A whole zebrafish cDNA library was screened for a clone encoding a protein responsive to formoside. Pools of bacteria containing library clones were plated across 25 150 mm LB agar plates containing ampicillin. An aliquot of the bacteria on each of these plates was stored, and the rest was midiprepped as a plate. The resulting cDNA was linearized with *Pac I* and transcribed into cRNA. These pools of cRNA were microinjected into *X. laevis* oocytes along with cRNA encoding the CFTR and β_2AR. These oocytes were tested *via* an electrophysiological bioassay, which utilizes TEVC to probe for an increase in CFTR current in response to formoside. The cDNA library clones corresponding to the cRNA- injected oocytes that responded to the application of formoside (fraction A) were sub-fractionated by replating the corresponding bacterial aliquot onto 120 100 mm LB agar plates containing ampicillin. These plates were replica plated with velveteen. The original plates were stored at 4^{o}C, and the cDNA clones from every three replica plates were miniprepped as a pool. Each cDNA pool was placed in a grid and aliquots were combined in columns and rows. The combined pools of cDNA were *in vitro* transcribed into cRNA, microinjected into oocytes, and tested *via* the bioassay for an electrophysiological response to formoside. The cDNA pool corresponding to where a positive column and row overlapped (A9) was linearized, *in vitro* transcribed into cRNA, microinjected into oocytes, and tested *via* the bioassay for an electrophysiological response to formoside. Turquoise columns and rows denote a weaker response in the bioassay than blue. 306 bacterial colonies from the original A9 plate were individually inoculated into 5 ml of LB broth containing ampicillin and

incubated overnight at 37^{o}C with shaking at 225 rpm. Aliquots from each growth were stored at -80^{o}C. Samples from every 6 growths were combined as a pool, miniprepped, and placed in another grid. These cDNA pools were combined in columns and rows, linearized, *in vitro* transcribed into cRNA, microinjected into oocytes, and tested *via* the bioassay for an electrophysiological response to formoside. As before, the cDNA pool corresponding to where a positive column and row overlapped (A9-f4) was linearized, *in vitro* transcribed into cRNA, microinjected into oocytes, and tested *via* the bioassay for an electrophysiological response to formoside. The bacteria corresponding to the six cDNA clones that comprise fraction A9-f4 were individually inoculated into LB broth containing ampicillin. The cDNA was miniprepped, linearized, *in vitro* transcribed into cRNA, microinjected into oocytes, and tested *via* the bioassay for an electrophysiological response to formoside. Parallel to these experiments, these six cDNA clones were sequenced. Clone A9-f4-230, which contains a novel cDNA insert, was positive *via* this bioassay and appears to encode a formoside-responsive protein.

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