MOLECULAR MECHANISMS BY WHICH SALVINORIN A BINDS TO AND ACTIVATES THE KAPPA-OPIOID RECEPTOR

by

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<table>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT&lt;sub&gt;2A&lt;/sub&gt;</td>
<td>5-hydroxytryptamine 2A (serotonin-2A) receptor</td>
</tr>
<tr>
<td>7TM</td>
<td>seven transmembrane</td>
</tr>
<tr>
<td>AC</td>
<td>adenylate cyclase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>β&lt;sub&gt;2&lt;/sub&gt;AR</td>
<td>β&lt;sub&gt;2&lt;/sub&gt;-adrenergic receptor</td>
</tr>
<tr>
<td>b&lt;sub&gt;n&lt;/sub&gt;</td>
<td>Bieman modified Roepstorff and Fohlman nomenclature peptide ion</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3', 5'-cyclic monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>cloned DNA</td>
</tr>
<tr>
<td>CHCA</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>CID</td>
<td>collision-induced dissociation</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter (10^-2 meter)</td>
</tr>
<tr>
<td>CNBr</td>
<td>cyanogen bromide</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DEA</td>
<td>Drug Enforcement Administration</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified essential medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOR</td>
<td>δ-opioid receptor</td>
</tr>
<tr>
<td>DTT</td>
<td>1,4-dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EL1</td>
<td>first extracellular loop</td>
</tr>
<tr>
<td>EL2</td>
<td>second extracellular loop</td>
</tr>
<tr>
<td>EL3</td>
<td>third extracellular loop</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FTICR</td>
<td>Fourier transform ion cyclotron resonance mass spectrometer</td>
</tr>
<tr>
<td>Gα</td>
<td>G protein alpha subunit</td>
</tr>
<tr>
<td>Gαs</td>
<td>alpha subunit of stimulatory G protein for adenylyl cyclase</td>
</tr>
<tr>
<td>Gαi</td>
<td>alpha subunit of inhibitory G protein for adenylyl cyclase</td>
</tr>
<tr>
<td>Gβ</td>
<td>G protein beta subunit</td>
</tr>
<tr>
<td>Gγ</td>
<td>G protein gamma subunit</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine-nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein-coupled receptor kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HEPES</td>
<td>4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
</tbody>
</table>
IL1  first intracellular loop
IL2  second intracellular loop
IL3  third intracellular loop
INRC  International Narcotics Research Conference
IP3  inositol-1,4,5-trisphosphate
IRES  internal ribosome entry site
JNK  c-Jun N-terminal kinase
kDa  kiloDalton
KOR  κ-opioid receptor
LSC  liquid scintillation counting
LSD  lysergic acid diethylamide
MALDI  matrix assisted laser desorption/ionization
MAPK  mitogen-activated protein kinase
MD  molecular dynamics
MOR  μ-opioid receptor
MS  mass spectrometry
MS/MS  tandem mass spectrometry
µg  microgram
µl  microliter
mg  milligram
ml  milliliter
mM  millimolar
MTS  methanethiosulfonate
MTSEA  2-aminoethylmethanethiosulfonate
MW    molecular weight
m/z   mass to charge ratio
NA    nucleus accumbens
NCBI  National Center for Biotechnology Information
NIDA  National Institute on Drug Abuse
nM    nanomolar
NMDA  $N$-methyl-D-aspartate
ORL1  opioid receptor-like (an orphan receptor)
PAGE  polyacrylamide gel electrophoresis
PBS   phosphate buffered saline
PCR   polymerase chain reaction
PDB   protein data bank
PDSP  Psychoactive Drug Screening Program
PI    phosphoinositide
PI3K  phosphatidylinositol 3 kinase
PIP$_2$ phosphatidylinositol biphosphate
PKA   protein kinase A
PKC   protein kinase C
PLC$\beta$ phospholipase $C\beta$
PMF   peptide mass fingerprinting
PTX   pertussis toxin
rpm   rotations per minute
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFU</td>
<td>relative fluorescence units</td>
</tr>
<tr>
<td>SA</td>
<td>sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid)</td>
</tr>
<tr>
<td>SCAM</td>
<td>substituted cysteine accessibility method</td>
</tr>
<tr>
<td>S. divinorum</td>
<td><em>Salvia divinorum</em></td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>S/N</td>
<td>signal to noise ratio</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
</tr>
<tr>
<td>vDW</td>
<td>van der Waals’</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>$y_n$</td>
<td>Bieman modified Roepstorff and Fohlman nomenclature peptide ion</td>
</tr>
</tbody>
</table>
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Salvinorin A, the most potent naturally-occurring hallucinogen, has gained great attention since the κ-opioid receptor (KOR) was identified as its principal molecular target (1). However, the molecular mechanisms by which salvinorin A — a small-molecule agonist — binds to and activates KOR was unclear. To understand these mechanisms, three aims were proposed for my dissertation research; correspondingly, I will report our findings in three parts (Chapter 3, Chapter 4 and Chapter 5) in this dissertation.

The primary goal (Chapter 3) is to identify the binding site of salvinorin A in KOR. A combination of site-directed mutagenesis and molecular modeling was applied to determine the structural features of KOR essential for the binding of Salvinorin A (2). Meanwhile, a series of naturally-occurring and synthetic salvinorin A derivatives was designed and assayed to compare their binding and functional properties (3-6).

The subsequent goal (Chapter 4) is to investigate KOR’s conformational change during the activation process. In this part of the dissertation research, over-expression of Gα16 and Gαi2 were used to increase the coupling ratio between KOR and the Gα subunits (7). The substituted cysteine accessibility method (SCAM), utilizing the specific reaction between the thiolate groups (-S⁻) and 2-aminoethylmethanethiosulfonate (MTSEA), was
applied to detect the conformational changes of the receptor (7). Intriguingly, these G protein-dependent conformational changes significantly increased the binding affinity of salvinorin A.

In PART III (Chapter 5), our goal is to further verify ligand-receptor interactions by designing a series of ligands capable of covalently binding to KOR. From our earlier work using the SCAM approach, we demonstrated that C3157.38 was both water accessible and highly reactive to methanethiosulfonate (MTS) reagents (7). Thus far, two compounds — RB-48 and RB-64 (both with pM potency and extraordinary selectivity for KOR) — have emerged as being suitable for affinity-labeling KOR. Our preliminary mass spectrometry data was consistent with C3157.38 as the labeling site.

Collectively, this research project has revealed the molecular mechanisms by which a small-molecule agonist selectively binds to and activates a Class A GPCR.
CHAPTER 1: Introduction

1.1 G Protein-Coupled Receptors

1.1.1 G Protein-Coupled Receptor Overview

G protein-coupled receptors (GPCRs) are membrane-spanning proteins that can detect extracellular stimuli and initiate intracellular responses. Human GPCRs are the largest receptor superfamily with roughly 1000 members, which are grouped into three major classes according to the authoritative list by the International Committee of Pharmacology Committee on Receptor Nomenclature and Classification (NC-IUPHAR) (8). This list is updated constantly on the IUPHAR website (www.iuphar-db.org). The representative receptors for these three major classes are rhodopsin (Class A), the secretin receptor (Class B), and the metabotropic glutamate receptors (Class C). The rhodopsin-like Class A is the largest and currently most studied. GPCRs can recognize highly distinct stimuli or ligands, including photons, odorants, small-molecules, peptides and proteins (9, 10). Consequently, GPCRs represent the largest known human gene-family and account for 4-5% of the human genome (11-13). Nearly 30% of the marketed prescription drugs target GPCRs (14). To understand how GPCRs transduce signals has been a major goal in life science for the last few decades (15).

1.1.2 G Protein-Coupled Receptor Structures

The defining structural feature of GPCRs is their seven transmembrane spanning domains (7TM) linked by three intracellular and three extracellular loops, which forms a helical
bundle. Therefore GPCRs are also referred to as 7TM or heptahelical receptors. This 7TM structure of GPCRs was first predicted by Unwin and Henderson in 1975 based on electron diffraction data obtained from bacteriorhodopsin (16). In 2000, this 7TM structure was validated by the first high-resolution crystal structure of a mammalian GPCR (bovine rhodopsin, resolution 2.80 Å, 1f88) (17, 18). In this structure, rhodopsin is tightly locked into the inactive state by the covalently-bound ligand, 11-cis-retinal. This inactive-state structure of rhodopsin provides a critical template for GPCR homology modeling and has led to a deeper understanding of all Class A GPCRs (10, 19-22). More recently, the structure of rhodopsin in the photoactivated intermediate state was also achieved (23). In 2007, the first high-resolution structure of a human GPCR (β2-adrenergic receptor (β2AR)-T4 lysozyme fusion protein, resolution 2.40 Å, 2rh1) was solved (24-27). To restrain the flexible internal loops of β2AR, the authors either engineered the receptor with a T4 lysozyme in the third intracellular loop or coupled it with an antibody. Although the overall structures are similar to rhodopsin, there are apparent differences in the second extracellular loop and the E/DRY interactions between the cytoplasmic ends of transmembrane TM3 and TM6. The highly conserved E/DRY motif is known to play a critical role in regulating GPCR conformations (28). Also significant helical shifts are observed as compared to rhodopsin. In the high-resolution structure (24, 25), β2AR binds to a diffusible ligand — carazolol. Interestingly, functional characterization of this engineered β2AR shows both the wild-type-like binding to an inverse agonist (carazolol), and an increased affinity for agonists, which is a profile similar to that of constitutively active mutants. The snapshot of these receptors is only the first step; future studies incorporating agonist-bound and G protein- or arrestin-
associated GPCR structures will provide further insights into the mechanisms of GPCR signaling.

1.1.3 G Protein-Coupled Receptor Function

GPCRs transmit signals from extracellular stimuli to intracellular G proteins through conformational changes, which demonstrate the allosteric nature and conformational plasticity of GPCRs (29, 30). The G proteins, composed of alpha (Gα), beta (Gβ), and gamma (Gγ) subunits, exist in a heterotrimeric complex when inactive. Signaling is initiated by ligand-GPCR (complex) interaction, which leads to conformational changes of the receptor and these structural changes promote the exchange of guanine-nucleotide on the Gα subunit. The ligand bound receptor promotes Gα to release the constitutively-bound guanosine diphosphate (GDP) and in turn bind to guanosine triphosphate (GTP). The GTP-bound Gα subunit then dissociates from the heterotrimeric G protein-complex, while Gβ and Gγ remain associated. Both the GTP-bound Gα subunit and Gβγ dimer can modulate a number of downstream effectors (Figure 1.1), including phospholipases (e.g., phospholipase C (PLC)) and nucleotide cyclases (e.g., adenylate and guanylate cyclases). These effectors will further produce second messengers (e.g., Ca^{2+} and 3’-, 5’-cyclic adenosine monophosphate (cAMP)) and eventually cause a series of biochemical and electrical events in the living cells (31-34).

GPCR signaling is precisely regulated by a series of cellular processes, including phosphorylation, desensitization, internalization, downregulation, recycling and degradation (35, 36). It is well-known that a long period of exposure to the ligands causes
GPCR desensitization. Usually phosphorylation is thought to be the first step of desensitization, followed by internalization and downregulation. Several protein kinases have been shown to phosphorylate the intracellular domains of GPCRs following agonist exposure, including the second messenger-dependent kinases such as protein kinase A (PKA) and protein kinase C (PKC), as well as specific G protein-coupled receptor kinases (GRKs) (37-41). In the classic model of β2AR signaling (Figure 1.2) (42), the binding of agonist to receptor results in the rapid phosphorylation of β2AR, which promotes the association of arrestin. The binding of arrestin to the receptor not only attenuates β2AR signaling due to the G protein uncoupling, but also induces internalization by clathrin-mediated endocytosis. The internalized GPCRs can traffic within the cell to the lysosome for degradation, or recycle to the plasma membrane in a process known as resensitization. An alternative pathway of GPCR internalization was also suggested to occur via caveolae (43-45).

Numerous theories have been proposed to explain the mechanism of GPCR activation and agonist effects (46-48). In the basic two-state receptor theory, only agonist and receptor were thought to play an active role for signaling; whereas G proteins are only considered to couple to GPCR after agonist-GPCR binding. However, in real biological systems, GPCR signaling is much more complex. Without the consideration of GPCR-accessory proteins, it is difficult to explain the multiple binding and signaling properties of GPCRs. Accumulating evidence suggests that at least some GPCRs are actually pre-coupled to G proteins, which allosterically affects ligand binding (29, 49, 50). Also there is evidence showing some GPCRs can signal without the participation of G proteins (51).
In addition, there are other challenges in understanding GPCR structure and function, for example, the existence and significance of homo- and/or heterodimers (21, 49, 52, 53), conformational plasticity (30, 54, 55) and functional selectivity (54, 56). Even with some spectacular advances in recent years, GPCR signaling is still far from being completely understood.

Figure 1.1 GPCR signaling. GPCR activated by an agonist transduces signal to the G protein, which promotes the exchange of GDP to GTP in Ga subunit and dissociation of Ga subunit from Gβγ. Both the GTP-bound Ga subunit and the Gβγ dimer can activate various effectors. Typically Ga_s stimulates adenylyl cyclase (AC) and increases cAMP level, whereas Ga_i inhibits AC and decreases cAMP level. Ga_q family activates PLC, which cleaves phosphatidylinositol biphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP3). The Gβγ dimer can activate many signaling molecules, such as phospholipases, ion channels and lipid kinases. [Modified from Figure 1 in Ref (57).]
Figure 1.2 GPCR signaling regulation based on β2AR model (42). Following receptor activation, GPCRs are phosphorylated by kinases, which lead to rapid desensitization due to G protein uncoupling. Then arrestin binds to the phosphorylated third intracellular loops and carboxy-terminal of the activated GPCRs. Desensitized receptors are internalized through clathrin-coated vesicles. These clathrin-coated vesicles can fuse with early endosomes where the receptors are dephosphorylated and recycled back to the plasma membrane or may be guided to lysosomes for degradation. [Modified from Figure 1.1 in Ref(58).]

1.2 Opioid Receptors

1.2.1 Opioid Receptor Overview

Opioid receptors belong to the rhodopsin-like Class A GPCRs (59, 60). Based on the early studies of ligand binding, opioid receptors were classified into three subtypes: µ-opioid receptor (MOR), δ-opioid receptor (DOR) and κ-opioid receptor (KOR) (61, 62). MOR and KOR were named after the prototypic drugs, i.e. morphine and ketocyclazocine respectively (63, 64). DOR (δ, for deferens) was named because it was discovered in guinea-pig ileum and mouse vas deferens (64). Later, an orphan receptor
(opioid receptor-like, ORL1) was placed within the opioid family because of a high degree of structural homology ( > 60%) to other opioid receptors (65). However, the opioid antagonist, naloxone, which binds to MOR, DOR and KOR receptors with differing affinities, does not have significant affinity for ORL1. From a pharmacological perspective, ORL1 is structurally related to the endogenous opioid systems, yet is pharmacologically distinct. All the discussions in this dissertation will focus on the main subtypes of opioid receptors, i.e., MOR, DOR and KOR. The nomenclature MOR, DOR and KOR are recommended by the International Narcotics Research Conference (INRC). There are several revisions of the terminology for opioid receptors in history. Originally opioid receptors were named by using the Greek letters µ, δ and κ, then the MOR, DOR and KOR recommended by INRC, and more recently using MOP, DOP and KOP by IUPHAR. Because of its wide acceptance in opioid field, the INRC version is used throughout this dissertation.

The human opioid receptor genes were discovered and cloned in the early 1990s (66-69). The opioid genes are located on three different chromosomes and all have multiple introns. Generally, opioid receptors are about 60% homologous, with the most conserved amino acids found in the transmembrane domains and intracellular loops (70). Subdivisions of the individual receptor subtypes (µ1, µ2, δ1, δ2, κ1, κ2 and κ3) were thought to exist because of the different pharmacological properties exhibited by ligands at the same receptor subtype (59). However, cloning efforts for these putative subdivisions have not confirmed any new genes. Several mechanisms could explain the subdivision-like pharmacological profiles: receptor splice variants, single nucleotide
polymorphisms (SNP), heterodimerization and interactions with different accessory proteins \((60, 71, 72)\).

Studies examining opioid receptor mRNA expressions have determined that all three classic opioid receptors are expressed throughout the central nervous system, peripheral sensory and autonomic nervous systems. Traditionally, opioids have been though to exert antinociceptive effects via the central nervous system (CNS). Now it is accepted that the opioid receptors’ action can also be peripheral and local. The presence of opioid receptors in dorsal horn and peripheral nerve terminals provides an opportunity to design peripheral analgesics without severe CNS side effects. In addition to the analgesia, opioid receptors can regulate many other physiological functions. MOR can regulate respiratory and cardiovascular functions, mood, thermoregulation, hormone secretion and immune functions \((73)\). KOR causes dysphoria with limited physical dependence liability, while euphoria, respiratory depression and physical dependence mainly involve MOR and DOR. Additionally MOR and KOR are found in the wall of the gastrointestinal tract \((74-77)\). KOR was also suggested to be expressed on CD4+ lymphocytes and monocular phagocytes, the major immune cell types infected by HIV, because of the suppression of HIV-1 expression by KOR selective agonists \((78)\). Data from opioid receptor knockout animals have confirmed many of these activities of opioid receptors \((70)\).

**1.2.2 Opioid Receptor Function**

Opioid receptors are prototypical \(\text{G}_{i/o}\) coupled receptors and thus pertussis toxin (PTX) sensitive. Opioid receptors are known to promiscuously couple to all \(\text{G}_i/\text{G}_{o}\) subtypes
and the PTX-insensitive Ga\textsubscript{z} protein. Like many other Ga\textsubscript{i}/Ga\textsubscript{o} coupled receptors, the opioid receptors can inhibit adenylyl cyclases and Ca\textsuperscript{2+} channels, and stimulate K\textsuperscript{+} channels. Lately the opioid receptors have been shown to regulate the mitogen-activated protein kinase (MAPK) cascade. The inhibition of adenylyl cyclases leads to the decrease of cAMP production, which reduces the level of neurotransmitter release by attenuating the activity of cAMP-dependent PKA (79, 80). There is accumulating evidence that the release of glutamate, \(\gamma\)-aminobutyric acid (GABA) and glycine throughout the CNS can be inhibited by opioid receptors (81). For example, KOR agonists are dysphoric because of the direct inhibition of dopamine release from nerve terminals in the nucleus accumbens (NA). Although the predominant action of opioids in the nervous system is inhibitory, in several brain regions important for either supraspinal analgesia (e.g. periaqueductal grey (PAG)) or euphoria (e.g. ventral tegmental area (VTA)) are excitatory. It is now accepted that opioid-induced excitations are due to a disinhibition mechanism (Figure 1.3), which is the inhibition of inhibitory neurotransmitters.

Other than the Ga subunit, opioid receptors also can signal through G\(\beta\gamma\) subunits (82) to cause the activation of K\textsuperscript{+} conductance, inhibition of Ca\textsuperscript{2+} channels and modulation of the N-methyl-D-aspartate (NMDA) receptors. It is suggested that opioid receptors can regulate the PLC\(\beta\) signaling pathway and Ca\textsuperscript{2+} mobilization via G\(\beta\gamma\), without involving Ga\textsubscript{q} (83). However, other mechanisms may still play a role; for example, both DOR and KOR were shown to stimulate PLC\(\beta\) by coupling to Ga\textsubscript{16} protein (84). The activation of MAPKs can also occur through the G\(\beta\gamma\) subunits (85). G\(\beta\gamma\) subunits primarily regulate the MAPKs through their effector-mediated pathways. It is very likely that opioid
receptors are able to mediate cellular responses by several mechanisms and the relative importance of each mechanism may depend on the cellular milieu.

**Figure 1.3** Opioid receptors regulate the dopaminergic activity in VTA through the disinhibition mechanism. Stimulation of opioid receptors reduces γ-aminobutyric acid (GABA) transmission, which increases the firing rate of dopaminergic neurons via disinhibition. [Modified from Figure 2 in Ref (86).]

1.2.3 Opioid Receptor Signaling Regulation
Opioid receptors are subject to agonist-activated phosphorylation by GRKs and activation of the arrestin-mediated endocytic pathway, as discussed previously (Figure 1.2, Section 1.1.3). The mechanism of desensitization is that agonist-activated receptors are first phosphorylated by GRKs, which then facilitates arrestin binding and prevents the receptor from coupling to G proteins (42, 87, 88). Arrestin-bound receptors are internalized and sorted in early endosomes and then may traffic to either lysosomes for downregulation or into endosomes for dephosphorylation and recycling, also known as resensitization. The major phosphorylation sites are in the carboxyl tail and intracellular loops of the opioid receptors. Besides GRKs, other protein kinases might be involved in the phosphorylation of the opioid receptors, such as ERK1/ERK2. Receptor internalization also appears to be agonist and receptor type dependent. An interesting example is the case of morphine and DAMGO; even though both are high affinity MOR agonists, DAMGO, but not morphine, can induce MOR phosphorylation (89). Recent studies have shown that opioid receptors, like some other G protein-coupled receptors, can form functional homo- or heterodimers (90, 91). It is still a challenge to characterize different forms of opioid receptor complexes, including the homo- and heterodimers.

1.3 Opioids

1.3.1 Endogenous Opioids

The term opioid encompasses all morphine-like agonists as well as endogenous opioid peptides. Opioid receptors are activated physiologically by their endogenous opioid peptides (Table 1.1). Endogenous opioids play a critical role in modulating pain, perception, mood, reward and some autonomic nervous system functions. Two closely
related endogenous opioids (Met-enkephalin and Leu-enkephalin) were first identified by Hughes et al. in 1975 [NOTE: The name of enkephalin is derived from the Greek enkephalos, meaning in the head]. The precursor protein, proenkephalin, gives rise to the endogenous opioids after proteolytic cleavage (59). Met-enkephalin and Leu-enkephalin have differential affinities for opioid receptors, from high affinity to low affinity in order of DOR>MOR>>KOR. Other precursor proteins and products are subsequently found and characterized (Table 1.1). Proopiomelanocortin generates β-endorphin, which is an agonist at both MOR and DOR, with little affinity for KOR. Prodynorphin can produce multiple dynorphin peptides (dynorphinA and B, α-neo-endorphin, and β-neo-endorphin), which have highest affinity for KOR but also bind to MOR and DOR. Endomorphin-1 and -2, whose precursor is still unknown, show high selectivity for MOR (92). All these peptides are full agonists for their cognate receptors, but none of them is exclusively selective (65) and none of these peptides have apparent affinity for ORL1. Similarly, the recently discovered ORL1 peptide agonist, nociceptin/orphanin-FQ, has no apparent affinity for MOR, DOR and KOR.

Table 1.1 Endogenous opioid peptides*

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Opioid peptide product</th>
<th>Amino acid sequence</th>
<th>Receptor type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proenkephalin</td>
<td>[Met]-enkephalin</td>
<td>YGGFM</td>
<td>MOR and DOR (93)</td>
</tr>
<tr>
<td></td>
<td>[Leu]-enkephalin</td>
<td>YGGFL</td>
<td>MOR and DOR (93)</td>
</tr>
<tr>
<td>Proopiomelanocortin</td>
<td>β-endorphin</td>
<td>YGGFM TSEKSQTPLVTLF KNAIKNAYK</td>
<td>MOR and DOR (94, 95)</td>
</tr>
<tr>
<td>Prodynorphin</td>
<td>dynorphin A</td>
<td>YGGFL RRIRPKLKWDNQ</td>
<td>KOR(96)</td>
</tr>
</tbody>
</table>
13

<table>
<thead>
<tr>
<th>Pronociceptin and orphanin-FQ</th>
<th>nociceptin and orphanin-FQ</th>
<th>FGGFTGARKSARKLANQ</th>
<th>ORL1(95, 97)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
<td>endomorphin-1</td>
<td>YPWF-NH₂</td>
<td>MOR (92)</td>
</tr>
<tr>
<td></td>
<td>endomorphin-2</td>
<td>YPFF-NH₂</td>
<td>MOR (92)</td>
</tr>
<tr>
<td>Prodermorphin and prodeltorphin</td>
<td>dermorphin</td>
<td>Y(D)AFGYP-S-NH₂</td>
<td>MOR (71)</td>
</tr>
<tr>
<td></td>
<td>deltorphin</td>
<td>Y(D)MFHLMD-NH₂</td>
<td>DOR (71)</td>
</tr>
</tbody>
</table>

*adapted from Table 1 in Ref (71) and Figure 3 in Ref (59)

1.3.2 Exogenous Opioids

Opium, the extract of the poppy plant, has been used medicinally for hundreds of years. In the early 1800s, the active pharmacological ingredient of the poppy plant was identified and named morphine after Morpheus, the Greek god of dreams (98, 99). Morphine and synthetic morphine-like alkaloids, MOR agonists, can effectively reduce severe pain. However, MOR agonists have severe side effects including respiratory depression, tolerance and dependence. The major goal of opioid research is to discover new analgesic drugs without all or some of those side effects, thereby avoiding opioid addiction. Two approaches have been applied to design and improve opioid ligands: 1) modifying morphine and 2) modifying endogenous peptides. The latter approach would also target nonpeptide peptidomimetics for better bioactivity and selectivity. After over 50 years of synthetic efforts, thousands of compounds based on natural alkaloid structures have been developed. Sadly, it has now become clear that analgesic potency and dependence liability are inseparable for MOR agonists. The progressive simplification of morphine structure is shown in Figure 1.4. From the morphinans, the
benzomorphans, the piperidines, to the phenylpropylamines, numerous compounds with unique properties are still widely used in the clinic (Table 1.2).

**Figure 1.4** Chemical backbones of morphine-derived molecules. [Adapted from Figure 4 in Ref (71) and Figure 4 in Ref (59).]

**Table 1.2** Select list of endogenous opioid peptides and morphine derivatives.

<table>
<thead>
<tr>
<th>Name and structure</th>
<th>Precursor</th>
<th>Receptor type</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>-</td>
<td>MOR</td>
<td>Selective agonist</td>
</tr>
</tbody>
</table>
Heroin

Morphine

Codeine

Morphine

Fentanyl

Piperidine

Sufentanil

Piperidine

Methadone

Phenylpropylamine

Enkephalin

DMAGO

Morphine

Morphine

Naloxone

MOR, DOR, KOR

Selective agonist

Selective agonist

Selective agonist

Selective agonist

Selective agonist

Selective agonist

Selective agonist

Selective antagonist
Naltrexone

Morphine MOR Non-selective antagonist

Tyr-D-Ala-Gly-Phe-D-Leu-OH
DADLE
Tyr-D-Ser-Gly-Phe-D-Leu-Thr-OH
DSLET
Tyr-D-Pen-Gly-Phe-D-Pen-OH
DPDPE

Enkephalin DOR Selective agonist

Tyr-D-Ala-Gly-Phe-D-Leu-OH
DADLE
Tyr-D-Pen-Gly-Phe-D-Pen-OH
DPDPE

Enkephalin DOR Selective agonist

TAN-67
Morphine DOR Selective agonist

Naltrindole (NTI)
Morphine DOR Selective agonist

N-Benzyl naltrindole (BNTI)
Morphine DOR Selective agonist
Arylacetamide  KOR  Selective agonist

Benzomorphan  KOR  Selective agonist

Naltrindole  KOR  Selective agonist

Naltrindole  KOR  Selective antagonist

Naltrindole  KOR  Selective antagonist
1.3.2.1 MOR Ligands

Thousands of morphine analogs have been synthesized, some representative compounds are shown in Table 1.2. Here is a brief introduction of some typical morphine-like compounds. Etorphine is a nonselective agonist for all opioid receptors and is a potent analgesic with severe side effects. Etorphine is only used for immobilizing large animals (100). Buprenorphine, a partial MOR agonist and a KOR antagonist, is a powerful analgesic for the treatment of moderate and severe pain (101). Nalorphine is an antagonist at the MOR and an agonist at KOR (102). Nalorphine is a strong analgesic with limited respiratory depression; however, it has dysphoric and psychotomimetic effects. Fentanyl, and analogues such as sufentanil and alfentanil, are among the most potent MOR agonists and widely used for surgical anaesthesia (60, 102). Another very important morphine derivative is methadone, which is widely used to treat opioids addiction and withdrawal (103). Naloxone and naltrexone are non-selective antagonists for all opioid receptors (104).

1.3.2.2 DOR Ligands

Most DOR ligands are tools for opioid research, having no medicinal use. Two selective DOR agonists, [D-Ala2, D-Leu5]-enkephalin (DADLE) and [D-Ser2, Leu5]-enkephalin (DSLET), are peptides. Conformationally constrained cyclic peptides such as [D-Pen2, D-Pen5]-enkephalin (DPDPE) is a potent DOR agonist (60). The nonpeptide selective DOR antagonist, naltrindole (NTI), is an analogue of naltrexone. NTI leads to the discovery of the benzyl derivative (N-benzynaltrindole (BNTI). These compounds are currently used as selective DOR antagonists. Other nonpeptide DOR agonists include
morphinan derivatives TAN-67. The benzylpiperazine derivative SNC-80 represents a new series of piperazine agonists (105). DOR agonists show both antinociceptive and antidepressant-like activities in animal behavior models (106, 107), although the proconvulsant effects limit their therapeutic use.

1.3.2.3 KOR Ligands

Similar to DOR ligands, KOR ligands are also mainly used for research; however, some novel KOR ligands show potential for medicinal applications (108). Chemically, KOR agonists can be categorized in five classes: the endogenous peptides (dynorphin), the benzomorphans, the arylacetamides (prototype U50488 and U69593), the benzodiazepine derivatives and lastly salvinorin A. The benzomorphan compounds, which include ethylketocyclazocine and bremazocine, show marginal KOR selectivity. They have been rejected from clinical development due to psychotomimetic and dysphoric effects, despite low dependence liability (109). Because it was thought that the severe side effects of opioid drugs were due to the lack of selectivity, there was some optimism in truly selective KOR agonists. However, this hope failed after testing a novel class of selective KOR agonists — the arylacetamide derivatives including U50488, U69593 and enadoline (110). Another similar compound, TRK-820, is a morphinan derivative with stronger analgesic effect. Recently, salvinorin A, the main active ingredient of Salvia divinorum (S. divinorum), was identified as a novel KOR agonist (1). Salvinorin A has no structural resemblance to any known opioid ligands (Table 1.2). In recent years, a number of KOR antagonists have been designed based on the DOR antagonist NTI (111). The bivalent NTI ligand, nor-binaltorphimine (nor-BNI), has been identified as a highly active and
selective KOR antagonist. Additional derivatives, such as 5’-GNTI and 6’-GNTI, have been designed based on nor-BNI. Because adverse CNS effects are associated with many KOR agonists, recent efforts have focused on peripherally acting agents (112-114).

1.3.3 Opioids’ Medical Use and Abuse

Opioids are the most effective analgesics (103, 115). Conventionally, opioids, such as the MOR full agonists (morphine, hydromorphone, oxycodone, hydrocodone, etc.), have been used to treat both moderate and severe pain. Although these drugs have their limitations, no other group of drugs has been found to provide better pain relief. To meet various clinical needs, both immediate-release (short-acting) and controlled-release (long-acting) opioids are produced. For postsurgical pain, short-acting opioids with duration of 2 to 4 hours are preferred. For example, the ultra-short-acting analogue of fentanyl, remifentanil, is rapidly metabolized by blood and tissue esterases with a half-life shorter than 10 min (116). Conversely, cancer pain, chronic and nonmalignant pain require long-acting opioids with a duration of 12-24 hours (117).

Opioid abuse has accompanied their medical uses since the beginning. Many opioids are on the controlled substance list of the Drug Enforcement Administration (DEA). For example, heroin is on schedule I, morphine and fentanyl are on schedule II. It is also well-known that opioid drugs cause tolerance and physical dependence. Of these three opioid receptors, MOR is responsible for the reinforcing effect of opioids. MOR agonists induce euphoria by indirectly enhancing dopamine in the NA. The mechanism is by inhibiting GABA release within the VTA (Figure 1.3), thus disinhibit the dopaminergic
neurons that project to NA. Similar to cocaine, amphetamine and nicotine, morphine can cause long-term potentiation (LTP) of glutamatergic transmission onto VTA dopaminergic neurons; however, the mechanisms are currently unclear (118). LTP is thought to underlie learning and memory and LTP induction by opioid drugs may explain opioid addiction. Other mechanisms could also play a role here, such as changes at the level of the opioid receptors (phosphorylation, desensitization, interactions with other receptors and G protein-uncoupling), the alteration of gene expression and changes in neuronal circuits (70). There is still controversy about the role of desensitization in morphine tolerance (72, 81, 87).

Another phenomenon associated with opioid dependence is the severe withdrawal effect after chronic opioid use. Biochemically, there is a down-regulation of MOR and an up-regulation of cAMP-PKA pathway. It is believed that withdrawal is caused by the enhancement of cAMP production and consequently increased neurotransmitter release (81). MOR agonists, such as methadone, are the main pharmacological treatments for opioid withdrawal. Methadone produces similar analgesia and side effects as morphine, but also has a long duration of action and slow elimination; meanwhile, it presents slow and relatively mild withdrawal effects (119, 120). The related compound, L-alpha-acetyl-methadol (LAAM), having a longer action than methadone, was approved by the FDA for opioid abuse treatments in 2002.

1.4 The History of Salvia Divinorum and Salvinorin A

Research
*S. divinorum*, or Diviner’s sage, is a powerful psychoactive herb belonging to the Lamiaceae (mint) family. *S. divinorum* was first recorded in print in 1939 (121), and introduced to western countries by Wasson and Hofmann in 1962 (122, 123). Before then it had been used as an ritual entheogen by Mazatec shamans for centuries (124). The origin of the *S. divinorum* is not very clear. A commonly accepted opinion is that *S. divinorum* is a cultigen created by Mazatec people, not occurring in the wild state. *S. divinorum* was used traditionally to produce a hallucinogenic experience essential for divination and healing. Over the past two decades, *S. divinorum* has been increasingly used for recreational purpose due to the easy access through the Internet. Some Internet-based companies sell live *Salvia* plants, dried leaves, as well as extracts. Even though *S. divinorum* has some hallucinogenic effects like lysergic acid diethylamide (LSD), it also differs from the common recreational drugs, such as LSD, marijuana or mushroom (containing psilocybin) (125). *S. divinorum* is legal in most countries and in the majority of states within the USA (www.erowid.org). In 2003 DEA placed *S. divinorum* on the list of drugs of concern. Many scientists who see the importance of salvinorin A in research support regulating *Salvia* as an entheogen rather than as a LSD-like hallucinogen (www.sagewisdom.org). Generally entheogen is a psychedelic substance used in a religious context to bring on a spiritual experience (126-128). It is of great interest to keep *Salvia* and its major active component — salvinorin A available in opioid research, which already showed great potential for medicinal applications and as a research tool to explore the function of opioid receptors.
The neoclerodane diterpene salvinorin A is the primary psychoactive component of *S. divinorum*. Salvinorin A was first isolated from *S. divinorum* leaves and chemically characterized in 1982 (129). Initial attempts in the 1990s to identify the molecular target of salvinorin A failed. Salvinorin A was submitted to the National Institutes of Mental Health sponsored Psychoactive Drug Screening Program (PDSP) in 2001 and screened against more than 50 targets including GPCRs, ion channels and transporters. Surprisingly, salvinorin A was found to only bind to KOR and not even its close family members MOR and DOR (1). Interestingly, it has no actions at the 5-HT2A receptor, the principal molecular target for classic hallucinogens such as mescaline and LSD. Neither does salvinorin A bind to NMDA and PCP sites. The most convincing proof that the KOR is the site of action is that the effects of salvinorin A are blocked by selective KOR antagonists in mice (130). The scientific research of salvinorin A accelerated after the discovery of KOR as the molecular target. Salvinorin A has been confirmed as a highly selective and potent KOR agonist with convincing proof both in vitro and in vivo (1, 125), some of which is shown in Table 1.3.

Salvinorin A is unique in its structure (Table 1.2) and function (Table 1.3). Unlike other known opioid-receptor ligands, salvinorin A is not an alkaloid — it does not contain a basic nitrogen atom (1, 131). Therefore, it represents the only known psychoactive diterpene and non-nitrogenous hallucinogen. Also, salvinorin A is the most potent naturally-occurring psychoactive compound with smoking doses as low as 200 µg. The effective dose of salvinorin A in humans approaches that of the synthetic hallucinogen LSD and 4-bromo-2,5-dimethoxy-phenylisopropylamine (DOB). In addition, the
antinociceptive effect of salvinorin A was examined in both regular and KOR knockout mice by tail-flick latencies or hot-plate assays. The antinociceptive effect diminishes within 30 minutes after intraperitoneal injections (IP) of salvinorin A (1-4 mg/kg) (132). Several publications investigated the analgesic effects of salvinorin A (133-135). As a KOR agonist, salvinorin A could also have dysphoric and depressive-like effects (136, 137). Depressant-like effect of salvinorin A was observed in a forced swimming test (137), which is a well-accepted rodent behavior model for the evaluation of antidepressant drug effects. IP injection of salvinorin A in rats increases immobility in the forced swim test. On the other hand, KOR antagonists could have antidepressant-like effects in vivo (138, 139). Additional studies in mice show that salvinorin A indeed decreases the dopamine level in the caudate putamen, but not in NA (130). These observations confirm that salvinorin A acts on KOR and provide a new treatment of depression by modulating KOR signaling pathways. Other therapeutic potentials of salvinorin A and related compounds are treating cocaine addiction and psychiatric disorders associated with hallucinations, such as schizophrenia and Alzheimer’s disease (108, 140-144). The toxicological study of salvinorin A showed no signs of organ damage in rodents after a two-week treatment (145).

Table 1.3 Pharmacological effects of salvinorin A in select animal studies*

<table>
<thead>
<tr>
<th>Effects</th>
<th>Dose*</th>
<th>Methods</th>
<th>Antagonist*</th>
<th>Animal Model</th>
<th>Drugs With Similar Effect</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decreases basal dopamine level in caudate</td>
<td>1.0 mg/kg and 3.2 mg/kg, IP</td>
<td>Microdialysis and HPLC</td>
<td>Blocked by nor-BNI, 10 mg/kg</td>
<td>(C57BL/6J)</td>
<td>U69593, U50488 and R84760</td>
<td>(130)</td>
</tr>
<tr>
<td>Effect</td>
<td>Dose/Conditions</td>
<td>Model</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>----------------------------</td>
<td>----------------</td>
<td>-----------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Induces place aversion and decreases locomotion</td>
<td>1.0 mg/kg and 3.2 mg/kg, IP</td>
<td>U69593, U50488 and TRK-820</td>
<td>(130)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkeys trained to discriminate U69593 from vehicle by food reinforcemnt</td>
<td>0.001-0.032 mg/kg, SC</td>
<td>Rhesus monkey</td>
<td>U69593</td>
<td>(146)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pharmacokinetic elimination $t_{1/2} = 56.6 \pm 24.8 \text{ min}$</td>
<td>0.032 mg/kg, IV</td>
<td>Rhesus monkey</td>
<td>-</td>
<td>(147)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose dependent and time dependent antinociception</td>
<td>0.5-4 mg/kg, IP</td>
<td>Nor-BNI 10 mg/kg</td>
<td>mice</td>
<td>-</td>
<td>(132)</td>
<td></td>
</tr>
<tr>
<td>Inhibits enteric cholinergic transmission</td>
<td>1 pM-1 µM, incubation with tissue</td>
<td>Nor-BNI, 30 nM and Naloxone 1 µM</td>
<td>Guinea-pig</td>
<td>-</td>
<td>(135)</td>
<td></td>
</tr>
<tr>
<td>Dose dependent antinociception</td>
<td>14-23 nmol, IT</td>
<td>Nor-BNI, 6.8 nmol; but not NTI and β-funaltrexamine</td>
<td>mice</td>
<td>-</td>
<td>(149)</td>
<td></td>
</tr>
<tr>
<td>Produces drug discrimination</td>
<td>1-3 mg/kg, IP</td>
<td>Nor-BNI, 4.5 nM, ICV</td>
<td>rats</td>
<td>U69593</td>
<td>(150)</td>
<td></td>
</tr>
<tr>
<td>Mixing stimulating and depressive effects</td>
<td>0.1-10 µg/kg, IM</td>
<td>Nor-BNI, 10 mg/kg; Rimonabant, 1 mg/kg</td>
<td>zebrafish</td>
<td>-</td>
<td>(151)</td>
<td></td>
</tr>
</tbody>
</table>
Dr. Bryan Roth’s lab is one of the major forces pushing the study of salvinorin A forward since its discovery of KOR as the molecular target in 2002 (1). Through the efforts of several research groups, we now have a better understanding of the actions of salvinorin A on KOR. Salvinorin A has also been proven to be a valuable research tool to study opioid receptors. The long term goal of salvinorin A research is to discover new therapeutic agents without the sedative and hallucinogenic effects. In the following chapters, a combination of molecular pharmacology, biochemistry, chemistry, computer modeling, mass spectrometry and animal behavior methods have been applied to the study of salvinorin A. Specifically, I have proposed three specific aims for my dissertation research: **specific aim 1** was designed to identify the binding site of salvinorin A in KOR; **specific aim 2** was designed to investigate KOR’s conformational change during the activation process; **specific aim 3** was to further verify salvinorin A - KOR interactions by designing a series covalently-bound KOR ligands based on salvinorin A’s structure. During the past four and half years, eight research papers (2-7, 152, 153) and one review paper (108) related to my work have been published in peer-reviewed journals. Step by step, the molecular mechanisms by which salvinorin A binds to and activates KOR were revealed. Correspondingly, I will report our findings in three parts (Chapter 3, Chapter 4 and Chapter 5) in this dissertation. The future direction of salvinorin A/KOR research will be discussed in chapter 6.
Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals

2.1.1.1 Commercial chemicals

Standard reagents were purchased from Sigma-Aldrich (St. Louis, MO). [\(^{3}\text{H}\)]diprenorphine (54.9 Ci/m mole), [\(^{3}\text{H}\)]U69593 (41.7 Ci/m mole) [\(^{3}\text{H}\)]DADLE (44.3 Ci/m mole), and [\(^{3}\text{H}\)]DAMGO (56.8 Ci/m mole) were purchased from PerkinElmer-LifeScience, Inc. MTSEA was obtained from Anatrace, Inc. In binding site study (2), two sources of salvinorin A were used: Biosearch and the S. divinorum Research and Information Center, Malibu, CA. For the conformational study (7), salvinorin A was kindly provided by Dr. Thomas Prisinzano (University of Iowa). Naloxone and U69593 were purchased from Sigma-Aldrich. Dynorphin A (1-13) was purchased from either Sigma-Aldrich or Bachem Bioscience, Inc.

2.1.1.2 Synthesized salvinorin A derivatives

This part of the work was done by collaborating with two medicinal chemistry labs, Dr. Jordan K. Zjawiony (University of Mississipi) and Dr. Mark A. Rizzacasa (The University of Melbourne, Australia). There are 55 salvinorin A derivatives have been tested in our lab (Table 3.6 in Chapter 3). The chemical syntheses and characterizations for half of these compounds have been published in recent papers (2-6), are not repeated here.
2.1.2  cDNA Constructs

2.1.2.1 Sub-cloning of hKOR into pUniversal-signal

Dr. Wesely Kroeze in Dr. Bryan Roth’s lab constructed a pIRES-neo (Clontech, Palo Alto, CA) based, membrane protein expression vector—pUniversal-signal(154). It contains a Kozak sequence (CACCATG) to enhance translation(155); an N-terminal hemagglutinin signal sequence to enhance the translocation of receptor to the plasma membrane; a FLAG epitope tag. FLAG-tagged human KOR was subcloned into the vector pUniversal-Signal. The presence of the correct KOR sequence was verified by automated dsDNA sequencing (Genomics Core facility, Case Western Reserve University) before use.

2.1.2.2 Generation of KOR mutants by site-directed mutagenesis

Regular Mutants — Mutations were introduced using the Quickchange mutagenesis kit from Stratagene according to the manufacturer's recommendations and verified by automated dsDNA sequencing. Most mutants are listed in Table 3.1 (Chapter 3).

Mutants for SCAM — Site-directed mutagenesis was based on the C315738S background of the human KOR in the vector pcDNA3.1 following the procedure of the Quickchange mutagenesis kit. About 40 mutants (TM7, TM6 and EL2) were made for the SCAM study (Figure 4.2, Chapter 4).

2.1.3 Cells

2.1.3.1 Transient transfection and cell membrane preparation
Cells (HEK 293T, Gα<sub>16</sub> and Gα<sub>i2</sub> Cells) were grown in 10 cm culture dishes in medium with 10% fetal calf serum in a humidified atmosphere consisting of 5% CO<sub>2</sub> and 95% air at 37 °C. Cells were transfected with either the wild-type, C315<sup>7.38S</sup> hKOR single mutant, or a double mutant hKOR DNA incorporating the C315<sup>7.38S</sup> mutation (12 µg/10 cm dish) using EasyTransgater (America Pharma Source). After a total of 48 hours of transfection, cells were harvested for experiments by detaching with Versene solution (Invitrogen). HEK 293T cells are a highly transfectable derivative of the HEK (human embryonic kidney) cell line, which has the temperature sensitive gene for SV40 T-antigen.

The transient transfected or stable cells were scraped and centrifuged at 1000g for 5 min, then cell membranes were lysed in standard binding buffer (50 mM Tris, 0.1 mM EDTA, 10 mM MgCl<sub>2</sub>, pH 7.4) and cell lysates were centrifuged at 14000g for 20 min to get membrane pellets. The membrane pellets were washed in the standard binding buffer and kept at -80 °C until use in binding assays.

2.1.3.2 Stable expression of G proteins

Stable cell lines expressing the human Gα<sub>16</sub> and Gα<sub>i2</sub> were obtained by transfecting the G protein expression vectors (pcDNA3.1, UMR cDNA Resource Center) into HEK 293 cells and selecting in 900 µg/mL G418. Those cell lines were maintained and transfected in 450 µg/mL G418. The expression of Gα<sub>16</sub> and Gα<sub>i2</sub> were characterized with anti-human Gα<sub>16</sub> and Gα<sub>i2</sub> polyclonal antibodies (Cell Sciences, Inc.).

2.2 Methods
2.2.1 Radioligand Binding Assays

2.2.1.1 Competition binding assay

The binding assay for KOR uses either \[^3\text{H}\]diprenorphine or \[^3\text{H}\]U69593 (PerkinElmer-LifeScienceInc) as radioligand. \(K_i\) determinations were performed by using 10 concentrations of unlabeled ligand spanning an appropriate dose range (~10^5 to 10^{-3} nM). All assays were conducted in triplicate or duplicate using polypropylene standard (8 x 12 format) 96 deep well (1 mL/well) plates as binding reaction containers. Reactions were in 250 µL and contained 175 µL of standard binding buffer, 25 µL of test drug or buffer (for total binding), 25 µL of \[^3\text{H}\]diprenorphine (0.15 ~ 0.25 nM final concentration), and 25 µL of membrane receptors. Nonspecific binding was defined by Naloxone at a final concentration of 10 µM (SigmaAldrich). The 96-well plates were incubated in the dark at room temperature for 90 min. Filters were presoaked in 0.3% PEI in 50 mM Tris buffer (4 °C, pH 7.4). The binding reaction was terminated by rapid filtration under vacuum by a Brandel Harvester (Brandel). Each well was washed three times with 0.5 mL of cold 50 mM Tris buffer (pH 7.4). Filters were dried and placed into 6.5 ml scintillation vials (Laboratory Products Sales Inc). To each vial, 4 ml Ecoscint A biodegradable scintillation solution (National Diagnostics) was added. After being capped, those scintillation vials were counted in a liquid scintillation counter (PerkinElmer-LifeScienceInc). Each vial was counted for about 2 min. The raw data were analyzed by Prism (GraphPad Software, Inc.) to generate \(K_i\) values reported as the mean ± standard error of the mean (SEM).

2.2.1.2 Saturation binding assay
Saturation binding was used to determine $K_d$ and $B_{max}$ Values for WT KOR and mutants. Membranes were prepared from transfected HEK 293T cells. Saturation binding of $[^3]H$diprenorphine was performed at eight concentrations of $[^3]H$diprenorphine ranging from 0.03 nM to 3 nM. Binding was carried out in standard binding buffer at room temperature for one hour in triplicate in a volume of 250 µL with about 30 µg of membrane protein. Naloxone (10 µM) was used to define nonspecific binding. Protein concentrations of membranes were determined by the Bradford protein assay method with BSA as the standard. Binding data were analyzed with Prism (GraphPad Software, Inc.).

2.2.2 SCAM

2.2.2.1 MTSEA reaction

Transfected cells were incubated with 2ml/plate Versene solution (Invitrogen) for 2 min, detached and pelleted at 1000g at 4 °C. After being washed with cold Kreb buffer (130 mM NaCl, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.3 mM CaCl$_2$, 1.2 mM MgSO$_4$, 10 mM glucose, and 25 mM HEPES at pH 7.4), the pellets were centrifuged and resuspended. The cell suspension was incubated with freshly prepared 1 mM MTSEA in 0.125 mL at room temperature for 5 min. The reaction mixtures were quenched by ice-cold 0.8% BSA in Kreb’s buffer, then pelleted and washed with regular cold buffer. After centrifugation, the pellets were resuspended and 100µL aliquots were used for $[^3]H$diprenorphine binding.

Rationale: The MTS reaction mechanism is a nucleophilic reaction (Fig 2.1), nucleophilic sulfhydryl group (SH or likely S$-$) will replace methanethiosulfonate group and build a
new disulfide bond. SCAM utilizes the specific reaction between SH and methanethiosulfonate reagents (MTS) to detect the secondary structure information of the receptor protein (Fig 2.1). After the chemical modification by MTS, an observable inhibition of the receptor function is taken as evidence for this reaction, for example the interruption of normal ligand-receptor binding or change of activity. Mutation affects surface expression and surface expression level does affect the SCAM result. This is the theoretical limitation of SCAM, because the inhibition is calculated by $B_{max}$ values (Equation 2.1). For this concern, we made Cys mutations for the whole helix 7 residues. And our conclusion was drawn based on the SCAM pattern changes of 23 consecutive residues rather than a few specific ones.

![Figure 2.1 Nucleophilic reaction between MTSEA and Cys side chain –SH (S').](image)

**2.2.2.2 SCAM radioligand binding assay**

The setup for the SCAM radioligand binding assay was similar to the above competition binding assay (section 2.2.1.1). However, each reaction contained 100 µL of standard binding buffer, 25 µL of naloxone at a final concentration of 10 µM (or buffer for total binding), 25 µL of $[^3]$Hdiprenorphine (0.2~0.4 nM final concentration), and 100 µL of membrane receptors. After counting, the raw data were analyzed by GraphPad Prism to
determine the inhibition number. The inhibition number was calculated using Equation 2.1(156):

\[
\text{Inhibition} = \left[1 - \frac{B_{\text{max}(\text{MTSEA})}}{B_{\text{max}(\text{NO MTSEA})}}\right]
\]  

(2.1)

where \(B_{\text{max}(\text{MTSEA})}\) is the specific binding after the MTSEA reaction and \(B_{\text{max}(\text{NO MTSEA})}\) is the specific binding without MTSEA. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by Dunnett’s post test (using \(p < 0.01\) as the level of significance, Figure 4.2 in Chapter 4).

### 2.2.2.3 Determination of second-order rate constants

The second-order rate constants of the reactions between the KOR Cys mutants and MTSEA was determined [estimated using a pseudo-first-order equation 2.2] to gain quantitative information on Cys sensitivity, according to the published method (157, 158). Cells expressing a KOR mutant receptor were incubated with indicated concentrations of MTSEA (mostly 0.01, 0.25, 1.0 and 2.0 mM) for 5 min. The results were fit to Equation 2.2:

\[
Y = Ne^{-kt} + \text{plateau}
\]

(2.2)

where \(Y\) is the fraction of the initial binding, \(N\) is the extent of inhibition, \(k\) is the second-order rate constant (M\(^{-1}\)s\(^{-1}\)), \(c\) is the concentration of MTSEA (M), \(t\) is the incubation time (300 s) and \(\text{plateau}\) is the fraction of residual binding at saturating concentrations of MTSEA. The data were analyzed by the built-in kinetic function of GraphPad Prism.

### 2.2.2.4 Ligand protection against the MTSEA reaction
This experiment is an extension of the MTSEA reaction as shown above (section 2.2.2.1). Only one step was added: detached cells were incubated with indicated concentrations of naloxone (or U69593) for 20 min before MTSEA reaction. The calculation of protection is using \( Protection = \frac{1 - B_{\text{max}}(\text{Naloxone+MTSEA})}{B_{\text{max}}(\text{MTSEA})} \) according to ref (156).

2.2.3 Functional Assays

2.2.3.1 Intracellular calcium mobilization (Ca\(^{2+}\) flux)

Functional assays using WT and mutant KORs were performed as previously detailed (159). Both the stable line rKOR Cell and mutant hKOR cells (co-transfected with Ga\(_{16}\) into HEK-293) were plated the night before the experiment at about 50,000 cells/well (100 µL/well of poly-lysine treated 96-well assay plates). Cells were incubated at 37 °C overnight. The regular media was changed to serum free and indicator free media at least one hour before loading reagent buffer. 1X Reagent loading buffer was diluted from 10X component B loading buffer with a final 2.5 mM probenecid (inhibitor of the anion-exchange protein) at pH 7.4 (by 1 M NaOH). One hour before assay, the serum free buffer was exchanged to dye-containing reagent buffer (30 µL/well) for 96-well assay plate. After incubating at 37 °C for one hour, another 30 µL/well loading buffer was added to the 96-well assay plate right before starting the assay. Drug compounds plate was prepared in advance with appropriate drug concentration gradient: 0, 1, 10, 30, 100, 1000, 10000 nM. Hydrophilic drugs were dissolved directly with loading buffer. Hydrophobic drugs were dissolved in DMSO first and diluted with loading buffer to the appropriate concentration. The 200µL 96-well tips (Molecular Devices Corp) and drug compounds plate (96-well polypropylene deep well plate 2.0mL) should be incubated to
37 °C before starting the FlexStationII to collect data. The samples were excited at 485 nm, and emission was detected at 525 nm. Initial volume was 60µL, and the pipettor height was set to 80 µL to transfer a volume of 30 µL at a rate of 1 (corresponds to a rate of 26 µL/second). Drug compound transfer was started after 20 seconds of background reading. Run time was set to 80 seconds to get 53 reads with a 1.52 seconds interval. The raw data were analyzed by GraphPad Prism give EC50 and E_max values.

2.2.3.2[^35S]GTPγS binding assay

Similar as the above binding membrane preparation (section 2.1.3.1), rKOR cells were detached in PBS, centrifuged at 1000g/10min, and washed with standard binding buffer. Fresh membrane pellets were used for[^35S]GTPγS binding assays. For each 96-well plate assay, 4-5 pellets (1 pellet from 1 10-cm dish) were needed. Ten different concentrations of test drugs in appropriate concentrations are made in the binding buffer (50 mM Tris·HCl, 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, pH 7.4). The setup of the[^35S]GTPγS assay is in 96 well sample plates (Wallac) designed for 1450 MicroBeta Counter (Perkin Elmer): 50 µl drug solution, 50 µl[^35S]GTPγS (PerkinElmer, 1250 Ci/mmol), 50 µl membrane plus 20 µM GDP (5 min incubation), incubating the mixture for 15 min, then 50 µl beads (FlashBlue, PerkinElmer, stock is 100 mg/ml, 110 µl stock solution/ plate). The plates were shaken for half an hour on Titer Plate Shaker (Lab-line Instruments, Inc.), spun down at 1000 rpm (~270g) for 2min, then counted with a MircoBeta Counter (PerkinElmer).

2.2.4 Computer Modeling
This part of the work was in collaboration with Dr. Richard Westkaemper (Virginia Commonwealth University). A short introduction is shown here, the details have been published in recent papers (2, 7, 160).

2.2.4.1 KOR Modeling for binding site (2)

Alignment of the rhodopsin and KOR sequences was performed manually by matching the highly conserved residues in transmembrane helices previously identified (161), (162). Amino acid side-chain geometries for the KOR receptor model were established from backbone-dependent libraries of rotamer preference using the program SCWRL (163). The PROTABLE facility within SYBYL was used to identify sites of unusual and sterically clashing side chain geometries that were interactively corrected as necessary. Model minimizations were performed using the Tripos Force Field, Gasteiger-Huckel charges with a distance-dependent dielectric constant = 4 and non-bonded cutoff = 8 Å to a gradient of 0.05 kcal/(mol·Å). The KOR model was further modified by minimization with an ensemble of known agonists docked into the receptor (164, 165). In order to explore the inherent flexibility of the EL2 loop and the effects of the EL2 loop in limiting ligand access to Y313, molecular dynamics (MD) simulations were performed. In preparation for the MD simulations, the N-terminus of the KOR was removed so as not to constrain the EL2 during the simulation. An MD simulation was performed for 100 ps using the default settings of the MD routine within SYBYL and maintaining all residues except the EL2 (Val195 to Asp223) as an aggregate to observe the behavior of the loop. An average KOR structure was then generated from individual MD conformations and was subsequently energy minimized. GOLD version 2.2 (166, 167) was used to dock
salvinorin A and 2-thiosalvinorin B into the MD-averaged and minimized KOR model and into the minimized MD snapshot model. Prior to docking, the CONCORD routine within SYBYL was used to assign initial conformations to salvinorin A and 2-thiosalvinorin B. A large active site radius of 25.0 Å about the Asp138 α-carbon atom was used to define the receptor site, thus allowing for the possibility of direct interaction of salvinorin A with distant residues, including Y313. The ligand-receptor complex was energy minimized to identify and resolve any remaining strain within the system.

2.2.4.2 KOR modeling for SCAM (7)

The hKOR model presented here was built using the coordinates of the recently obtained activated bovine rhodopsin crystal (‘B’ chain of PDB code = 2I37) (23) as the initial template. A loop search was performed to replace the missing three residues (A235 to Q237) in the IL3 of rhodopsin, and the IL3 (K231 to A241) was subsequently energy-minimized using the Tripos Force Field (Gasteiger-Hückel charges, distance-dependent dielectric constant = 4.0, nonbonded cutoff = 8 Å). The N- and C-termini of the hKOR (M1 to E33 and C322 to P327) were removed. Residues in the structurally conserved transmembrane helical and IL1 regions were mutated to their cognate residues in the hKOR. The rotated extracellular portion of TM2 was incorporated into the model by replacing residues L84 to Y102 in bovine rhodopsin with residues A106 to S123 from a previously described ‘TM2-rotated’ hKOR-salvinorin A interaction model(168). The importance of Q115 as a probable H-bonding interaction site for salvinorin A was incorporated into this ‘TM2-rotated’ hKOR model. Loop searches were then performed to replace the remaining bovine rhodopsin segments in the model with...
hKOR segments. The sequence was renumbered and SCWRL(169) was used to place the sidechains onto the hKOR model backbone.

In order to reproduce the characteristic features of the binding site in the previously described hKOR model(168) in which the agonist salvinorin A was bound, additional refinement of the model was carried out. First, in order to accommodate the ligand in the binding site, the EL2 was raised out of the binding cavity, enlarging it. This was accomplished by replacing the EL2 (S192 to W221) with the one from the previously described hKOR model(2, 168) in which molecular dynamics (MD) was used to enlarge the binding site cavity. Additionally, in order to reorient Y3137.36 and Y3207.43 for consistency with the previously-proposed salvinorin A-hKOR interaction model, the coordinates of the atoms in the extracellular portion of TM7 (L3097.32 to N3227.45) were replaced with the coordinates of the corresponding residues in the earlier hKOR model(168). Next, the sidechain conformations of key residues in the hKOR were modified to match those in the rhodopsin template, as these sidechains had been assigned conformations by SCWRL that differed significantly from those of the original bovine rhodopsin template. As a final step in the refinement of the hKOR, the agonist salvinorin A was placed into the binding site in a manner previously described (2), and the receptor-ligand complex energy-minimized without constraints. The stereochemical integrity of the final hKOR receptor model was verified by PROCHECK and the ProTable facility within SYBYL 7.3.
2.2.5 Large Quantity of KOR Expression and Purification

2.2.5.1 KOR expression

HEK293 T cells were grown in 15 cm culture dishes in medium with 10% FBS in a humidified atmosphere consisting of 5% CO₂ at 37 °C. To obtain large quantity of KOR protein for MS, cells were transfected with the WT KOR — pcDNA3.1(+) FLAG-KOR-His₆ (25 μg/15 cm dish), using Fugene 6 transfection reagent (Roche). This pcDNA3.1(+) FLAG-KOR-His₆ vector was made by Dr. Timothy A. Vortherms, a former postdoc in our lab. It contains an N-terminal FLAG tag and a C-terminal His₆ tag. The FLAG and His₆ tagged KOR was used for RB-64 labeling and subsequent mass spectrometric analysis. Another CHO cell line stably expressing hKOR without any tags was used for quantifying the RB-64 (a salvinorin A analog showed covalently-bound potential) and other drugs’ labeling ability. This CHO cell line was made by Joe Rittiner, a graduate student in our lab. The expression of KOR in these cells has been characterized by various radioligand binding. Our current efforts are making stable Flp-in HEK293 cell lines (Invitrogen) expressing WT FLAG-KOR-His₆ and mutants, which allow receptor proteins expression at a consistent level.

2.2.5.2 Labeling KOR with RB-64 or RB-48

After a total of 48 hours of transfection, cell media was removed and the transfected cells were washed by cold PBS, then the cells were labeled with RB-64 or RB-48 (5ml-10 μM/3hr for 10cm dish, 25 ml-20 μM/10hr for 15cm dish) in ice-cold PBS. Cells were
detached and centrifuged. Cell pellets were washed with standard binding buffer twice centrifuged at 15,000g for 20 min at 4 °C.

**2.2.5.3 Anti-FLAG M2 affinity purification**

The above cell pellets were resuspended in 1 ml TTSEC lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and 2% Triton X-100, pH 7.4, one tablet of protease inhibitor cocktail was freshly dissolved in 25 ml buffer). Membrane solubilization was completed by gently shaking and incubating on ice for 30 min. The membrane solution was centrifuged at 4 °C for 30 min at 15,000g to obtain a clear supernatant. The supernatant was collected and mixed with pre-washed and equilibrated anti-FLAG M2 affinity resin (#F2426, Sigma). For every two 15 cm dishes’ supernatant, 60 µl of 50% M2 affinity resin slurry was added. After 1 h of rotation at 4 °C, the suspension was centrifuged at 4 °C for 1 min at 800g, and the supernatant was removed. The resin was washed four times with TTSEC buffer and centrifuged in the same conditions as above. Then the resin was washed once again with TTSEC B buffer (50 mM Tris-HCl, 150 mM NaCl, 2% Triton X-100, pH 7.4). Following the final wash, KOR proteins were eluted with 100 µl of 200 µM FLAG peptide (#F3290, Sigma) in TTSEC B buffer for every two 15 cm dishes’ supernatant.

**2.2.5.4 Ni\(^{2+}\) affinity purification of KOR**

The above eluted supernatant was adjusted to contain 10 mM imidazole. And for every two 15 cm dishes’ supernatant, 20 ul of pre-washed, equilibrated Ni\(^{2+}\)-NTA Superflow
resin (Qiagen) was added. The resin was pre-washed four times with 1 ml of TTSEC B buffer containing 20 mM imidazole. The mixture was incubated for 2 hr at 4 °C with gentle agitation and centrifuged at room temperature for 1 min at 800g, and then the supernatant was removed. The resin was washed six times with 1ml TTSEC B buffer. His6-tagged KOR was eluted twice from the resin with 25 ul of TTSEC B containing 250 mM imidazole for 1 min at 4 °C. The elutions were combined in a 1.5 ml eppendorf tube and concentrated to about 10% of the original volume using Microncon centrifugal filter tubes (Millipore) with MW 10 kDa cutoff. The eluted KOR was collected and stocked in low retention tube.

2.2.5.5 SDS-PAGE and western blot analysis

Samples were treated in Laemmli sample buffer containing 1% β-mercaptoethanol at 65 °C with for 8 min. KOR proteins were resolved on 10% Tris-HCl gel. Proteins were transferred to nitrocellulose membrane (Bio-Rad), and the membranes were prepared for immunodetection following the general western blotting protocol. After 1 hr blocking in milk, the membrane was incubated for 1 hr at room temperature with corresponding primary antibodies (1:1000 dilution for anti-FLAG (#F7425, Sigma) and 1:250 for anti-KOR antibody (#44-302G, Biosource)). After washing three times with TSET buffer, the membranes were incubated in a 1:1500 dilution of peroxidase labeled anti-rabbit IgG-HRP (#PI1000, Vector). Protein bands were visualized using the Supersignal west pico chemiluminescent substrate (Thermo).
2.2.5.6 SDS-PAGE and Coomassie staining

Similar as the above SDS-PAGE procedure, concentrated KOR sample was treated in Laemmli sample buffer containing 1% β-mercaptoethanol and incubated at 65 °C for 8 min. KOR proteins were resolved on 4-20% Tris-Glycine gel (Invitrogen). The SDS-PAGE gel was stained with 0.12% R-250 Coomassie (Bio-Rad) in 10% acetic acid for 15 min on an orbital shaker at 40 rpm and destained with 10% acetic acid. The gel was saved in 1% acetic acid in a sealed plastic bag, and submitted to the proteomics center at UNC, Chapel Hill. All the above procedure should be done in a clean hood to avoid airborne contamination.

2.2.6 Mass Spectrometric Analysis

This part of the work was performed in collaboration with the UNC-Duke Michael Hooker Proteomics Center at UNC, Chapel Hill following their standard protocols (proteomics.unc.edu/protocol.shtml).

A brief introduction of MALDI-MS is provided here. Matrix assisted laser desorption/ionization (MALDI) and electrospray (ESI) are two soft ionization techniques that can ionize low volatility, high molecular weight molecules, such as proteins and peptides. MALDI was invented by two teams independently in 1988 (170). The organic compound, so called matrix, was used to absorb energy from a laser pulse and assist the ionization of big molecules (Equation 2.3). Proteins and peptides are ionized and detected predominantly in form — MH⁺. The ultraviolet or infrared laser requires matrix
molecules to have high UV or IR absorbance, such as benzoic and cinnamic acid derivatives. MALDI is often combined with a time-of-flight (TOF) mass analyzer. Ions are accelerated in an electric potential field, where the velocity of an ion is proportional to its \((m/z)^{1/2}\). A detector at the end of the flight tube will produce a signal when the ion strikes. The arriving time can be used to calculate the ion’s m/z ratio.

\[
\text{Matrix} + h\nu \rightarrow \text{Matrix}^+, \quad \text{Matrix}^+ + M \rightarrow (\text{Matrix} - H)^- + MH^+
\]

(2.3)

where M represents protein or peptide.

The detected peptide mass can be used to map the protein sequence and further determine the protein identification, so called “bottom up” MS. Peptide mass fingerprinting (PMF) is a technique to identify an unknown protein by matching the observed fragment peptide masses to the theoretical peptide masses generated from a protein database. The popular protein sequence databases include the National Center for Biotechnology Information (NCBI) and Swiss-prot database (Swiss-Prot). Mascot (www.matrixscience.com) is a search engine for protein identifications. PMF has been successfully used for post-translational modifications. Post-translational modification widely exists in biological systems, including glycosylation, phosphorylation, prenylation, acylation and proteolysis. In our study the modification is caused by covalently-bound-ligand labeling, a significant mass shift should be observed by mass analyzer.

### 2.2.7 Prepulse Inhibition Animal Study

This part of the work was collaborating with Dr. William C. Wetsel (Duke University).
Mice — Adult naïve male and female C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were used in all experiments. Animals were maintained under a 14:10 hr light/dark cycle in a humidity- and temperature-controlled room with water and laboratory chow supplied. All experiments were conducted in accordance with NIH guidelines and under an approved protocol from the Institutional Animal Care and Use Committee at Duke University.

Salvinorin A and RB-64 Disrupt Prepulse Inhibition (PPI) in C57BL/6J Mice — Mice were administered vehicle (10% Tween 80 in Milli-Q water; 8 ml/kg, i.p.) or one of four doses of salvinorin A(0.25, 0.5, 1.0, 2.0 mg/kg, i.p.) or RB-64 (0.005, 0.01, 0.05, 0.1 mg/kg, i.p.) immediately before placement into the PPI apparatus (Med-Associates). After 5 min acclimatization to 62 dB white-noise, animals were administered 84 test trials, beginning and ending with 10 trials each of startle-only stimuli (40 msec 120 dB white-noise burst). The remaining 64 trials were randomized between the following trial types: 8 startle-only trails, 8 trials without any stimuli (null trials), 16 trials with prepulse stimuli (4, 8, 12, and 16 dB above the 62 dB background, 4 of each intensity, 20 msec in length) that were not paired with startle stimuli (prepulse-only trials), and 32 trials of prepulse stimuli (4, 8, 12, and 16 dB above the 62 dB background, 8 at each intensity) paired with the 120 dB startle stimulus given 100 msec following the onset of the prepulse stimulus. Trials were separated by a variable interval (8-15 sec) and total test-time lasted 26-30 min for each animal.
Statistical analysis — The data were analyzed with SPSS 11 programs (SPSS Inc) and are presented as means ± standard error of the mean. Differences in treatment effects on null activity, baseline startle responses, and overall PPI were analyzed with ANOVA, with the main effects of dose nested within compound. Repeated measures ANOVA (RMANOVA) was used to examine the effects of salvinorin A and RB-64 on prepulse-dependent PPI, with prepulse intensity (4, 8, 12, and 16 dB) as the within subjects effect, and compound and dose as the between subjects effects (dose nested within compound). Differences between treatment groups were determined with Bonferroni corrected pairwise comparisons. In all cases, \( p < 0.05 \) was considered statistically significant.
Chapter 3: The Binding Site of Salvinorin A (Modified from Ref(2))

3.1 Introduction and Rationale

Salvinorin A is the only known non-nitrogenous opioid receptor agonist which is highly selective for KOR and has no significant activity at DOR, MOR or ORL1, nor other tested GPCRs, neurotransmitter transporters, or ion channels (1). Because of its unique structure and selectivity for a single Class A GPCR, the salvinorin A-KOR complex provides a model system for exploring the atomic features responsible for small-molecule selectivity among highly homologous receptors. However, the binding site of KOR is not well understood even though KOR has been identified as the molecular target of salvinorin A. Our primary goal for salvinorin A study is to identify its binding site in KOR.

Combined molecular modeling/mutagenesis studies can provide testable models for ligand interactions and activation mechanisms (171, 172) and these approaches have been used successfully for biogenic amine (173, 174) and peptide receptors (1). However, this combined method seems to work more successfully for ligands (either small molecule or peptidergic) which have ionic and hydrogen-bond-type interactions with conserved charged residues (e.g. Asp, Glu) or residues capable of forming hydrogen bonds (e.g. Tyr, Arg) for anchoring and orienting ligands in the binding pocket (see ref (171) for reviews). Since salvinorin A possesses no ionizable groups, ionic interactions can not provide stability in the binding pocket, although it is conceivable that hydrogen-bond and hydrophobic interactions could be involved. Salvinorin A is a triangle-like molecule with
three rotatable functional groups extending out its backbone, acetoxy group on C-2 position, methyl ester on C-4 position and furan group on C-12 position (Figure 3.1). Our initial hypothesis is that due to the hydrophobic nature of salvinorin A, all three functional groups of salvinorin A can interact with KOR residues through van der Waals’ forces and/or hydrogen-bonding. It is noticeable that all three groups are hydrophobic and contain a hydrogen acceptor — oxygen. To test this hypothesis, we designed mutants of KOR which lose either van der Waals’ forces or hydrogen bonds, or both (Table 3.1). By this means, we can separate van der Waals’ forces from hydrogen bonds, and have a clear view of how salvinorin A binds to KOR.

![Figure 3.1 The pharmacophore of salvinorin A. [Modified from Figure 5 in ref (2).]](image)

However, the limitation of mutagenesis is that the mutation itself may cause significant structural change of the receptor and affect the binding affinity, so other biophysical methods are required to validate the mutagenesis results. In order to fully understand the
mechanisms for how salvinorin A binds to and activates KOR, the research process will take several steps: 1) analyzing salvinorin A’s pharmacophore, and using computer modeling to predict the key residues that are believed to contribute in salvinorin A binding to KOR (i.e. Tyr313, Gln115, Tyr 312 and Tyr139); 2) make mutations of these residues and determine if these mutations affect salvinorin A binding and function by competition radioligand binding and functional studies (e.g. Ca$^{2+}$ mobilization); 3) predict the orientation of salvinorin A in the binding site by examining specific functional groups’ interactions with KOR, such as the interaction between substituted Cys and the free sulfhydryl group of a salvinorin A analog; 4) use all the experimental data as constraints to refine the docking/modeling and predict a comprehensive binding site; 5) re-examine KOR binding site through extensive salvinorin A’s SAR study; 6) re-examine KOR binding site by comparing with other structural studies, like X-ray crystallography, NMR, spin labeling and SCAM (will be discussed in Chapter 4); 7) Based on all the information obtained, novel salvinorin A analogs with special properties could be designed (as discussed in Chapter 5).

In this chapter, we will focus on the above steps 1-5. All the mutations of KOR residues made to identify the binding site are summarized in Table 3.1, including unpublished data and data from recent papers (for $K_i$ values see Table 3.2 and Table 4.6).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Mutation</th>
<th>Hypothesized Effects</th>
<th>Observed Effects On Binding</th>
<th>Ref</th>
<th>Binding Site Residue?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sal A</td>
<td>U69</td>
<td>Dyn A</td>
</tr>
<tr>
<td>Y139</td>
<td>A</td>
<td>Decrease of</td>
<td>Minor</td>
<td>Minor</td>
<td>NO</td>
</tr>
<tr>
<td>Protein</td>
<td>Amino Acid</td>
<td>Effect Description</td>
<td>E1</td>
<td>E2</td>
<td>E3</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Y312</td>
<td>A</td>
<td>Decrease of hydrophobic interaction and loss of hydrogen bond</td>
<td>Minor</td>
<td>Minor</td>
<td>NO</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>loss of hydrogen bond</td>
<td>Minor</td>
<td>Medium</td>
<td>NO</td>
</tr>
<tr>
<td>Y313</td>
<td>A</td>
<td>Decrease of hydrophobic interaction and loss of hydrogen bond</td>
<td><strong>Strong</strong></td>
<td>Medium</td>
<td>Minor</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>loss of hydrogen bond</td>
<td>Minor</td>
<td>Minor</td>
<td>NO</td>
</tr>
<tr>
<td>Y119</td>
<td>A</td>
<td>Decrease of hydrophobic interaction and loss of hydrogen bond</td>
<td><strong>Strong</strong></td>
<td>Medium</td>
<td>Minor</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>loss of hydrogen bond</td>
<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
</tr>
<tr>
<td>Y320</td>
<td>A</td>
<td>Decrease of hydrophobic interaction and loss of hydrogen bond</td>
<td><strong>Strong</strong></td>
<td><strong>Strong</strong></td>
<td>Minor</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>loss of hydrogen bond</td>
<td><strong>Strong</strong></td>
<td>Very Strong</td>
<td>Medium</td>
</tr>
<tr>
<td>Y66</td>
<td>A</td>
<td>Decrease of hydrophobic interaction and loss of hydrogen bond</td>
<td>Minor</td>
<td>Minor</td>
<td>Minor</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>loss of hydrogen bond</td>
<td>Minor</td>
<td>Medium</td>
<td>Minor</td>
</tr>
<tr>
<td>E209</td>
<td>M</td>
<td>Loss of ionic interaction</td>
<td>Minor</td>
<td>Minor</td>
<td>Minor</td>
</tr>
<tr>
<td></td>
<td>Q</td>
<td>Disrupt of ionic interaction and hydrogen bond</td>
<td>Minor</td>
<td>Minor</td>
<td>Minor</td>
</tr>
</tbody>
</table>
### 3.2 Results

#### 3.2.1 Receptor-based Binding Site Study

**3.2.1.1 Key residues in the binding site were identified by a combined mutagenesis/computer-modeling method**

We performed site-directed mutagenesis studies and characterized the binding and functional parameters of the mutated KORs. All of the mutants were expressed at high levels in HEK293 T cells and all had comparable affinities for the antagonist \[^{3}H\]diprenorphine (Table 3.2) and natural agonist dynorphin A (1-13), with the
exceptions of the Y139A (8-fold attenuation), Y119F and Y320A (6-fold attenuation each) and Y119A (4-fold attenuation). These results demonstrate that the mutations studied did not dramatically alter KOR receptor expression nor significantly perturb the topology of the receptor since binding for the high-affinity antagonist and endogenous agonist dynorphin were not greatly attenuated (e.g. < 10-fold change in affinities).

Table 3.2 Affinity (Kᵢ, nM) of salvinorin A, U69593 and dynorphin A (1-13) binding to the WT KOR and mutants transiently expressed in HEK293 T cells

<table>
<thead>
<tr>
<th></th>
<th>Kᵢ (nM)ᵇ</th>
<th>Sal A</th>
<th>U69</th>
<th>Dyn A</th>
<th>ratioᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>0.25</td>
<td>31.6±6.5</td>
<td>11.7±3.4</td>
<td>1.75±0.35</td>
<td></td>
</tr>
<tr>
<td>Y139A</td>
<td>0.26</td>
<td>53.9±13.9</td>
<td>20.6±4.0</td>
<td>1.98±0.41</td>
<td></td>
</tr>
<tr>
<td>Y139F</td>
<td>0.59</td>
<td>193±38</td>
<td>101±29</td>
<td>13.3±4.6</td>
<td></td>
</tr>
<tr>
<td>Y312A</td>
<td>0.55</td>
<td>88.6±10.9</td>
<td>52.6±11.3</td>
<td>1.79±0.28</td>
<td></td>
</tr>
<tr>
<td>Y312F</td>
<td>0.18</td>
<td>65.1±11.0</td>
<td>53.0±12.5</td>
<td>1.39±0.22</td>
<td></td>
</tr>
<tr>
<td>Y313A</td>
<td>0.72</td>
<td>694±106</td>
<td>107±32</td>
<td>4.10±0.78</td>
<td></td>
</tr>
<tr>
<td>Y313F</td>
<td>0.26</td>
<td>63.3±15.2</td>
<td>37.0±5.0</td>
<td>0.93±0.14</td>
<td></td>
</tr>
<tr>
<td>Y119A</td>
<td>0.19</td>
<td>342±40</td>
<td>59.9±2.4</td>
<td>6.71±0.89</td>
<td></td>
</tr>
<tr>
<td>Y119F</td>
<td>0.32</td>
<td>233±66</td>
<td>90.7±5.3</td>
<td>11.3±4.8</td>
<td></td>
</tr>
<tr>
<td>Y320A</td>
<td>0.92</td>
<td>380±103</td>
<td>195±34</td>
<td>3.15±0.82</td>
<td></td>
</tr>
<tr>
<td>Y320F</td>
<td>0.82</td>
<td>301±75</td>
<td>276±88</td>
<td>9.68±2.84</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Saturation binding of ³H-Diprenorphine to the wild type and the mutants was performed of two or three independent experiments. Data represent in Mean without ±SEM, modified from table 2 of Ref(2).
ᵇ The affinity constants (Kᵢ) of the different compounds were determined in competition binding assays with ³H-diprenorphine and increasing concentrations of unlabeled compounds. Each value is the mean of three or four independent experiments (see Chapter 2 for details).
ᶜ For each compound ratio is Kᵢ (mutant)/Kᵢ (wild-type).
In four out of ten of the studied KOR mutants, the affinity for salvinorin A was not significantly altered from WT KOR (Table 3.2). However, a dramatic decrease in the affinity of salvinorin A was obtained by introducing a single mutation, Y313A on TM7 ($K_i = 694$ nM, 22-fold decrease) — a smaller effect was seen for U69369 binding and no significant effect was seen for dynorphin A (1-13). To elucidate the molecular mechanism(s) responsible for salvinorin A’s selective interaction with Tyr313, we examined a Y313F mutation which should maintain hydrophobic interactions and abolish hydrogen-bond-type interactions between the –OH of Tyr313 and salvinorin A. Surprisingly the Y313F mutation caused no loss of the binding affinity, indicating that hydrophobic interactions between salvinorin A and Tyr313 provide stabilization of salvinorin A in the binding pocket.

Of the other tested mutations, the mutations at the Tyr119 and Tyr320 loci had significant effects while mutations at the Tyr139 locus had modest effects (<10-fold change) on salvinorin A’s affinity for KOR. It appears that the primary mode of interaction at Tyr119 and Tyr320 is via H-bonding: in each case the majority of the affinity was lost on the mutation from Tyr to Phe; little additional perturbation resulted from the Tyr to Ala mutation. The affinities of U69593 and dynorphin A (1-13) were, in general, only modestly affected by the various mutations, with exception of Y320A and Y320F. Interestingly, the single mutation which had the greatest effect on salvinorin A’s affinity (Y313A) attenuated U69593’s affinity 9-fold and had an insignificant effect on dynorphin A (1-13) affinity (Table 3.2).
We next determined the agonist potencies and efficacies of salvinorin A, U69593 and dynorphin A (1-13) at wild-type and mutant KORs (Table 3.3). As expected, the Y313A mutation significantly attenuated salvinorin A’s potency to activate KOR (6-fold), while the Y119A mutation decreased agonist potency 9-fold. Surprisingly, several other mutations attenuated salvinorin A’s potency for activating KORs: Y139A (5-fold) and Y312A (4-fold). These results were intriguing since Y139A and Y312A did not affect binding of salvinorin A to the KOR. The most severe effect was found by mutations Y320A and Y320F. They abolished agonist-induced activation of KOR by all three tested compounds: salvinorin A, U69593 and dynorphin A (1-13). Aside from the Y320 mutations, Y139A (3-fold) and Y119A (4-fold), dynorphin A (1-13) tolerated mutations without losing agonist efficacy. U69593 showed a dramatic loss of potency for activation for the Y312A (19-fold) and Y119A (15-fold) mutations. As with the other compounds, U69593’s agonist potency was diminished 6-fold by Y139A. In contrast to salvinorin A (6-fold), Y313A only has a 2-fold effect on U69593’s ability to activate KOR.

Table 3.3 Agonist potency (EC$_{50}$, nM)$^a$ and relative agonist efficacy (normalized E$_{max}$)$^a$ of salvinorin A, U69593 and dynorphin A (1-13) for the WT KOR and mutants transiently expressed in HEK293 cells.

<table>
<thead>
<tr>
<th>KOR</th>
<th>EC$_{50}$ (nM)</th>
<th>ratio$^c$</th>
<th>E$_{max}$</th>
<th>EC$_{50}$ (nM)</th>
<th>ratio$^c$</th>
<th>Relative E$_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SalA</td>
<td>U69</td>
<td>DynA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild Type</td>
<td>45.8±8.1</td>
<td>3232±728</td>
<td>23.6±6.4</td>
<td>1.13±0.19</td>
<td>343±54</td>
<td>1.53±0.26</td>
</tr>
<tr>
<td>Y139A</td>
<td>240±44</td>
<td>5</td>
<td>4893±795</td>
<td>140±38</td>
<td>1.13±0.29</td>
<td>1044±258</td>
</tr>
<tr>
<td>Y139F</td>
<td>47.0±22.2</td>
<td>1</td>
<td>5265±952</td>
<td>13.3±3.6</td>
<td>1.00±0.15</td>
<td>369±98</td>
</tr>
<tr>
<td>Y312A</td>
<td>189±46</td>
<td>4</td>
<td>4667±589</td>
<td>451±225</td>
<td>0.93±0.12</td>
<td>416±86</td>
</tr>
<tr>
<td>Y312F</td>
<td>68.3±22.1</td>
<td>1</td>
<td>4308±526</td>
<td>98.0±33.4</td>
<td>1.03±0.09</td>
<td>172±39</td>
</tr>
</tbody>
</table>

$^a$Values are means±SD.

$^c$Ratio of EC$_{50}$ or E$_{max}$ values for each KOR mutant relative to the WT KOR.
<table>
<thead>
<tr>
<th></th>
<th>EC50 (μM)</th>
<th>Relative Emax</th>
<th>EC50 (μM)</th>
<th>Relative Emax</th>
<th>EC50 (μM)</th>
<th>Relative Emax</th>
<th>EC50 (μM)</th>
<th>Relative Emax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y313A</td>
<td>275±53</td>
<td>6</td>
<td>5124±705</td>
<td>4</td>
<td>2</td>
<td>1.17±0.12</td>
<td>233±44</td>
<td>1</td>
</tr>
<tr>
<td>Y313F</td>
<td>49.6±15.6</td>
<td>/</td>
<td>5127±747</td>
<td>1</td>
<td>2</td>
<td>0.97±0.42</td>
<td>91±3.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Y119A</td>
<td>434±163</td>
<td>9</td>
<td>5004±718</td>
<td>4</td>
<td>15</td>
<td>0.80±0.26</td>
<td>1290±634</td>
<td>4</td>
</tr>
<tr>
<td>Y119F</td>
<td>43.5±14.7</td>
<td>/</td>
<td>3880±770</td>
<td>1</td>
<td>2</td>
<td>0.80±0.15</td>
<td>276±99</td>
<td>1</td>
</tr>
<tr>
<td>Y320A</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Y320F</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*EC50 and relative Emax were determined from calcium flux assays as described under Methods in Chapter 2. The results represent the average of three independent experiments with normalized Emax values in Mean ± SEM form. Modified from Table 3 of Ref(2).*

bND=no detectible agonist activity; mutants Y320A and Y320F have no response to salvinorin A, U69593 or dynorphin A (1-13).

c For each compound ratio is EC50(mutant)/EC50(wild-type).

### 3.2.1.2 Specific ligand-receptor interactions determine the orientation of salvinorin A in the binding site

To fully elucidate the orientation of salvinorin A, we examined the binding of 2-thiol salvinorin B at wt and Cys-substituted KOR mutants. We reasoned that Cys-substitutions should show enhanced affinity for 2-thionylsalvinorin if the Cys residue was in close proximity to the –SH moiety of 2-thiosalvinorin B. Since the KOR has a single Cys in the binding pocket (Cys315; (175)) we initially characterized C315S. As shown in Table 5, the C315S did not significantly alter the binding of either salvinorin A or salvinorinyl-2-thiol. Based on the salvinorin A binding model, we constructed several Cys substitutions in the background of C315S including Y119C, I294C, E297C, L309C, S310C and Y313C (Table 3.4) of which Ile294, Glu297, Leu309, Ser310 and Tyr313 are unique to KORs. As predicted by the salvinorin A binding model, the Y313C mutation preserved the affinity of 2-thiosalvinorin B because the –SH group is predicted to be in close proximity to the Cys substitution at the Y313 locus in the C315S background. In contrast, the affinity of salvinorin A for the Y313C-C315S double mutant decreased by 15.8-fold,
presumably because the 2-acetoxy group of salv inorin A cannot form interactions with C313 as effectively or as strongly as can 2-thiosalvinorin B. Additionally, I294 and E297, when mutated to Cys, gave rise to significantly enhanced affinities for both salv inorin A and 2-thiosalvinorin B.

**Table 3.4** Effect of Cys-substitution mutations\(^a\) on salv inorin A and 2-thiosalvinorin B binding to KORs.

<table>
<thead>
<tr>
<th>KOR</th>
<th>Salv inorin A</th>
<th>2-thiosalvinorin B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_i) (nM)</td>
<td>(K_i) (nM)</td>
</tr>
<tr>
<td>Wild Type</td>
<td>61 ± 23</td>
<td>161 ± 59</td>
</tr>
<tr>
<td>C315S</td>
<td>38 ± 18</td>
<td>202 ± 72</td>
</tr>
<tr>
<td>C315S-Y313C</td>
<td>596 ±41</td>
<td>261 ± 53</td>
</tr>
<tr>
<td>C315S-Y119C</td>
<td>178 ± 54</td>
<td>226 ± 49.5</td>
</tr>
<tr>
<td>C315S-I294C</td>
<td>8 ± 4</td>
<td>45 ± 5.8</td>
</tr>
<tr>
<td>C315S-E297C</td>
<td>2.5</td>
<td>37</td>
</tr>
<tr>
<td>C315S-L309C</td>
<td>16 ± 14</td>
<td>87 ± 66</td>
</tr>
<tr>
<td>C315S-S310C</td>
<td>49</td>
<td>261</td>
</tr>
<tr>
<td>C315S-Y320C</td>
<td>NA(^e)</td>
<td>NA(^e)</td>
</tr>
<tr>
<td>C315S-Y66C</td>
<td>85 ± 20</td>
<td>240 ± 25</td>
</tr>
</tbody>
</table>

\(^a\) The double mutants were based on C315 (7.38), which is conserved among the opioid receptor family and is differentially accessible (Ref(176)). Modified from Table 4 of Ref(2).

\(^b\) The affinity constants \((K_i)\) of the different compounds were determined in competition binding assays with \(^3\)H-diprenorphine and increasing concentrations of unlabeled compounds. Each value is the mean of three or two independent experiments (see Experimental Section for details).

\(^c\) For each compound ratio is \(K_i\) (mutant)\(\)\(/K_i\) (C315S).

\(^d\) For each compound ratio is \(K_i\) (salvinor A)\(\)\(/K_i\) (2-thiosalvinorin B).

\(^e\) NA=NO affinity

**3.2.1.3 Molecular modeling to visualize and predict the binding site**
By docking techniques (Chapter 2 for details), the complete binding site of salvinorin A was predicted. These studies were performed in collaboration with Dr. Richard Westkaemper (Virginia Commonwealth University) (2). The closest sites of interaction are between salvinorin A and TM1–3, TM6, TM7 and the extracellular loop 2 of the receptor model. In our model, the 2-acetoxy group of salvinorin A is positioned between the EL2 loop and the top of TM7 near Tyr313 and the furan ring hydrogen-bonds with Tyr320 in TM7 and Tyr119 in TM2. The 4-methyl ester group is oriented toward the top of TM6. All of the mutated residues in the current model point approximately toward the central cavity, with the exceptions of Leu309 and Ser210 while Tyr312 and Tyr313 are located more remotely near the top of the binding cavity. Table 3.5 lists the distances between heavy atoms of salvinorin A and nearby amino acid residues in the KOR. Mutations of some of the residues within a distance of 4 Å have been shown by others to affect the binding of conventional KOR ligands. These include Asp138, the putative ammonium-binding residue (177); Cys210, the EL2 loop disulfide-forming Cys (178) and Ile294, which has been implicated in a SCAM study (175). While present in the binding cavity, Tyr139 was not predicted to interact significantly with docked ligand.

<table>
<thead>
<tr>
<th>3.0Å</th>
<th>3.5 Å</th>
<th>4.0 Å</th>
<th>4.5 Å</th>
<th>5.0 Å</th>
</tr>
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<tbody>
<tr>
<td>NONE</td>
<td>I135</td>
<td>Y66</td>
<td>M112</td>
<td>Y139</td>
</tr>
<tr>
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<td>D138</td>
<td>Y119</td>
<td>A298</td>
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<td></td>
<td>E209</td>
<td>V134</td>
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<tr>
<td></td>
<td>C210</td>
<td>S211</td>
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<tr>
<td></td>
<td><strong>I294</strong></td>
<td><strong>L212</strong></td>
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<tr>
<td></td>
<td><strong>Y313</strong></td>
<td><strong>F214</strong></td>
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</table>

*Table 3.5 Residues within specified distances between the salvinorin A and the KOR receptor for the proposed binding mode. Receptor atoms may be part of the backbone or the side chain.*
Table 3.1

<p>| | | | |</p>
<table>
<thead>
<tr>
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<tr>
<td>I316</td>
<td></td>
<td>E297</td>
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<tr>
<td>Y320</td>
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<td>Y312</td>
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* Highlighted residues have been validated by mutagenesis study (table 3.1). [Modified from Table 1 of Ref(2)].

Figure 3.2 Modeling salvinorin A-KOR interactions. The proposed binding mode of salvinorin A in the KOR was looking through the extracellular side of the helical bundle. Tyr313 and Tyr320 in TM7 are nearest to the viewer. The KOR is color-coded based on assignment of secondary structure using the Kabsch-Sander (36) algorithm (red=helix, blue=beta sheet, violet=turn, yellow=coil). The ligand and important nearby residues are rendered as capped stick figures. [Modified from Figure 5c from ref(2).]

3.2.2 Ligand-based Binding Site Study

This part of the work was done by collaborating with two medicinal chemistry labs, Dr.
Jordan K. Zjawiony (University of Mississippi) and Dr. Mark A. Rizzacasa (The University of Melbourne, Australia). In this dissertation, I only present the data of the salvinorin A derivatives that have been synthesized by our collaborators and tested in Dr. Bryan Roth’s lab; for details please refer to the refs (179, 180). The covalently-bound salvinorin A compounds will be discussed in chapter 5.

This extensive SAR study was to identify key functional groups in the salvinorin A structure. All salvinorin A analogs were screened in competition radioligand binding assays with cloned opioid receptors (Table 3.6). Compounds with submicromolar affinity were also screened for functional activities using calcium mobilization (or Ca\(^{2+}\) flux) assay. The results suggest that the acetoxy group on C-2 position, methyl ester on C-4 and furan ring on C-12 are required for salvinorin A binding, while the lactone (C-17) and ketone (C-1) functionalities are not (5, 159). For all the terpenoids isolated from *S. Divinorum*, salvinorin A appears to be the only one to have activity at KOR, whereas other salvinorins show negligible affinity. Extending the alkyl chain and adding bulky aromatic groups to C-2 were found to diminish, if not totally abolish, the affinity for KOR (159). The acetoxy group is therefore the optimal alkyl chain length. Bioisosteres of salvinorin A were also developed and evaluated in binding and functional assays. Replacement of the oxygen with sulfur on C-2 produced the most similar analog of salvinorin A. The C-2 thioacetate isostere indeed produced comparable activity to salvinorin A, but nitrogen substitution had a diminishing effect. Intermediates, which lack a β-carbonyl at C-2 displayed moderate affinity (6). The difference in affinity of sulfur and nitrogen may be attributed to the presence of two lone pairs of electrons, just like
salvinorin A itself, in thioacetate which may play a role in receptor recognition. A hydrogen bond acceptor at C-2 may facilitate high affinity binding, but is not critical in receptor recognition when considering the affinities of intermediates. In fact, a C-2 methoxymethyl ether analog of salvinorin A was reported to have a 7-fold increase in potency for KOR using [3H]diprenorphine as the radioligand (179). It has also been shown that replacing the acetate group with a formate group lowers KOR affinity (5).

The stereoisomer derivatives of salvinorin A were also examined (4, 5). Sulfur analogs of salvinorin A and B with natural (α) and inverted (β) configurations at carbon C-2 were designed. Thiolanalogs with the same configuration at C-2 as in natural salvinorin A showed higher affinity to KOR than their corresponding epimers. The novel class of salvinorin A derivatives, hemiacetal, were recently tested. The dimethyl ester derivative of the hemiacetal was found to bind to both KOR and MOR with a 9-fold selectivity for KOR (3). However, after acetylation, compound 52 is practically devoid of affinity (Table 3.6).

<table>
<thead>
<tr>
<th>No.</th>
<th>Structure Derivative Name</th>
<th>Chemical Formula</th>
<th>Molecular Weight</th>
<th>Binding affinity ( Ki ), nM (Receptor, Hot ligand, Reference)</th>
<th>Functional activity ( Ec50 ), nM, Emax, (Receptor, Assay, Ref)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Salvinorin A</td>
<td>C23H28O8</td>
<td>432.46</td>
<td>( 4 \pm 1 ) (rKOR, ([3H]U69,(5))) 39.6 ± 14.5 (hKOR, ([3H]Dipre,(2))) 239439 (hDOR, ([3H]DADLE)) 274324 (hMOR, ([3H]DAMGO))</td>
<td>( 46 \pm 8 ) 100% (rKOR, (Ca^{2+}) flux, (5)) 17 ± 6 100% (rKOR, ([35S]GTP\gamma S))</td>
</tr>
</tbody>
</table>

Table 3.6 Salvinorin A derivatives tested in Dr. Bryan Roth’s lab.*
<table>
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<tr>
<th></th>
<th>Structure</th>
<th>8-epi-salvinorin A</th>
<th>C23H28O8</th>
<th>432.46</th>
<th>163 ± 50 (rKOR, [3H]U69,(5))</th>
<th>244 ± 102 78 % (hKOR, Ca(^{2+}) flux, (5))</th>
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<tbody>
<tr>
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<table>
<thead>
<tr>
<th></th>
<th>R=H</th>
<th>Salvinorin B</th>
<th>C21H26O7</th>
<th>390.43</th>
<th>8672 (rKOR, [3H]U69,(5))</th>
<th>Not active at 10 μM</th>
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<tr>
<td>3</td>
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<table>
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<tr>
<th></th>
<th>R=CHO</th>
<th>2-salvinorinylformate</th>
<th>C22H26O8</th>
<th>418.44</th>
<th>18 ± 2 (rKOR, [3H]U69,(5))</th>
<th>315 ± 35 108 % (hKOR, Ca(^{2+}) flux, (5))</th>
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<table>
<thead>
<tr>
<th></th>
<th>R1=R2=Ac</th>
<th>Salvinorin C</th>
<th>C25H30O9</th>
<th>474.50</th>
<th>1022 ± 262 (rKOR, [3H]U69,(5))</th>
<th>Not active at 10 μM</th>
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<table>
<thead>
<tr>
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<th>R1=H, R2=Ac</th>
<th>Salvinorin D</th>
<th>C23H28O8</th>
<th>432.46</th>
<th>&gt;10000 (rKOR, [3H]U69,(5))</th>
<th>Not active at 10 μM</th>
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<tbody>
<tr>
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<table>
<thead>
<tr>
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<th>R1=Ac, R2=H</th>
<th>Salvinorin E</th>
<th>C23H28O8</th>
<th>432.46</th>
<th>&gt;10000 (rKOR, [3H]Dipre, (5))</th>
<th>Not active at 10 μM</th>
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<tbody>
<tr>
<td>7</td>
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<table>
<thead>
<tr>
<th></th>
<th>R1=R2=Ac</th>
<th></th>
<th></th>
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<tr>
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</tr>
<tr>
<td></td>
<td><strong>Dihydrosalvinorin C</strong></td>
<td><strong>C25H32O9</strong></td>
<td><strong>476.52</strong></td>
<td><strong>1125 ± 365</strong></td>
<td><strong>(rKOR, [3H]U69,(5))</strong></td>
<td><strong>Not active at 10 μM</strong></td>
</tr>
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</tr>
<tr>
<td>9</td>
<td><strong>R1=Ac, R2=H</strong></td>
<td><strong>Dihydrosalvinorin E</strong></td>
<td><strong>C23H30O8</strong></td>
<td><strong>434.48</strong></td>
<td><strong>156 ± 18</strong></td>
<td><strong>(rKOR, [3H]U69,(5))</strong></td>
</tr>
<tr>
<td>10</td>
<td><img src="image1.png" alt="Structure 10" /></td>
<td><strong>13,14,15,16-tetrahydrosalvinorin A</strong></td>
<td><strong>C23H32O8</strong></td>
<td><strong>436.50</strong></td>
<td><strong>59 ± 11</strong></td>
<td><strong>(rKOR, [3H]U69,(5))</strong></td>
</tr>
<tr>
<td>11</td>
<td><img src="image2.png" alt="Structure 11" /></td>
<td><strong>17-salvinorinyl lactol</strong></td>
<td><strong>(predominantly β anomer)</strong></td>
<td><strong>C23H30O8</strong></td>
<td><strong>434.48</strong></td>
<td><strong>6 ± 1</strong></td>
</tr>
<tr>
<td>12</td>
<td><img src="image3.png" alt="Structure 12" /></td>
<td><strong>17-deoxysalvinorin A</strong></td>
<td><strong>C23H30O7</strong></td>
<td><strong>418.48</strong></td>
<td><strong>6 ± 2</strong></td>
<td><strong>(rKOR, [3H]U69,(5))</strong></td>
</tr>
<tr>
<td>13</td>
<td><img src="image4.png" alt="Structure 13" /></td>
<td><strong>8,17-didehydro-17-deoxysalvinorin A</strong></td>
<td><strong>C23H28O7</strong></td>
<td><strong>416.46</strong></td>
<td><strong>6 ± 2</strong></td>
<td><strong>(rKOR, [3H]U69,(5))</strong></td>
</tr>
<tr>
<td></td>
<td><img src="https://example.com/structure1.png" alt="Chemical Structure" /></td>
<td><strong>18-hydroxysalvinorin A</strong>&lt;br&gt;C22H28O7&lt;br&gt;404.45</td>
<td><strong>347 ± 53</strong>&lt;br&gt;(rKOR, [3H]U69,(5))</td>
<td><strong>&gt;10000</strong>&lt;br&gt;Antagonist-like&lt;br&gt;(hKOR, Ca(^{2+}) flux, (5))</td>
<td></td>
<td></td>
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<tr>
<td>14</td>
<td><img src="https://example.com/structure2.png" alt="Chemical Structure" /></td>
<td><strong>1-deoxysalvinorin A</strong>&lt;br&gt;C23H30O7&lt;br&gt;418.48</td>
<td><strong>18 ± 2</strong>&lt;br&gt;(rKOR, [3H]U69,(5))</td>
<td><strong>141 ± 43</strong>&lt;br&gt;122%&lt;br&gt;(hKOR, Ca(^{2+}) flux, (5))</td>
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<tr>
<td>15</td>
<td><img src="https://example.com/structure3.png" alt="Chemical Structure" /></td>
<td><strong>2α-salvinorinyl thiol</strong>&lt;br&gt;C21H26O6S&lt;br&gt;406.49</td>
<td><strong>157 ± 40</strong>&lt;br&gt;(hKOR, [3H]Dipre, (2))&lt;br&gt;54.5 ± 25.7&lt;br&gt;(rKOR, [3H]U69, (4))</td>
<td><strong>287 ± 85</strong>&lt;br&gt;89%&lt;br&gt;(hKOR, Ca(^{2+}) flux,(4))</td>
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<td></td>
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<tr>
<td>16</td>
<td><img src="https://example.com/structure4.png" alt="Chemical Structure" /></td>
<td><strong>2β-salvinorinyl thiol</strong>&lt;br&gt;C21H26O6S&lt;br&gt;406.49</td>
<td><strong>151 ± 53</strong>&lt;br&gt;(rKOR, [3H]U69, (4))</td>
<td><strong>123 ± 30</strong>&lt;br&gt;106%&lt;br&gt;(hKOR, Ca(^{2+}) flux,(4))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td><img src="https://example.com/structure5.png" alt="Chemical Structure" /></td>
<td><strong>2α-salvinorinyl thioacetate</strong>&lt;br&gt;C23H28O7S&lt;br&gt;448.53</td>
<td><strong>18.4 ± 7.9</strong>&lt;br&gt;(rKOR, [3H]U69, (4))&lt;br&gt;8 ± 1&lt;br&gt;(rKOR, [3H]U69,(6))</td>
<td><strong>4.77 ±2.72</strong>&lt;br&gt;107%&lt;br&gt;(hKOR, Ca(^{2+}) flux,(4))</td>
<td></td>
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<tr>
<td></td>
<td>Structure</td>
<td>Formula</td>
<td>rKOR, [3H]U69,</td>
<td>hKOR, Ca²⁺ flux</td>
<td>Notes</td>
<td></td>
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<tr>
<td>19</td>
<td><img src="image" alt="2β-salvinorinyl thioacetate" /></td>
<td>C₂₃H₂₈O₇S</td>
<td>546 ± 140</td>
<td>&gt;2000</td>
<td>71%</td>
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<tr>
<td></td>
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<td>(rKOR, [3H]U69, (4))</td>
<td>(hKOR, Ca²⁺ flux,(4))</td>
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<tr>
<td>20</td>
<td><img src="image" alt="Salvinorinyl-2-chloride" /></td>
<td>C₂₁H₂₅ClO₆</td>
<td>608 ± 103</td>
<td>3347 ± 1115</td>
<td>76%</td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td>(rKOR, [3H]U69, (6))</td>
<td>(hKOR, Ca²⁺ flux,(6))</td>
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<tr>
<td>21</td>
<td><img src="image" alt="Salvinorinyl-2-trichloroacetate" /></td>
<td>C₂₃H₂₅Cl₃O₈</td>
<td>375 ± 42</td>
<td>1012 ± 244</td>
<td>101%</td>
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<td></td>
<td></td>
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<td>(rKOR, [3H]U69, (6))</td>
<td>(hKOR, Ca²⁺ flux,(6))</td>
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<tr>
<td>22</td>
<td><img src="image" alt="Salvinorinyl-2-trifluoroacetate" /></td>
<td>C₂₃H₂₅F₃O₈</td>
<td>211 ± 37</td>
<td>825 ± 204</td>
<td>103%</td>
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<td>(rKOR, [3H]U69, (6))</td>
<td>(hKOR, Ca²⁺ flux,(6))</td>
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<tr>
<td>23</td>
<td><img src="image" alt="Salvinorinyl-2-bromide" /></td>
<td>C₂₁H₂₅BrO₆</td>
<td>261 ± 67</td>
<td>2704 ± 480</td>
<td>59%</td>
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<td>(rKOR, [3H]U69, (6))</td>
<td>(hKOR, Ca²⁺ flux,(6))</td>
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<tr>
<td>24</td>
<td><img src="image" alt="2-salvinorinylamide" /></td>
<td>C₂₃H₂₉NO₇</td>
<td>86 ± 22</td>
<td>78 ± 1</td>
<td>87%</td>
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<td>(rKOR, [3H]U69, (6))</td>
<td>(hKOR, Ca²⁺ flux,(6))</td>
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<td>25</td>
<td><img src="image" alt="2-salvinorinylazide" /></td>
<td>C₂₁H₂₅N₃O₆</td>
<td>140 ± 28</td>
<td>&gt;4000</td>
<td>Antagonist-like</td>
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<td>(rKOR, [3H]U69, (6))</td>
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<td>(hKOR, Ca²⁺ flux,(6))</td>
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<td>26</td>
<td><img src="image" alt="Salvinorinyl-2-propionate" /></td>
<td></td>
<td>32.63</td>
<td>4.7</td>
<td>100%</td>
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<td>(rKOR, [3H]Bremazocine, (159))</td>
<td>(hKOR, cAMP, (159))</td>
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<tr>
<td>No.</td>
<td>Formula</td>
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<td>Affinity</td>
<td>Activity</td>
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<td>27</td>
<td>C24H30O8</td>
<td>446.49</td>
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<td>Not active at 10 μM</td>
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<td>28</td>
<td>C26H34O8</td>
<td>474.54</td>
<td>&gt;10000</td>
<td>(rKOR, [3H]Bremazocine, (159))</td>
<td>Not active at 10 μM</td>
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<td>29</td>
<td>C24H30O9</td>
<td>462.49</td>
<td>&gt;10000</td>
<td>(rKOR, [3H]Bremazocine, (159))</td>
<td>Not active at 10 μM</td>
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<td>C24H27Cl3O9</td>
<td>565.82</td>
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<td>31</td>
<td>C28H38O8</td>
<td>502.60</td>
<td>3199</td>
<td>(rKOR, [3H]Bremazocine, (159))</td>
<td>40 34% (hKOR, cAMP, (159))</td>
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<tr>
<td>32</td>
<td>C24H33NO6</td>
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<td>&gt;10000</td>
<td>(rKOR, [3H]U69)</td>
<td>Not determined</td>
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<td>No affinity</td>
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<td>34</td>
<td>C21H27NO6</td>
<td>389.44</td>
<td>391 ± 71</td>
<td>(rKOR, [3H]U69)</td>
<td>Not determined</td>
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<td>35</td>
<td>C25H32O8</td>
<td>460.52</td>
<td>17.2 ± 3.2</td>
<td>(rKOR, [3H]U69)</td>
<td>Not determined</td>
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</tr>
<tr>
<td>No.</td>
<td>R3=</td>
<td>Molecular Structure</td>
<td>EC50 (μM)</td>
<td>(rKOR, [3H]U69)</td>
<td>(rKOR, [3H]Dipre)</td>
<td>(rKOR, [35S]GTPγS)</td>
</tr>
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<tr>
<td>36</td>
<td>2-salvinorinyl valerate&lt;br&gt;C26H34O8&lt;br&gt;474.54</td>
<td>103 ± 26</td>
<td>(rKOR, [3H]U69)</td>
<td>Not determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>2-salvinorinyl hexanoate&lt;br&gt;C27H36O8&lt;br&gt;488.57</td>
<td>103 ± 30</td>
<td>(rKOR, [3H]U69)</td>
<td>Not determined</td>
<td></td>
<td></td>
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<tr>
<td>38</td>
<td>RB-64&lt;br&gt;C24H27NO8S&lt;br&gt;489.53</td>
<td>0.59 ± 0.21&lt;br&gt;39 ± 11</td>
<td>(rKOR, [3H]U69)&lt;br&gt;(rKOR, [3H]Dipre)</td>
<td>0.077 ± 0.016&lt;br&gt;(rKOR, [35S]GTPγS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>RB-65&lt;br&gt;C24H30O9&lt;br&gt;462.48</td>
<td>20 ± 1&lt;br&gt;430 ± 50</td>
<td>(rKOR, [3H]U69)&lt;br&gt;(rKOR, [3H]Dipre)</td>
<td>Not determined</td>
<td></td>
<td></td>
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<tr>
<td>40</td>
<td>αβ-chloro-propionyl&lt;br&gt;-salvinorin (RB-55)&lt;br&gt;C24H29ClO8&lt;br&gt;480.93</td>
<td>22 ± 10&lt;br&gt;190 ± 70</td>
<td>(rKOR, [3H]U69)&lt;br&gt;(rKOR, [3H]Dipre)</td>
<td>Not determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>RB-59&lt;br&gt;C24H27NO8&lt;br&gt;457.47</td>
<td>74 ± 34&lt;br&gt;1010 ± 200</td>
<td>(rKOR, [3H]U69)&lt;br&gt;(rKOR, [3H]Dipre)</td>
<td>Not determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>RB-55-1&lt;br&gt;C24H29ClO8&lt;br&gt;480.93</td>
<td>30 ± 15&lt;br&gt;270 ± 50</td>
<td>(rKOR, [3H]U69)&lt;br&gt;(rKOR, [3H]Dipre)</td>
<td>Not determined</td>
<td></td>
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<tr>
<td>43</td>
<td>RB-55-2&lt;br&gt;C24H29ClO8&lt;br&gt;480.93</td>
<td>58 ± 32&lt;br&gt;4290 ± 2920</td>
<td>(rKOR, [3H]U69)&lt;br&gt;(rKOR, [3H]Dipre)</td>
<td>Not determined</td>
<td></td>
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<tr>
<td>44</td>
<td>RB-65&lt;br&gt;C24H30O9&lt;br&gt;462.48</td>
<td>1970 ± 890</td>
<td>(rKOR, [3H]U69)</td>
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<tr>
<td></td>
<td>Molecular Structure</td>
<td>Comments</td>
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</tbody>
</table>
| 45 | ![Image](image) | RB-66  
C23H26Cl2O8  
501.35  
3.43 ± 1.50 X 10^{-5}  
2.10 ± 0.84  
(rKOR, [^3H]U69)  
32 ± 15  
(rKOR, [^3H]Dipre)  
0.19 ± 0.01  
102%  
(rKOR, [^35S]GTPyS) |
| 46 | ![Image](image) | RB-48  
C23H27ClO8  
466.91  
1.46 ± 0.86  
(rKOR, [^3H]U69)  
Not determined |
| 47 | ![Image](image) | RB-50  
C23H27BrO8  
511.35  
Being tested  
Not determined |
| 48 | ![Image](image) | LP-0511  
C24H29ClO8  
480.94  
0.27 ± 0.24  
(rKOR, [^3H]U69)  
41 ± 8  
(rKOR, [^3H]Dipre)  
Not determined |
| 49 | ![Image](image) | 2-acetylamino-4-salvinorinylamide  
C22H28N2O6  
416.47  
224.0 ± 84.0  
(rKOR, [^3H]U69)  
Not determined |
| 50 | ![Image](image) | 2-amino-4-salvinorinylamide  
C20H26N2O5  
374.43  
143.3 ± 26.9  
(rKOR, [^3H]U69)  
Not determined |
<table>
<thead>
<tr>
<th></th>
<th>Structure</th>
<th>Description</th>
<th>Formula</th>
<th>Molecular Weight</th>
<th>(rKOR, [3H]U69)</th>
<th>(hMOR, [3H]DAMGO)</th>
<th>Notes</th>
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<td>51</td>
<td><img src="image" alt="Hemi-acetal" /></td>
<td>Hemi-acetal</td>
<td>C20H26O8</td>
<td>394.42</td>
<td>NA for KOR and MOR</td>
<td>Not determined</td>
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<td>52</td>
<td><img src="image" alt="Diester" /></td>
<td>Diester</td>
<td>C22H30O8</td>
<td>422.47</td>
<td>219±59</td>
<td>Not determined</td>
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<td>53</td>
<td><img src="image" alt="Acetate" /></td>
<td>Acetate</td>
<td>C24H32O9</td>
<td>464.51</td>
<td>6003 ± 1242</td>
<td>Not determined</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td><img src="image" alt="Hydroxyester" /></td>
<td>Hydroxyester</td>
<td>C22H30O8</td>
<td>422.47</td>
<td>1991±70</td>
<td>Not determined</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td><img src="image" alt="Acetylated hemi-ketal" /></td>
<td>“Acetylated hemi-ketal” (RB-58)</td>
<td>C24H32O9</td>
<td>464.51</td>
<td>6000 ± 1200</td>
<td>Not determined</td>
<td></td>
</tr>
</tbody>
</table>

* For unpublished data, there is no reference listed.
3.3 Discussion

In the mutagenesis studies, we identified key residues in KORs responsible for the high binding affinity and efficacy of salvinorin A. Surprisingly, we discovered that salvinorin A was stabilized in the binding pocket by interactions with tyrosine residues in helix 7 (Tyr313 and Tyr320) and in helix 2 (Tyr119). By contrast, the prototypical nitrogenous KOR agonist U69593 and the endogenous peptidergic agonist dynorphin A (1-13) showed differential requirements for these three residues for binding and activation.

These results are important because they demonstrate that salvinorin A’s exquisitely potent and efficacious interactions with KORs are due to novel modes of binding within a common three-dimensional space shared by structurally diverse agonists, each of which utilizes different residues for binding and activating KORs. We also employed a novel approach whereby we examined the effects of Cys-substitution mutagenesis on the binding of salvinorin A and an analogue with a free sulfhydryl group — salvinorinyl-2-thiol. We discovered that residues predicted to be in close proximity — especially Tyr 313 — to the free thiol of salvinorinyl-2-thiol when mutated to Cys showed enhanced affinity for 2-thiosalvinorin B. Taken together, these findings imply that the diterpenoid salvinorin A utilizes unique residues within a commonly shared binding pocket to selectively activate KORs.

In the salvinorin A binding model described here, the mutated residues point toward a central cavity, although some are clearly less sterically accessible to bound ligands — particularly Tyr312 and Tyr313. Neither Y312A nor Y312F mutation affects salvinorin A’s affinity for the KOR. This is consistent with the proposed model, which indicates
only a weak interaction with Tyr312. Interestingly, the Y313A mutation has a large effect on affinity (22-fold) while Y313F has no significant effect. The results of the Y313A and Y313F mutations are consistent with Tyr313 stabilizing the ligand via a hydrophobic-type interaction and with our docked salvinorin A model which predicts a direct hydrophobic interaction with Tyr313. Though potentially accessible to small molecules, Tyr313 was positioned in a more remote area at the top of the binding cavity interacting with residues in the EL2. Tyr313 was previously proposed to provide a hydrogen bond to the 2-acetoxy carbonyl of salvinorin A based on docking studies performed using a de novo model developed by Mosberg’s group (181). The current model that is based explicitly on the experimental rhodopsin crystal structure shows a substantial difference in the disposition of Tyr313. The fact that Y313F has no effect on ligand affinity but Y313A reduces affinity by 22-fold has compelled us to modify our original model wherein we proposed that Tyr313 interacted with salvinorin A primarily via hydrogen-bonding type interactions. Based on our current findings, we propose that a hydrophobic interaction is more likely. In the proposed salvinorin A binding model, the 2-acetoxy group of salvinorin A provides the requisite hydrophobic interaction with Y313, so that salvinorin A retains affinity for the Y313F mutation, but loses affinity (22-fold decrease) for the Y313A mutation.

Mutations at other residues also had significant effects on the binding of salvinorin A. Although accessible, the closest Tyr139 side chain-ligand distance is at least 4 Å away — consistent with the weak effect of either Tyr139 mutation. In our proposed binding model, it is possible for the 17-oxo group to form a weak hydrogen bond with Tyr139;
the donor-hydrogen-acceptor (D-H-A) angle is roughly 130° when the ligand carbonyl oxygen-Tyr139 side chain oxygen distance is 3.0 Å. However, in order for this hydrogen bond to be formed, the Tyr139 \( \tau_1 \) torsion angle must assume values that give rise to higher-energy eclipsed conformations, consistent with the modest 6-fold increase in the \( K_i \) observed for the Y139F mutation. The Y139A mutation (only 2-fold decrease in affinity) suggests that an amino acid side chain that has a small hydrophobic group, rather than a large hydrophobic group, is beneficial when the interaction takes place with a hydrophilic group on the ligand. Both mutations of Tyr320 result in loss of affinity for salvinorin A by about 10-fold, suggesting hydrogen bond involvement. Our proposed model interacts with Tyr320 via hydrogen bonding with the furanyl substituent of salvinorin A. The interatomic distance between the furan oxygen atom in salvinorin A and the Tyr320 side chain oxygen is 3.0 Å. Mutation of Tyr119 to either Phe or Ala decreases the affinity of salvinorin A for the KOR, but to a somewhat lesser extent than for the analogous Tyr320 mutations, again indicating that hydrogen bond interactions are involved. As with Tyr320, our proposed salvinorin A model interacts with Tyr119 via hydrogen bonding with the furanyl substituent of the ligand (Figure 3.1). It places the furan ring oxygen atom somewhat farther away from Tyr119 (3.6 Å) than from Tyr 320 (3.0 Å), potentially explaining the slightly greater decrease in binding affinity for the Tyr320 mutation when compared to the analogous Tyr119 mutation.

To further explore the interactions of the salvinorins with the KOR, we employed a novel approach whereby we combined Cys-substitution mutagenesis with an evaluation of the binding of salvinorinyl-2-thiol and salvinorin A. We reasoned that if residues mutated to
Cys were in close proximity to the thiol of 2-thiosalvinorin B, there should be an enhancement (or at least a retention) of 2-thiosalvinorin B’s affinity for the KOR, while the affinity for salvinorin A would likely decrease. An inspection of the salvinorin A binding model disclosed that Tyr313, when mutated to Cys, would yield a Cys residue that is predicted to be in close proximity to the free thiol of salvinorinyl-2-thiol. In addition, mutating other nearby residues in a similar fashion would produce Cys residues nearer to other positions on the salvinorin molecule. If our proposed model is correct, mutations at these non-Tyr313 positions should affect the binding of salvinorin A and salvinorinyl-2-thiol roughly equally, since salvinorin A and salvinorinyl-2-thiol differ only at the 2-position. The results of these experiments are presented in Table 3.4 and agree with the predictions.

In our model of the KOR (Figure 3.2), the side chain of Cys315 is located in the interface between helices TM6 and TM7, so it is not surprising that the mutation C315S did not result in a statistically significant change in the binding affinity for either salvinorin A or salvinorinyl-2-thiol (Table 3.4). The most significant result from the Cys-substitution mutation studies is that the double mutant C315S-Y313C KOR has 15.8-fold less affinity for salvinorin A than does the C315S single mutant KOR, whereas the same double mutant KOR’s affinity for 2-thiosalvinorin B is largely unaffected compared to the single-mutant C315S KOR. This would suggest that it is indeed the 2-position of the salvinorins that interact with Tyr313, since an SH-acetoxy interaction (in the case of salvinorin A) would be very weak and would increase the K_i significantly, whereas an SH-SH interaction (in the case of 2-thiosalvinorin B) would be stronger due to
hydrophobic interactions and/or disulfide bond formation, and would allow the double mutant to retain affinity for 2-thiolsalvinorin B. We believe disulfide bond formation is unlikely since preliminary studies have demonstrated that prolonged exposure to 2-thiosalvinorin B does not lead to an irreversible loss of binding (data not shown). Our proposed model also positions the 4-methyl ester group in very close proximity to Ile294. The double mutations C315S-I294C and C315S-E297C each affect both salvinorins roughly equally, increasing the affinity of both by 5- to 10-fold. The nature of these interactions is unclear, since both hydrophobic and hydrophilic hydrogen bond acceptor regions are present. However, visual inspection of space-filling models reveals that the terminal methyl of the ester group at the 4-position of salvinorin A can interact with hydrophobic portions of the Ile294 side chain. Mutation to Cys would retain some hydrophobic interaction potential, and perhaps more importantly, would remove some of the steric bulk in the region, allowing the 4-position to more effectively associate with the side chains at positions 294. The double mutations involving Leu309 and Ser310 did not significantly alter the binding affinity of either salvinorin, and this is consistent with our proposed model complex in that these residues are not part of the ligand binding site.

Taken together, these findings support a mode of binding whereby salvinorin A and 2-thiosalvinorin B interact with the KOR via residues that are not utilized by conventional KOR peptide and non-peptide agonists (e.g. dynorphin A (1-13) and U69593, respectively). These residues most likely line a putative binding pocket that overlaps in three-dimensional space with that used by nitrogenous KOR agonists. Thus, compounds like U69593 are predicted to bind in approximately the same three-dimensional space but
do so by utilizing different residues. It is likely that salvinorin A’s extraordinary selectivity and potency for the KOR is due to the fact that it uses these unconventional and generally non-conserved residues for ligand binding. Residues with which our proposed model interacts (namely Ile294 and Tyr313) are unique to the KOR. In addition, since there is no relatively strong salt bridge anchoring salvinorin A into the binding pocket, and since there are many hydrogen bonding and lipophilic interaction sites within the pocket, it would not be unexpected for the KOR to recognize salvinorin A via more than one binding mode. It is possible that these various models, taken together, could collectively give rise to the observed affinity and/or activation of the KOR, and that no individual bound conformation would be totally responsible for the observed effects.
CHAPTER 4: The Conformational Changes of KOR Induced by G protein-Coupling (Modified from Ref (7))

4.1 Introduction and Rationale

Recently crystal structures of the human β2AR have been obtained using two different approaches to stabilize receptor protein and increase polar surface area (24, 25, 182). Together with the rhodopsin structure (17, 18, 183), high-resolution GPCR X-ray structures represent an important breakthrough in understanding the molecular mechanism of how GPCRs function. However, to form a crystal, a GPCR has to be locked in a single conformational state. This is a significant limitation since there is a large amount of functional and biophysical evidence showing that GPCRs are conformationally complex and dynamic. GPCRs are very likely to adopt conformations specific for the bound ligand and the associated signaling protein (e.g. G proteins, arrestins). Although ligand-induced/stabilized conformational changes in GPCRs are well documented (184, 185), there is little direct evidence for G protein-induced conformational changes in any GPCR. Our goal is to investigate the potential conformational changes in KOR induced by G protein-coupling and how these conformational changes affect agonist interactions at molecular level.

First, we needed to design a cellular system where G proteins can induce conformational changes in KOR. It has been reported that G proteins are present in 10-100 molar excess compared to GPCRs(186, 187), the overexpression of KOR, 2 pmol/mg as compared to
less than 20 fmol/mg in native brain tissue, can consume the free G proteins and leave a significant portion of KOR uncoupled (Figure 4.1). In an analogous way, the overexpression of G protein changes this ratio with the consequence that the percentage of GPCR which is ‘precoupled’ to G proteins increases(188). To investigate this possibility, the effects of over-expressing Gα subunits (Gα16 or Gαi2) with the κ-opioid receptor (KOR) were examined (Figure 4.1). Since opioid receptors are capable of coupling with the pertussis toxin sensitive Gα–subunits Gβγ and the pertussis toxin insensitive Gα and Gα16 subunits(189, 190), two types of G proteins were chosen for study: Gαi2 which has demonstrated preferential coupling to KOR and the promiscuous Gα subunit Gα16.

Second, we need an appropriate approach to examine the conformational changes of KOR. There are several methods have been used to interrogate GPCR conformational changes (Table 4.1). Every method has its limitations and advantages. On the whole, the substituted cysteine accessibility method (SCAM) fits our research goal better than the others. Therefore the SCAM approach was used along with the sulphhydryl reagent (2-aminoethyl)methane thio sulfonate (MTSEA) to probe conformational changes which might occur in transmembrane domains 6 (TM6), 7 (TM7) and extracellular loop 2 (EL2) of the KOR in various G protein backgrounds. Also, a kinetic study was applied to confirm the conformational changes identified by SCAM.

To our knowledge, this is the first direct study investigating how G protein alpha subunits induce different conformational changes in their cognate GPCRs. This research will help
us gain deeper insight into GPCR signaling and function of receptor complex, especially the intriguing phenomena of “functional selectivity”. For many years it has been documented that GPCR agonists display functional selectivity or, the ability of ligands (both agonists and antagonists) to differentially modulate signaling pathways depending on the cellular milieu (191-195). One possible explanation for the phenomenon of functional selectivity is that G proteins differentially shift the conformation of GPCRs from the ground state to a series of activated states. Since G proteins are heterogeneously distributed in various cell compartments and show cell type and developmental expression patterns (196-198), it has been suggested that the cellular expression profile of G proteins and effectors will affect the pattern of activation of downstream signaling pathways (199-201). According to this model, different agonist molecules preferentially sample some conformational changes over others, leading to the establishment of an agonist-preferred G protein-coupling pathway. G proteins have recently been shown to precouple with receptors specifically before the addition of agonists (187, 202) leading, perhaps, to distinct conformations. Moreover, recent reviews of the G protein-dependent pharmacology of ligands suggest the promise for designing conformationally selective ligands (203), i.e. ligands that bind preferentially to particular GPCR conformations.
Figure 4.1 Ga subunit overexpression was used to stabilize receptor conformations in the various active states. In each cell system, KOR exists in both G protein-coupled and uncoupled forms. However, KORs are more likely to be in the G protein-coupled form when G protein alpha subunits (such as Gα16 and Gα12) are overexpressed. For clarification, abbreviations were used to describe the three cell systems: KOR, KOR•Gα16 and KOR•Gα12.

Table 4.1 The Comparison of Common Biochemical and Biophysical Methods Used for GPCR Conformation Study

<table>
<thead>
<tr>
<th>Method</th>
<th>Mechanism</th>
<th>Conclusion</th>
<th>Limitation</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spin Labeling</td>
<td>Nitroxide radicals labeling Cys, conformational changes detected by Electron paramagnetic resonance (EPR)</td>
<td>Rhodopsin activation involves TM3 and TM6 movements.</td>
<td>Introduced Cys mutations may change the whole structure; not much details other individual residues</td>
<td>(204)</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Fluorophore coupling to Cys, and nearby Trp quenching the fluorescence, conformational changes detected by Fluorescence spectroscopy.</td>
<td>Full activation of β2AR needs disrupting ionic lock.</td>
<td>Introduced Cys mutations may change the whole structure; fluorescence may be affected by native aromatic residues; not much details other individual residues</td>
<td>(205)</td>
</tr>
<tr>
<td>SCAM</td>
<td>Methanethiosulfonate modifying Cys, detected by Radioligand binding</td>
<td>TM6 rotating/tilting associated with β2AR activation</td>
<td>Introduced Cys mutations may change the whole structure</td>
<td>(206)</td>
</tr>
<tr>
<td>Metal Ion Chelating</td>
<td>Cu²⁺ or Zn²⁺ chelating with Asp, His, Cys; conformational changes detected by functional assay (cAMP production).</td>
<td>β2AR, TM6 and TM7 move apart with large amplitude.</td>
<td>Introduced His, Cys mutations may change the whole structure; not much details for other individual residues</td>
<td>(207)</td>
</tr>
<tr>
<td>Disulfide Crosslinking</td>
<td>N-ethylmaleimide crosslinking two close Cys residues, conformational changes detected by MW shift on Western Blot.</td>
<td>Muscarinic agonists trigger a separation between the cytoplasmic regions between TM1 and TM7, inverse agonists increase the proximity.</td>
<td>Introduced mutations may change the whole structure; crosslinking is not specific reaction; not much details other individual residues</td>
<td>(208)</td>
</tr>
<tr>
<td>Region Specific Antibody</td>
<td>Antibody can recognize the middle and distal part of N terminus, conformational changes detected by antigen-antibody interaction.</td>
<td>MOR undergoes conformational changes following receptor activation, antibodies differentiate ligands with varying efficacies.</td>
<td>Only global changes for N-terminus, no details for TMs; antigen-antibody interaction is not specific.</td>
<td>(209)</td>
</tr>
<tr>
<td>NMR</td>
<td>⁹F-, or ¹⁵N-labeling select</td>
<td>Rhodopsin Try side</td>
<td>Very complex</td>
<td>(210)</td>
</tr>
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</table>
4.2 Results

4.2.1 Examine the Conformational Changes of KOR by SCAM

4.2.1.1 Characterize Cys mutants in TMs 6 and 7 and EL2 of KOR.

Prior to performing Cys-accessibility studies, a large number of Cys mutants were created. For these studies, 23 consecutive residues in TM7 (not including C315<sup>7.38</sup>), 6 residues in the upper part of TM6 and 11 residues in EL2 of KOR were mutated to Cys based on the C315<sup>7.38S</sup> background<sup>(211)</sup>.<br>

The table below provides equilibrium dissociation constant (<i>K<sub>d</sub></i>) and maximum binding capacity (<i>B<sub>max</sub></i>) values using [<sup>3</sup>H]diprenorphine for TM6 and TM7 Cys mutants, which have been previously reported by others<sup>(157, 158)</sup>. Our pattern of results was similar and showed only minor alterations in diprenorphine affinity (data not shown). For EL2 mutants, <i>K<sub>d</sub></i> and <i>B<sub>max</sub></i> values are summarized in Table 4.2. Relatively minor alterations in <i>K<sub>d</sub></i> values (0.13 nM ~ 0.63 nM) were found which are similar to wild type KOR (0.46 nM) while the <i>B<sub>max</sub></i> ranged 0.022 to 1.1 pmol/mg (Table 4.2). These results indicate that the Cys mutagenesis does not greatly alter antagonist binding affinity. The sole exceptions were the P327<sup>7.50C</sup> mutation which resulted in a non-expressed receptor protein as judged by radioligand binding studies, and the Y320<sup>7.43C</sup> mutation. Surface biotinylation and anti-FLAG immunoblotting confirmed that the P327<sup>7.50C</sup> mutant was not expressed and that the Y320<sup>7.43C</sup>, along with other selected mutants were expressed on the plasma membrane (data not shown). Taken together, these findings indicate the examined Cys mutations do not drastically affect the binding pocket.
for diprenorphine and that among the mutants evaluated surface expression is normal.

**Table 4.2** $K_d$ and $B_{max}$ values of [³H]diprenorphine binding to the WT KOR and EL2 Cys mutants

<table>
<thead>
<tr>
<th>EL2 Mutants$^a$</th>
<th>$K_d$ nM</th>
<th>$B_{max}$ pmol/mg</th>
<th>Ratio$^b$ $K_d$(mutant)/$K_d$(WT)</th>
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</thead>
<tbody>
<tr>
<td>WT KOR</td>
<td>0.46 ± 0.11</td>
<td>2.7 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>C315S-V205C</td>
<td>0.43 ± 0.07</td>
<td>1.1 ± 0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>C315S-D206C</td>
<td>0.29 ± 0.05</td>
<td>0.61 ± 0.28</td>
<td>0.6</td>
</tr>
<tr>
<td>C315S-V207C</td>
<td>0.60 ± 0.06</td>
<td>1.0 ± 0.2</td>
<td>1.3</td>
</tr>
<tr>
<td>C315S-I208C</td>
<td>0.49 ± 0.10</td>
<td>0.74 ± 0.13</td>
<td>1.1</td>
</tr>
<tr>
<td>C315S-E209C</td>
<td>0.63 ± 0.14</td>
<td>0.70 ± 0.14</td>
<td>1.4</td>
</tr>
<tr>
<td>C315S-S211C</td>
<td>0.38 ± 0.11</td>
<td>0.83 ± 0.17</td>
<td>0.8</td>
</tr>
<tr>
<td>C315S-L212C</td>
<td>0.13 ± 0.04</td>
<td>0.022± 0.002</td>
<td>0.3</td>
</tr>
<tr>
<td>C315S-Q213C</td>
<td>0.48 ± 0.03</td>
<td>0.99 ± 0.22</td>
<td>1.0</td>
</tr>
<tr>
<td>C315S-F214C</td>
<td>0.24 ± 0.07</td>
<td>0.023 ± 0.002</td>
<td>0.5</td>
</tr>
<tr>
<td>C315S-P215C</td>
<td>0.36 ± 0.13</td>
<td>0.39 ± 0.12</td>
<td>0.8</td>
</tr>
<tr>
<td>C315S-D216C</td>
<td>0.39 ± 0.08</td>
<td>0.87 ± 0.31</td>
<td>0.8</td>
</tr>
</tbody>
</table>

$^a$Saturation binding of [³H]diprenorphine to the wild type and the EL2 mutants was performed according to the procedure in Chapter 2 MATERIALS AND METHODS. Data represent in mean ±SEM from two to four independent experiments. Receptors are transiently expressed in HEK 293T cells.

$^b$The ratio is $K_d$(mutant)/$K_d$(WT).

### 4.2.1.2 SCAM elucidates conformational changes induced by G proteins

Co-overexpression of Gα subunits and GPCRs will likely modify the local G protein environment sensed by GPCRs, leading to potential conformational changes in the GPCRs. These conformational changes induced by Gα subunits can be reflected by a change in the pattern of SCAM-sensitive residues. Prior to determining potential changes in conformation, however, the basal MTSEA sensitivity patterns for the various Cys mutants needed to be determined.
In initial studies, 7 out of the 23 Cys mutants in TM7 of KOR were identified as being significantly more sensitive to the MTSEA reagent than the C315\textsuperscript{7.38}S ‘Cys-less’ KOR as judged by an analysis of variance in HEK 293T cells: S310\textsuperscript{7.33}, F314\textsuperscript{7.37}, I316\textsuperscript{7.39}, A317\textsuperscript{7.40}, L318\textsuperscript{7.41}, G319\textsuperscript{7.42} and Y320\textsuperscript{7.43} (Figure 4.2a). Upon stable over-expression of G\textsubscript{\alpha}\textsubscript{16}, an additional two residues—S311\textsuperscript{7.34}C and N326\textsuperscript{7.49}C—became sensitive (Figure 4.2a). Upon stable over-expression of the ‘specific’ G\textsubscript{\alpha} subunit G\textsubscript{\alpha}\textsubscript{i2} even more residues became sensitive (Y313\textsuperscript{7.36}, N322\textsuperscript{7.45}, S323\textsuperscript{7.46} and L329\textsuperscript{7.52}). In addition, the absolute magnitude of average inhibition induced by the MTSEA reagent increased to 0.20 upon G\textsubscript{\alpha}\textsubscript{i2} overexpression (Table 4.3). In addition to a global change in TM7 residue sensitivity, there was an interesting switch for a critical residue essential for salvinorin A binding—Y313\textsuperscript{7.36}C (2), which changed from being insensitive to sensitive upon G\textsubscript{\alpha}\textsubscript{i2} overexpression.

For TM6, only residues which had previously been determined to be ‘sensitive’ by other investigators were tested\textsuperscript{(158)}. It was found that the upper part of TM6 displayed only a limited change in the absolute amount of sensitivity from 0.04 in G\textsubscript{\alpha}\textsubscript{16} cells to 0.03 in G\textsubscript{\alpha}\textsubscript{i2} cells (Table 4.3). An interesting observation was that V296\textsuperscript{6.57}C became insensitive after G\textsubscript{\alpha}\textsubscript{i2} over-expression (Figure 4.2b). These findings are consistent with a model which implies that V296\textsuperscript{6.57} is a half-turn from I294\textsuperscript{6.55} and is facing either other TMs or lipids. EL2 also presented a significant change in overall inhibition (0.06 for G\textsubscript{\alpha}\textsubscript{16} and 0.17 for G\textsubscript{\alpha}\textsubscript{i2} respectively), with two residues L212 and F214 being identified as SCAM-sensitive residues, being somewhat more sensitive in the G protein over-expression settings (Table 4.3 and Figure 4.2c).
Table 4.3 Changes in inhibition upon the coupling of G proteins $G_{\alpha16}$ and $G_{\alpha12}$

<table>
<thead>
<tr>
<th>EL2 Mutants&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Inhibition number</th>
<th>± Inhibition number&lt;sup&gt;b&lt;/sup&gt;</th>
<th>TM7 Mutants&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Inhibition number</th>
<th>± Inhibition number&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KOR</td>
<td>KOR·$G_{\alpha16}$</td>
<td>KOR·$G_{\alpha12}$</td>
<td>KOR</td>
<td>KOR·$G_{\alpha16}$</td>
</tr>
<tr>
<td>C315&lt;sup&gt;7.38&lt;/sup&gt;S</td>
<td>0.05 ± 0.03</td>
<td>0.04</td>
<td>0.13</td>
<td>C315&lt;sup&gt;7.38&lt;/sup&gt;S</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>C315S-V205C</td>
<td>-0.13 ± 0.05</td>
<td>0.17</td>
<td>0.43</td>
<td>C315S-L309&lt;sup&gt;7.32&lt;/sup&gt;C</td>
<td>-0.13 ± 0.09</td>
</tr>
<tr>
<td>C315S-D206C</td>
<td>0.06 ± 0.09</td>
<td>0.01</td>
<td>0.14</td>
<td>C315S-S310&lt;sup&gt;7.33&lt;/sup&gt;C</td>
<td>0.32 ± 0.06</td>
</tr>
<tr>
<td>C315S-V207C</td>
<td>-0.11 ± 0.08</td>
<td>0.21</td>
<td>0.21</td>
<td>C315S-S311&lt;sup&gt;7.34&lt;/sup&gt;C</td>
<td>0.22 ± 0.06</td>
</tr>
<tr>
<td>C315S-L208C</td>
<td>0.24 ± 0.08</td>
<td>-0.17</td>
<td>0.07</td>
<td>C315S-Y312&lt;sup&gt;7.35&lt;/sup&gt;C</td>
<td>-0.17 ± 0.03</td>
</tr>
<tr>
<td>C315S-E209C</td>
<td>0.07 ± 0.02</td>
<td>0.01</td>
<td>0.26</td>
<td>C315S-Y313&lt;sup&gt;7.36&lt;/sup&gt;C</td>
<td>0.28 ± 0.08</td>
</tr>
<tr>
<td>C315S-S211C</td>
<td>0.05 ± 0.02</td>
<td>0.15</td>
<td>0.22</td>
<td>C315S-F314&lt;sup&gt;7.37&lt;/sup&gt;C</td>
<td>0.54 ± 0.04</td>
</tr>
<tr>
<td>C315S-L212C</td>
<td>0.57 ± 0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>C315S-I316&lt;sup&gt;7.39&lt;/sup&gt;C</td>
<td>0.46 ± 0.04</td>
</tr>
<tr>
<td>C315S-Q213C</td>
<td>0.003 ± 0.035</td>
<td>0.13</td>
<td>0.17</td>
<td>C315S-A317&lt;sup&gt;7.40&lt;/sup&gt;C</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>C315S-F214C</td>
<td>0.39 ± 0.09</td>
<td>0.05</td>
<td>0.08</td>
<td>C315S-L318&lt;sup&gt;7.41&lt;/sup&gt;C</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>C315S-P215C</td>
<td>0.22 ± 0.05</td>
<td>0.01</td>
<td>0.11</td>
<td>C315S-G319&lt;sup&gt;7.44&lt;/sup&gt;C</td>
<td>0.40 ± 0.06</td>
</tr>
<tr>
<td>C315S-D216C</td>
<td>0.06 ± 0.04</td>
<td>0.05</td>
<td>0.14</td>
<td>C315S-Y320&lt;sup&gt;7.43&lt;/sup&gt;C</td>
<td>0.65 ± 0.10</td>
</tr>
<tr>
<td>Average</td>
<td>0.06 ± 0.17</td>
<td>0.17</td>
<td>0.17</td>
<td>C315S-T321&lt;sup&gt;7.44&lt;/sup&gt;C</td>
<td>-0.01 ± 0.02</td>
</tr>
<tr>
<td>TM6 Mutants&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Inhibition number</td>
<td>± Inhibition number&lt;sup&gt;b&lt;/sup&gt;</td>
<td>C315S-S323&lt;sup&gt;7.46&lt;/sup&gt;C</td>
<td>0.12 ± 0.04</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>KOR</td>
<td>KOR·$G_{\alpha16}$</td>
<td>KOR·$G_{\alpha12}$</td>
<td>C315S-S324&lt;sup&gt;7.47&lt;/sup&gt;C</td>
<td>-0.07 ± 0.04</td>
</tr>
<tr>
<td>C315&lt;sup&gt;7.38&lt;/sup&gt;S</td>
<td>0.05 ± 0.03</td>
<td>0.04</td>
<td>0.13</td>
<td>C315S-L325&lt;sup&gt;7.48&lt;/sup&gt;C</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>C315S-E297&lt;sup&gt;6.58&lt;/sup&gt;C</td>
<td>0.46 ± 0.003</td>
<td>0.15</td>
<td>0.13</td>
<td>C315S-N326&lt;sup&gt;7.49&lt;/sup&gt;C</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>C315S-V296&lt;sup&gt;6.57&lt;/sup&gt;C</td>
<td>0.28 ± 0.06</td>
<td>0.14</td>
<td>0.05</td>
<td>C315S-P327&lt;sup&gt;7.50&lt;/sup&gt;C</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table 2: Effect of MTSEA pretreatment on [3H]diprenorphine binding

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Adj. Inh. Number</th>
<th>p-Value</th>
<th>Average Inh. Number</th>
<th>Average p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C315S-L295</td>
<td>0.67 ± 0.07</td>
<td>-0.16</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>C315S-L294</td>
<td>0.65 ± 0.06</td>
<td>0.10</td>
<td>0.14 ± 0.01</td>
<td>0.13</td>
</tr>
<tr>
<td>C315S-F293</td>
<td>0.46 ± 0.06</td>
<td>0.03</td>
<td>0.14 ± 0.03</td>
<td>0.11</td>
</tr>
<tr>
<td>C315S-I290</td>
<td>0.72 ± 0.06</td>
<td>-0.01</td>
<td>0.11 ± 0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>0.18 ± 0.05</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*aSCAM analysis revealed differential inhibition number changes in TM6, TM7 and EL2 of the KOR with various G protein coupling. MTSEA was used to react specifically with Cys side chains and the modified Cys show different inhibition ability of [3H]diprenorphine (~0.2nM) binding. The effects of MTSEA pretreatment on [3H]diprenorphine binding were expressed as inhibition number. Data shown represents the mean ±SEM of three to six experiments. Inhibition number was calculated according to Equation 1 in Chapter 2 MATERIALS AND METHODS.

**± Inhibition number** represents the difference of inhibition number between G protein-coupling and non-G coupling states. Negative sign (-) means decrease of inhibition number under G protein coupling; positive sign (omitted in Table 2) means increase of inhibition number.

c[3H]diprenorphine binding was undetectable for the C3157.38S-P3277.50C mutant.

---

**Figure 4.2a**

[Bar graph showing effects of different mutations on [3H]diprenorphine binding for KOR and KOR-Gαi2.

**Figure 4.2b**

[Bar graph showing effects of different mutations on [3H]diprenorphine binding for KOR and KOR-Gαi2.]
**[3H]Diprenorphine binding was undetectable for the C3157.38S-P3277.50C mutant.**
4.2.2 Kinetics Studies Confirmed that Conformational Changes Occur in KOR

The estimation of second-order rate constants of MTSEA reactions with sulphydryls was performed by using the pseudo-first-order method (see Chapter 2 Materials and Methods). Briefly, the extent of reaction after a fixed time with four concentrations of MTSEA (all in excess over the reactive sulphydryls) was determined. For kinetic studies, a slight fluctuation of reaction rate will affect the inhibition number dramatically due to the exponential relationship between the reaction rate and the inhibition effect. TM7 Cys mutants were chosen for kinetics study since they displayed larger average inhibition changes (0.2 under \( \text{G} \alpha_{i2} \) overexpression as discussed above). The reaction rate constants varied significantly depending on the milieu ranging from 0.5- to 3.1-fold changes (Table 4.4) upon \( \text{G} \alpha_{i6} \) overexpression. Most of the Cys mutants displayed no change in reactivity or became more reactive, with two notable exceptions (N322\(^{7.45}\)C and L329\(^{7.52}\)C). With the exception of N326\(^{7.49}\) in the \( \text{G} \alpha_{i6} \)-overexpressed system, residue positions closer to the extracellular side (i.e. top) of the KOR react more rapidly than do those near the intracellular side when \( \text{G} \alpha \) proteins are overexpressed. For \( \text{G} \alpha_{i2} \) conditions, a different pattern of reactivity was observed. Some mutants showed increased reactivity (Y313\(^{7.36}\)C, F314\(^{7.37}\)C, L318\(^{7.41}\)C and Y320\(^{7.43}\)C) while L329\(^{7.52}\)C displayed decreased reactivity.

This kinetic method was not applied to the sensitive residues of TM6 and EL2. Generally the second-order rate data were consistent with the SCAM data, showing a distinct
pattern of reaction rate constants for the sensitive mutants upon different G-protein coupling. We also examined the effect of pre-incubation with naloxone on protection against the MTSEA reagent. Our results were essentially similar to those previously published for both sensitive and insensitive residues (data not shown) \((157, 158)\). For example, pretreatment with U69593 can block the inhibition induced by MTSEA in N326\(^{7.49}\)C, giving a protection number 31%, close to reported result \(\sim 38\% \) (156). In \(\text{G}_{\alpha_{i2}}\) overexpressed cells, for S311\(^{7.34}\)C, Y313\(^{7.36}\)C, N322\(^{7.45}\)C, S323\(^{7.46}\)C, and L329\(^{7.52}\)C mutants, no protection was observed using U69593.

**Table 4.4** Second-order rate constants \((k, M^{-1}s^{-1})\) of MTSEA reaction with Cys mutants of KOR

<table>
<thead>
<tr>
<th></th>
<th>(k_{(KOR)})</th>
<th>(k_{(KOR-G\alpha_{16})})</th>
<th>(k_{(KOR-G\alpha_{i2})})</th>
<th>(k_{(KOR-G\alpha_{i2})}/k_{(KOR)})</th>
<th>(k_{(KOR-G\alpha_{16})}/k_{(KOR)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C315S-S310(^{7.33})C</td>
<td>8.5 ± 1.1</td>
<td>7.8 ± 2.4</td>
<td>7.7 ± 0.9</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>C315S-S311(^{7.34})C</td>
<td>4.7 ± 1.0</td>
<td>7.1 ± 1.3</td>
<td>4.7 ± 0.3</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>C315S-Y313(^{7.36})C</td>
<td>5.8 ± 1.7</td>
<td>7.1 ± 1.4</td>
<td>11.1 ± 3.2</td>
<td>1.2</td>
<td>1.9</td>
</tr>
<tr>
<td>C315S-F314(^{7.37})C</td>
<td>4.2 ± 0.5</td>
<td>9.7 ± 2.4</td>
<td>6.8 ± 1.7</td>
<td>2.3</td>
<td>1.6</td>
</tr>
<tr>
<td>C315S-I316(^{7.39})C</td>
<td>6.1 ± 1.5</td>
<td>14.1 ± 3.1</td>
<td>7.9 ± 2.1</td>
<td>2.3</td>
<td>1.3</td>
</tr>
<tr>
<td>C315S-A317(^{7.40})C</td>
<td>4.7 ± 1.0</td>
<td>5.9 ± 1.3</td>
<td>4.2 ± 1.4</td>
<td>1.3</td>
<td>0.9</td>
</tr>
<tr>
<td>C315S-L318(^{7.41})C</td>
<td>4.5 ± 0.9</td>
<td>5.0 ± 1.1</td>
<td>8.1 ± 1.6</td>
<td>1.1</td>
<td>1.8</td>
</tr>
<tr>
<td>C315S-G319(^{7.42})C</td>
<td>5.8 ± 1.4</td>
<td>5.6 ± 1.1</td>
<td>4.7 ± 1.4</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>C315S-Y320(^{7.43})C</td>
<td>5.2 ± 1.8</td>
<td>5.3 ± 1.7</td>
<td>8.6 ± 0.8</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>C315S-N322(^{7.45})C</td>
<td>10.1 ± 3.5</td>
<td>5.3 ± 1.1</td>
<td>11.6 ± 3.2</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>C315S-S323(^{7.46})C</td>
<td>4.7 ± 1.3</td>
<td>5.1 ± 0.8</td>
<td>6.1 ± 1.2</td>
<td>1.1</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Selected rates of Cys-mutant reaction with MTSEA were examined. Cells were treated with four concentrations of MTSEA, followed by quenching, washing [3H]diprenorphine binding. The second-order rate constants (k) were determined in triplicate. Data represent in mean ±SEM of three to eight independent experiments.

The ratio is $k_{(KOR \cdot G{\alpha}_{16})}/k_{(KOR)}$.

The ratio is $k_{(KOR \cdot G{\alpha}_{i2})}/k_{(KOR)}$.

4.2.3 Conformational changes induced by G protein-coupling have significant effects on agonist affinities.

To examine the consequences of these conformational changes induced by various G protein backgrounds, three KOR agonists (salvinorin A, U69593 and dynorphin A (1-13)) were tested against wild-type KOR transiently expressed in the three cell systems (Figure 1). Two different radioactive ligands (the antagonist [3H]diprenorphine and the agonist [3H]U69593) were also used. In both the G{\alpha}_{16} and G{\alpha}_{i2} environments, salvinorin A and U69593 demonstrated significantly enhanced affinities (Table 4.5) although, surprisingly, the endogenous peptide dynorphin A (1-13)'s affinity was almost unchanged. Using [3H]U69593 revealed a differential preference between salvinorin A and U69593 as well.

The largest effect (18-fold) was seen for salvinorin A in the G{\alpha}_{16} background using [3H]U69593 as radioligand.

<table>
<thead>
<tr>
<th>Tested Agonists</th>
<th>Radioactive label [3H]diprenorphine</th>
<th>Radioactive label [3H]U69593</th>
<th>Dynorphin A (1-13)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$ (nM)$^a$</td>
<td>ratio$^b$</td>
<td>$K_i$ (nM)$^a$</td>
</tr>
<tr>
<td>KOR</td>
<td>33 ± 8</td>
<td></td>
<td>69 ± 12</td>
</tr>
<tr>
<td>KOR·G{\alpha}_{16}</td>
<td>13 ± 4*</td>
<td>2.3</td>
<td>59 ± 8</td>
</tr>
</tbody>
</table>

Table 4.5 Differential effects of G protein overexpression on agonists (salvinorin A, U69593 and dynorphin A (1-13)) binding affinities ($K_i$, nM)
Radioactive label $[^3H]U69593$

<table>
<thead>
<tr>
<th>Tested Agonists$^c$</th>
<th>salvinorin A</th>
<th>U69593</th>
<th>Dynorphin A (1-13)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$ (nM)$^a$</td>
<td>ratio$^b$</td>
<td>$K_i$ (nM)$^a$</td>
</tr>
<tr>
<td>KOR</td>
<td>0.80 ± 0.33</td>
<td>1.0 ± 0.2</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>KOR-Gα$_{16}$</td>
<td>0.045 ± 0.019*</td>
<td>18</td>
<td>0.81 ± 0.13</td>
</tr>
<tr>
<td>KOR-Gα$_{i2}$</td>
<td>0.12 ± 0.06*</td>
<td>6.7</td>
<td>0.89 ± 0.15</td>
</tr>
</tbody>
</table>

$^a$The affinity constants ($K_i$, nM) of the different agonists were determined in competition binding assays with $[^3H]$diprenorphine (antagonist) or $[^3H]U69593$ (agonist) and increasing concentrations of agonists (from $10^{-5}$ nM to $10^4$ nM). Data represent three to six independent experiments in mean ±SEM.

$^b$The ratio is $K_i$(KOR)$/K_i$(KOR·Gα$_{16}$) or $K_i$(KOR)$/K_i$(KOR·Gα$_{i2}$).

$^c$Salvinorin A and U69593 are small-molecule agonists (MW ~400 Da); dynorphin A (1-13) is the short form of the endogenous peptide agonist (MW ~1600 Da).

* $p < 0.05$ vs KOR

### 4.2.4 Refining the Binding Site of Salvinorin A

It has been previously suggested that EL2 is critical for salvinorin A-KOR binding although no residues have been thus far identified. As the sensitive residues identified by SCAM, L212 and F214 have great potential to be critical residues for salvinorin A binding, mutagenesis was applied to them. Because they are quite bulky hydrophobic residues, L212A and F214A, were constructed to examine a potential steric hindrance effect. Interestingly, alanine substitution at L212 and F214 enhanced salvinorin A affinity $K_i$ values (1.7 nM and 1.0 nM respectively; Table 4.6) while alanine mutagenesis of neighboring residues (S211A and Q214A) had no effect. According to our recent model, the EL2 dips down into the hKOR binding pocket (Figure 4.3), placing L212 and F214 into the binding pocket. Some other critical residues (I294$^{6.55}$A and Q115$^{2.60}$A) were also examined. Q115$^{2.60}$A decreased the $K_i$ value 17 fold, consistent with the results reported by Kane et al. (212). The role of Q115$^{2.60}$ in salvinorin A binding is not well understood; however, a hydrogen bond with the furan oxygen of salvinorin A is suggested by our latest models (Figures 4.3a and 4.3b).
Table 4.6 Affinity ($K_i$, nM) of salvinorin A binding to WT KOR and mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_d$ (nM)$^a$</th>
<th>$B_{max}$ (pmol/mg)$^a$</th>
<th>$K_i$ (sal A) (nM)$^b$</th>
<th>$K_i$(Mutant)/$K_i$(WT)</th>
<th>ratio$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOR-WT</td>
<td>0.46 ± 0.10</td>
<td>2.0 ± 0.4</td>
<td>33 ± 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S211A</td>
<td>0.47 ± 0.10</td>
<td>2.7 ± 0.8</td>
<td>38 ± 5</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>L212A</td>
<td>0.28 ± 0.07</td>
<td>0.27 ± 0.08</td>
<td>1.7 ± 1.0</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Q213A</td>
<td>0.51 ± 0.15</td>
<td>0.76 ± 0.04</td>
<td>40 ± 12</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>F214A</td>
<td>0.46 ± 0.22</td>
<td>0.8 ± 0.7</td>
<td>1.0 ± 0.1</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>I294$^6.55$A</td>
<td>0.53 ± 0.18</td>
<td>0.9 ± 0.8</td>
<td>12 ± 6</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>E297$^6.58$A</td>
<td>0.91 ± 0.08</td>
<td>1.8 ± 1.2</td>
<td>15 ± 3</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Q115$^2.60$A</td>
<td>0.65 ± 0.10</td>
<td>2.5 ± 1.7</td>
<td>556 ± 231</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Saturation binding of [³H]diprenorphine to the wild type and the mutants was performed according to the procedure in MATERIALS AND METHODS. Data represent in mean ±SEM of two to four independent experiments.

$^b$The affinity constants ($K_i$) of the different compounds were determined in competition binding assays with [³H]diprenorphine and increasing concentrations of salvinorin A (from $10^{-5}$ nM to $10^4$ nM). Data represent the mean ± SEM of two to four independent experiments.

$^c$The ratio is $K_i$(Mutant)/$K_i$(WT).

After considering the SCAM data and the recently determined crystal structure of photoactivated bovine rhodopsin (23), an updated model for the activated form of the hKOR, as recognized by the agonist salvinorin A, is shown in Figures 4.3. The sidechain conformations in the TM helical regions of the ‘inactive state’ (PDB 1U19(183)) and ‘activated’ rhodopsin crystals are essentially the same; however, there is a subtle but distinct difference in the positions of the TM helices relative to one another. In the ‘activated’ crystal form, TM3 and TM6 are roughly 1 Å further apart from one another in the intracellular region as compared to the ‘dark state’. Based upon the experimental findings reported here and elsewhere(185, 187), the binding of the G protein complex is thought to induce an active or ‘active-like’ state that is more conducive to the binding of agonists than is the inactive state. This change upon activation (or binding of G protein) of the GPCR effectively extends the binding pocket to include a narrow region bounded by the intracellular regions of TM2, TM3, TM6 and TM7. As shown in Figure 4.3b, the residues of TM7 that line this narrow extended binding site are those residues that, under
conditions of G protein overexpression, were found to significantly affect the binding of ${}^3$Hdiprenorphine when those positions were subjected to the SCAM procedure. This implies that these residues become accessible to the relatively small MTSEA reagent when G proteins are overexpressed, and this is reflected in the ‘activated’ rhodopsin-based model.

**Figure 4.3** KOR model based on active rhodopsin structure. 3a. Ribbon diagram of an earlier hKOR model derived from the ‘dark’ rhodopsin crystal structure. 3b. Ribbon diagram of the activated hKOR model derived from the ‘light’ rhodopsin crystal structure. Ribbons are color-coded based on secondary structure: red = α helix, blue = β strand, violet = turn, yellow = coil. Selected sidechains of residues in TM6, TM7 and EL2 whose positions were tested for [${}^3$H]diprenorphine inhibition are shown as capped sticks. Sidechain color coding indicates the conditions under which significantly altered [${}^3$H]diprenorphine binding occurred relative to the C3157.38S reference (see Figures 4.2a-c): green = KOR, KOR$\alpha_{16}$ and KOR$\alpha_{12}$; yellow = KOR$\alpha_{16}$ and KOR$\alpha_{12}$; red = KOR$\alpha_{12}$ only; magenta = KOR and KOR$\alpha_{16}$. Sidechains containing grey atoms did not significantly alter [${}^3$H]diprenorphine binding under any of the three conditions or were not tested (*i.e.* C3157.38). Cyan indicates conserved proline residues. A Connolly channel plot delineating the binding pocket in each receptor is shown as a transparent grey surface (probe radius = 1.4 Å). TM6 and TM7 are closest to the viewer.
In summary, significant conformational changes were observed on TM7, the extracellular portion of TM6 and EL2. Eight SCAM-sensitive residues (S310\(^{2.33}\), F314\(^{7.37}\) to Y320\(^{7.43}\)) on TM7 presented a cluster pattern when the KOR was exposed to baseline amounts of G protein, and additional residues became sensitive upon over-expression of various G proteins. In TM7, S311\(^{7.34}\) and N326\(^{7.49}\) were found to be sensitive in G\(\alpha_{16}\)-overexpressed cells and Y313\(^{7.36}\), N322\(^{7.45}\), S323\(^{7.46}\) and L329\(^{7.52}\) in G\(\alpha_{i2}\)-overexpressed cells. In addition, the degree of sensitivity for various TM7 residues was augmented, especially in G\(\alpha_{i2}\)-overexpressed cells. A similar phenomenon was also observed for residues in TM6 and EL2. In addition to enhanced sensitivity of certain residues, our findings also indicated that a slight rotation was predicted to occur in the upper part of TM7 upon G protein overexpression. These relatively modest conformational changes engendered by G protein overexpression had both profound and differential effects on the abilities of agonists to bind to KOR. These data are significant because they demonstrate that G\(\alpha\) subunits differentially modulate the conformation and agonist affinity of a prototypical GPCR.

### 4.3 Discussion

In the way it is applied here, SCAM is a biochemical method capable of detecting averaged receptor conformations in a defined time period (5 min in these studies). Both static conformations as well as averaged conformational fluctuations during the period the system is exposed to the MTSEA reagent will be reflected in altered Cys reactivity. The final SCAM pattern of MTSEA sensitivity thus represents a summary of the multiplicity of conformations of KOR in the presence and absence of G\(\alpha\) subunit
overexpression. Our data in Table 4.3 demonstrate that the averaged inhibition values of SCAM-sensitive residues are significantly changed by \( \text{G} \alpha \) subunit overexpression—especially by \( \text{G} \alpha_{i2} \). This change in MTSEA sensitivity in the absence of agonist binding strongly supports the notion that \( \text{G} \alpha \) subunits can induce conformational changes in GPCRs. It has been conventionally postulated that following agonist exposure GPCR conformations are altered. Indeed, some novel methodological approaches have recently demonstrated agonist-induced conformational changes in several GPCRs (213-215). To our knowledge, our results are the first to demonstrate that G proteins can alter GPCR conformations in the absence of ligand and, in addition, these findings are consistent with a growing literature suggesting the existence of precoupled GPCR-G protein complexes (187, 216, 217).

In this study, the patterns of residue accessibility (see Figures 4.2a-c) were rationalized via the comparison of our hKOR model with inactive and active rhodopsin structures. Residues L309 to Y313 are located at the extracellular end of TM7. L309 and Y312 were never accessible (or undetectable by inhibition of binding) while S310 was always accessible and significantly affected the binding of diprenorphine. S311 became accessible upon binding of \( \text{G}\alpha_{16} \) or \( \text{G}\alpha_{i2} \). Y313 became accessible only on overexpression of \( \text{G}\alpha_{i2} \). In the regions comprising the extracellular portions of both TM6 and TM7, there are contiguous sequences of amino acids (F293 to L299 and F314 to Y320) that were accessible. This cluster pattern of accessibility has previously been observed in TM6 and TM7 for the MOR and DOR, as well as the KOR (157, 158). However, some of the most sensitive residues are not facing directly into the
binding pocket (Figure 4.2a). Further study is necessary to clarify this. To date, this pattern of accessibility has been generally attributed to the inherent flexibility of the helix and/or to the permeability of the MTSEA reagent through the lipid membrane (218). The over accessibility patterns are likely due to the disorder of this region of the helix, also dynamic movements and the proline kink at P3277.50 may play a role. In a recent study(157), Xu et al. also suggested that the large distance between TM1 and TM7 may allow MTSEA to penetrate the KOR TM1-TM7 interface to react with substituted Cyss. The most sensitive residues on TM6 (E2976.58, I2946.55 and I2906.51) and EL2 (L212 and F214) are pointing to the binding pocket, which is consistent with the current model.

A pseudo-first-order method was used for obtaining the second-order rate of MTSEA reactions. For the 13 TM7 Cys mutants examined (Table 4.4), most (S3117.34C, Y3137.36C, F3147.37C, I3167.39C, L3187.41C, Y3207.43C and N3267.49C) have increased or unchanged reaction rate constants under either Gαi2 or Gα16 overexpression systems, which are consistent with the SCAM data. S3117.34C, I3167.39C and N3267.49C show enhancement under Gα16 overexpression but not Gαi2. The differences may be caused by an unfavorable local environment change presented in the new conformations in the Gαi2 overexpression system, but could also be caused by the proposed inherent flexibility of the extracellular portion of TM7. Since the conformational change is not dramatic and those residues are located in the upper part of the helix, a longer time exposure of MTSEA could compensate and reveal a high SCAM inhibition. N3227.45C, S3237.46C and L3297.52C, whose access to MTSEA is presumably less than that of more extracellularly-located residues (even under conditions of G protein overexpression), and whose kinetic
reaction rates are generally less than those of more extracellularly-located residues, can still significantly affect the binding of diprenorphine when these mutants are reacted with MTSEA. L329 C is furthest down in the pocket and theoretically least accessible to MTSEA (see Figure 4.3b). It should be mentioned that the mechanism by which the MTSEA reaches buried Cys residues such as C329 is not perfectly clear, as some studies have indicated that MTSEA is lipid membrane-permeable. In general, the reaction rates with G protein overexpression are within 0.5- to 3.1-fold of the corresponding non-G protein overexpression systems, and there is no large increase of rate observed between different mutants. Analysis of SCAM second-order reaction rate constants had been used to predict which residues most likely (or most often) face the interior of the binding cavity. Some of the reaction rate constants reported here were lower than previously reported and are likely due to differences in experimental techniques. In addition, the reactivity of individual residues (in TM7) did appear to indicate a directional preference and α-helical periodicity (see Figure 4.4).

Figure 4.4 shows that TM7 may undergo a modest counterclockwise rotation (viewed from the extracellular side) when in the presence of overexpressed Gαi2, but the rotational preference for TM7 of the Gα16-overexpressed KOR is less clear; its pattern of reactivity is possibly due to the effects of MTSEA membrane permeability and/or rotational flexibility. However, it seems reasonable to expect that the KOR presents a consistent and complementary receptor conformation to approaching agonists, at least in the case of Salvinorin A and U69593, whose binding affinities increased by roughly the same amount in both Gα16 and Gαi2 overexpressed systems (see Table 4.5).
Figure 4.4 Second-order rate constants ($k, M^{-1}s^{-1}$) revealed a directional preference and $\alpha$-helical periodicity. The ratios ($k(G\alpha_{i6})/k(\text{non-}G\alpha)$ or $k(G\alpha_{i2})/k(\text{non-}G\alpha)$) of MTSEA reaction rates upon G protein-coupling were divided into three subgroups, highlighted by labeling the alpha carbon with different colors: Red: ratio > 1.5; green, 0.5 < ratio <= 1.5; blue, ratio <= 0.5. All of the residues presented inhibited the binding of $[^3H]$diprenorphine by greater than 35% upon G$\alpha_{i2}$ coupling, and were significantly different from the
background C3157.38S in SCAM pattern (see Figure 4.2a). The KOR backbone is represented by a thin ribbon in which the individual TMs are color-coded (TM1 = red; TM2 = orange; TM3 = yellow; TM4 = green; TM5 = cyan; TM6 = blue; TM7/Helix 8 = violet), and salvinorin A is rendered as capped sticks (carbon = grey; oxygen = red).

Compared to TM6 and TM7, the pattern of EL2 is relatively simple, with only two residues (L212 and F214) next to C210 — which likely forms a disulfide bond with C131 — identified as sensitive. The residues next to C210, L212 and F214 are likely not to be as conformationally flexible as the other loop residues, and are more likely to present a fixed direction pointing down into the binding pocket. Similar observations were reported for the D2-dopamine receptor EL2 (219), where two residues (I184 and N186) were found to be SCAM-sensitive. In the D2 receptor, I184 and N186 are adjacent to the disulfide bond-forming C182, just as L212 and F214 are adjacent to C210 in the KOR. The KOR modeling studies presented here led to the prediction that L212 and F214 would cause a large steric hindrance for ligand binding to KOR. A reasonable prediction was that a smaller hydrophobic residue will lessen the blockage and potentiate ligand binding. The profound increase of salvinorin A binding for KOR mutants (L212A and F214A) supports this model (Table 4.6).

In summary, our major finding is that $G\alpha_{i2}$ and $G\alpha_{16}$ differentially modulate the conformation of a prototypical family A GPCR — KOR. It was discovered that when $G\alpha_{i2}$ and $G\alpha_{16}$ were over-expressed together with KOR, a differential pattern of Cys-accessibility to MTSEA was observed. In conjunction with these apparent changes in KOR conformation there was a selective alteration in salvinorin A’s affinity. These results are consistent with the hypothesis that $G\alpha$ subunits differentially interact with
GPCRs in the absence of agonists and that such interactions lead to conformational changes which are reflected in altered ligand affinities. Together with a recent chimeric-receptor/SCAM study by Vrotherms et al. (160), showing TM2’s orientation varies among opioid receptor family members, these findings revealed the mechanism why salvinorin A binds to KOR with such high affinity as well as high selectivity: the availability of both the critical residues (Y313, Y320, Y119 and Q115 etc.) and the appropriate helical orientation and conformation of the TM domains where these critical residues are located in (such as TM7 and TM2).
Chapter 5: The Design and Application of Covalently-Bound Agonists to Probe KOR

5.1 Introduction and Rationale

Salvinorin A, the most potent natural hallucinogen, gained immediate interest since KOR was identified as the sole molecular target in 2002 (1). Salvinorin A represents an attractive lead compound for drug development as discussed in Chapter 3. During the past few years, more than 250 derivatives of salvinorin A were synthesized by exploring almost all the commonly seen functional groups at C-2, C-4 and C-12 positions (Figure 3.1). Some of these analogs presented unique pharmacological profiles, from full KOR agonist to partial DOR or MOR agonists and antagonists (5, 220-224). A recent paper showed that the modification of C-12 of the furan ring yielded analogs with promising KOR antagonistic activity (225). However, most of the 250 analogs showed moderate affinity or even completely lost affinity to KOR. The challenge now is how to gain precise knowledge about salvinorin A – KOR interactions and rationally design novel salvinorin A derivatives. Covalent binding, also referred to as affinity labeling or proximity-accelerated chemical coupling, emerged as a powerful approach to unambiguously characterize ligand’s binding site in recent years. This method takes advantage of the chemically reactive amino acids inside or close to the ligand binding site, most of the time the reactive residue is a nucleophile (e.g., Cys). Introduction of a Cys-preferred reactive group (i.e., an electrophile) into the ligand structure leads to a covalent reaction between the ligand and receptor if the chemical reaction conditions are favorable (Figure 5.1). The reactive Cys residues could already exist in the protein or be
introduced by site-directed mutagenesis, which provides the opportunity to study receptors that lack Cys residues inside their binding sites. Because of the specific recognition between the ligand and receptor, this affinity labeling is also expected to be highly selective. By adjusting the reactive functional group on the covalently-bound ligand, a wide range of labeling reaction rates and structurally diverse labeling products can be obtained. Halogen and isothiocyanate groups are commonly used for such covalently-bound ligand design due to their relatively high reactivity under mild physiological conditions. The successful applications of affinity labeling methods include an allosteric modulator (imidazobenzodiazepine) for \( \text{GABA}_A \) receptor (226), fluorescently tagged inhibitors for calcium-bound protein arginine deiminase 4 (PAD4) (227), selective kinase inhibitors (228, 229), estradiols for estrogen receptor (230-235), and antagonists for \( N \)-methyl-D-aspartate (NMDA) receptor (a ligand-gated cation channel) (236).

To design covalently-bound ligands, we had to find a chemically reactive amino acid inside the binding site of salvinorin A. Our earlier binding site study showed there was a direct interaction between the acetoxy group of salvinorin A and Y313\(^{7.36} \), a critical residue inside the binding pocket (2). Nearby, a water-accessible C315\(^{7.38} \) is highly reactive to methanethiosulfonate reagent, which has been shown in the SCAM study by Xu et al. (176). Because the C315\(^{7.38} \)S mutation only affects the binding affinity moderately, it is very possible that C315\(^{7.38} \) exists as a free Cys rather than as a contributor to global structure or disulfide-bonds. Therefore this free and chemically reactive C315\(^{7.38} \) is a potential nucleophile at physiological pH 7.4 and can provide an
anchoring point for affinity labeling (Figure 5.1). Our hypothesis is that salvinorin A analogs with an appropriate electrophilic group will possibly react with C315 if these two reactive groups are within close proximity. The product of affinity labeling, i.e., the modified KOR protein, can be characterized by both the traditional pharmacological and novel mass spectrometric techniques (230, 231).

![Figure 5.1](image_url)

**Figure 5.1** The mechanism for RB-64 labeling (covalently-bound to) KOR. The molecular weight shift after the substitution reaction is about 431.5.

To test our hypothesis, we designed a research plan involving several technically demanding steps, including: 1) design and synthesize chemically reactive salvinorin A derivatives which can label a Cys residue under physiological conditions, i.e., pH 7.4 and mild temperature; 2) express a large quantity of KOR protein in a purification-friendly construct (e.g., FLAG and His6 tagged); 3) optimize the labeling reaction between covalently-bound ligand and KOR, and apply the optimal conditions to a large quantity of KOR (e.g., 20-150 mm dish cells transiently expressing KOR, labeling with RB-64 for 3 hr/10µM at 4°C in PBS buffer); 4) isolate the RB-64 modified KOR protein by tandem affinity purification (i.e., M2 resin for FLAG tag, Ni2+-NTA resin for His6 tag); 5) concentrate the modified KOR protein and confirm the existence of KOR by western
blotting (i.e., anti-FLAG and anti-KOR antibodies), then use SDS-PAGE gel and Coomassie staining to separate and detect KOR from other associated proteins; 6) use an appropriate protease to perform in-gel digestion (i.e. Chymotrypsin to cover the TM7 region) and identify the labeling site (C315<sup>7.38</sup>) through mass spectrometry; 7) validate the affinity labeling site(s) by mutagenesis studies of the residues spatially close to C315<sup>7.38</sup>, such as Y313<sup>7.36</sup>, F314<sup>7.37</sup> and I316<sup>7.39</sup>.

Thus far, two compounds — RB-48 and RB-64 (both with pM affinities and extraordinary selectivity for KOR) — have emerged as suitable ligands for affinity labeling. RB-64, RB-48 and related compounds were synthesized by our collaborator Dr. Jordan K. Zjawiony (University of Mississippi). The structures and possible labeling mechanism of RB-64 is shown in Figure 5.1. Overall, this study validated our prior salvinorin A binding site in KOR (2), and provided even more precise information about the ligand-receptor interactions. Undoubtedly this study will further aid the rational design of chemically unique KOR ligands with therapeutic potential. Moreover, this study predicts a bright future for the application of mass spectrometry in GPCR research.

5.2 Results

5.2.1 Characterizing Covalently-Bound Ligands by Molecular Pharmacology Methods

5.2.1.1 RB-64 and RB-48 are potent KOR agonists

The binding affinities of RB-64 and RB-48 were defined by competition binding assays with both [<sup>3</sup>H]U69593 (selective agonist for KOR) and [<sup>3</sup>H]diprenorphine (non-selective...
antagonist for KOR). RB-64 and RB-48 showed affinities similar to salvinorin A in competing with $[^3$H]diprenorphine. However, $[^3$H]U69593 can differentiate RB-64 and RB-48: the latter showed a similar affinity (2.10 nM) as salvinorin A’s (1.85 nM); while RB-64 showed a moderate 3-fold increase (0.59 nM) in binding affinity as compared to salvinorin A. Both compounds presented high potency in the $[^3$S]GTPγS assay. Interestingly RB-64 had an EC$_{50}$ value down to 0.077 nM, much smaller than salvinorin A’s (17 nM) and RB-48’s EC$_{50}$ was in between them (0.19 nM).

<table>
<thead>
<tr>
<th></th>
<th>$K_i$ (nM)$^a$</th>
<th>$K_i$ (nM)$^b$</th>
<th>$EC_{50}$ (nM)$^c$</th>
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<tr>
<td>SalA</td>
<td>1.85± 1.49</td>
<td>21 ± 11</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>RB-48</td>
<td>2.10 ± 0.84</td>
<td>32 ± 15</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>RB-64</td>
<td>0.59 ± 0.21</td>
<td>39 ± 11</td>
<td>0.077 ± 0.016</td>
</tr>
</tbody>
</table>

$^a$ The affinity constants ($K_i$) of the different compounds were determined in competition binding assays with $[^3$H]U69593 and increasing concentrations of unlabeled compounds. Each value is the mean of three independent experiments.

$^b$ The affinity constants ($K_i$) of the different compounds were determined in competition binding assays with $[^3$H]diprenorphine.

$^c$ The functional potency ($EC_{50}$) of the different compounds were determined in $[^3$S]GTPγS assay. See Chapter 2 for details.

### 5.2.1.2 Covalent labeling of WT KOR and mutants with RB-64

Our earlier covalent labeling study of RB-64 and RB-48 showed that both ligands can covalently bind to KOR. The labeling result of RB-48 was not consistent due to its high reactivity and low stability. RB-64 was less reactive, but consistently demonstrated a higher labeling ability. A time course and dose response study of RB-64 labeling showed that 3hr/10µM (at 4°C in PBS buffer) was the optimal condition for effective RB-64
labeling without the non-specific effects caused by both higher concentrations and longer incubation times. At these conditions, a maximal 59% of KOR was labeled by RB-64, and 41% residual binding left (Figure 5.2a). As comparison, both salvinorin A and naloxone didn’t show any significant labeling of KOR in parallel experiments (Figure 5.2a). However, there was a slight drop in $B_{max}$ value for salvinorin A labeling, which was probably caused by inefficient washing (intact cells used) and non-specific hydrophobic interactions. Stronger reaction conditions, i.e., 10hr/20µM, achieved 82% labeling of KOR. To avoid non-specific effects associated with high dose of RB-64, we used the moderate labeling conditions (i.e., 3hr/10µM) in the irreversible binding study.

To precisely determine the labeling site of RB-64, WT KOR with native C315$^{7.38}$ and mutated KOR with mutations C315S, Y313C, F314C and I316C were tested using the RB-64 labeling assays. Interestingly, RB-64 preferred residues close to Y313$^{7.36}$ — F314$^{7.37}$ and C315$^{7.38}$ — but not I316$^{7.39}$, which was too far away. The residual KOR $B_{max}$ values for Y313$^{7.36}$ and I316$^{7.39}$ (> 200% after normalization) are much larger than their corresponding internal standards (100% refers to untreated KOR). And intriguingly, the labeling silent mutation C315S had no significant reactivity with RB-64 as compared to naloxone labeling, even though all three compounds (RB-64, salvinorin A and naloxone) decreased the $B_{max}$ of KOR moderately. The mechanism for this unexpected effect is not clear. Insufficient washing could be one of the reasons. By comparing the residual $B_{max}$ values, the reactivity of Cys mutants to RB-64 was lost for I316C, Y313C and C315S mutations. All these data are consistent with our prior model, in which C-2 position of
salvinorin A is within close contact to Y313$^{7,38}$, so residues C315$^{7,36}$ and F314$^{7,37}$ are in the most favorable position for labeling reaction.

Figure 5.2 RB-64 labeling. Irreversible reaction of RB-64 with (2a) WT KOR and (2b) Cys mutants (based on C315S background). (2a) Receptors were exposed for 3h/10µM
RB-64, sal A, naloxone and no drug treatment (vehicle DMSO only). (2b) 10 hr/20 µM RB-64, sal A, Naloxone and Blank (vehicle only) were used for labeling. After labeling reaction, the cell membranes were extensively washed (three times); the residual binding was determined by [³H]diprenorphine saturation binding. In (2b) $B_{max}$ values were expressed as a percentage of the internal control in which no drug, only vehicle was present in the PBS buffer. Each bar represents mean ± SEM for two to four independent experiments in which each sample was measured in triplicate. See Chapter 2 for details.

5.2.2 Characterizing Covalently-Bound Ligands by Mass Spectrometry

5.2.2.1 KOR expression, labeling and purification

To improve the efficiency of purification, a tandem tagged KOR, containing an N-terminal FLAG tag and C-terminal His₆ tag, was constructed in pcDNA3.1(+) vector and expressed in HEK293 T cells. Cells were harvested after being labeled by RB-64 for 10 hr/20µM. Cell lysates were purified by sequential Anti FLAG M2 and Ni²⁺-NTA affinity purification. The elute was concentrated via Millipore filtration tubes with MW 10 kDa cutoff. The presence of KOR proteins was confirmed by western blotting and Coomassie staining after electrophoretic separation (Figure 5.3). The final products in western blotting showed two KOR bands: 1) “mature type” KOR at 52 kDa and 2) “immature type” KOR at 47 kDa (Figure 5.3a). The distribution of KOR protein in two MW bands was also observed by Wannemacher et. al (237). The coomassie stained gel was then submitted to the UNC-Duke Michael Hooker Proteomics Center at UNC, Chapel Hill for mass spectrometric analysis. The correct protein bands (i.e., 52 and 47 kDa) were both excised from the gel, digested by chymotrypsin and examined by mass analyzers. Two mass spectrometers, AB 4800 MALDI TOF/TOF and Bruker Ultraflex MALDI TOF/TOF, were used for our study. Both mass analyzers can detect femtomole quantities of peptides and proteins.
Figure 5.3 Separation of KOR by SDS-PAGE. (3a) western blotting (10% SDS-PAGE) of purified FLAG-KOR-His-tagged receptor after two-step purification by anti-FLAG M2 and Ni²⁺ NTA resins. Double bands (52 kDa and 47 kDa) represent various degree of glycosylation of KOR protein (237). (3b) Coomassie stained 4-20% SDS-PAGE gel.

5.2.2.2 Mass spectrometric analysis

Peptide samples, containing KOR fragments after chymotrypsin digestion, were analyzed on automated AB 4800 MALDI TOF/TOF and Bruker Ultraflex MALDI TOF/TOF. Both instruments gave similar patterns of identified chymotryptic peptides. For each MALDI analyzer, a small amount of sample was co-crystallized with α-cyano-4-hydroxycinnamic acid (CHCA) and was analyzed over an m/z range of 600-4000. In Figure 5.5a, the spectrum of chymotrypsin is shown to define those peptides that represent autolysis products found in the experimental samples. The mass spectra generated from chymotryptic digestion of the 52 and 47 kDa KOR bands are shown in Figures 5.5b and 5.5c. The Mascot searches of these chymotryptic peptides identified human KOR
(accession number AAA63646, JC2338, Q8IWP3 human, Q499G4 human) as the only protein hit with significant scores found in both the higher and lower MW bands (Table 5.2). 19 matched peptides were found for high score JC2338 (Table 5.3), various oxidation states of the same peptides were omitted. The sequence coverage of KOR was 26%, including the potential anchoring site for RB-64 – C3157.38 (Figure 5.5 and Table 5.3).

**Table 5.2** Mascot searches of chymotryptic peptides from KOR (47 kDa) and KOR (52 kDa). Protein scores greater than 64 are significant ($p < 0.05$).

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<thead>
<tr>
<th>Accession Mass</th>
<th>Description</th>
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<tbody>
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<td>47 kDa</td>
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</tr>
<tr>
<td>1. JC2338</td>
<td>42631 77 kappa opioid receptor 1 - human</td>
</tr>
<tr>
<td>2. Q8IWP3_HUMAN</td>
<td>41087 71 DRG kappa 1 splice variant KOR 1A.- Homo sapiens (Human).</td>
</tr>
<tr>
<td>3. Q499G4_HUMAN</td>
<td>42617 70 Opioid receptor, kappa 1.- Homo sapiens (Human).</td>
</tr>
<tr>
<td>52 kDa</td>
<td></td>
</tr>
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<td>1. AAA63646</td>
<td>16607 77 HUMKOR NID: - Homo sapiens</td>
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<td>2. Q8IWP3_HUMAN</td>
<td>41087 77 DRG kappa 1 splice variant KOR 1A.- Homo sapiens (Human).</td>
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<tr>
<td>3. JC2338</td>
<td>42631 75 kappa opioid receptor 1 - human</td>
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<tr>
<td>4. Q499G4_HUMAN</td>
<td>42617 75 Opioid receptor, kappa 1.- Homo sapiens (Human).</td>
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Table 5.3 Detected KOR peptides from chymotryptic digestion by AB 4800 MALDI TOF/TOF

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<th>Expected monoisotopic [M]$^+$, Da</th>
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<td>1033.42</td>
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<td>168 - 173</td>
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<td>747.39</td>
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<tr>
<td>226 - 233</td>
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<td>999.53</td>
<td>MKICVFIF</td>
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<tr>
<td>232 - 240</td>
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<td>1017.63</td>
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<td>874.53</td>
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<td>1160.59</td>
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<tr>
<td>300 - 309</td>
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<td>930.44</td>
<td>GSTSHSTAAL</td>
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<tr>
<td>314 - 320</td>
<td>786.44</td>
<td>785.38</td>
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<td></td>
</tr>
<tr>
<td>370 - 380</td>
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<td>1256.65</td>
<td>LRIDIDGMNKPV</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>371 - 380</td>
<td>1160.56</td>
<td>1159.57</td>
<td>RDIDIDGMNKPV</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

MKTIIALSYIFCLVFA DYKKDDDDK MESPIQIFRGEPGPTCAPSA CLPPNSSAWF
Signal sequence     FLAG       10           20       30
PGWAEPSNGSAGSEDAQLEPAHISPAIPVIITAVYSSVVFVVGLVGNSLV
                                    40 50 60 70 80

MFVIIRYTKMKTAATNIYIFNLALADALVTNTMPFSTVYLMNSWPGVDVL
                              90 100 110 120 130

CKIVISIDYYNMFTSIFTLTMMSVDRYIAVCHPVKALDFRTPLKAKIINJ
                       140 150 160 170 180

CIWLLSSSVGISAIVLGGTKVRREDVDVIECSLQFPDDDSWWDLFMKICV
                               190 200 210 220 230

FIFAFVIPVLIIIIVCYTLMILRLKSVRLLSBSREKDRNLRITRLVLVV
                         240 250 260 270 280

AVFVCWTPHIHFILVEALGSTSHSTAAALSSYFCIALGYTNSSLNPILY
                           290 300 310 320 330

AFLDENFKRCFRDFCFPLKMRMRQSTSRVNRNTVQDPAYLRIDIDGMNKPV
                          340 350 360 370 380

HHHHHHH
His$_6$
**Figure 5.4** Sequence coverage of KOR by MALDI TOF/TOF. FLAG and His\textsubscript{6} tags are indicated with rectangular boxes. The solid black lines represent the chymotrypsin digested peptides detected by MS analysis. The thicker solid black lines indicate that the corresponding sequences were confirmed in MS/MS analysis by AB 4800 MALDI TOF/TOF. Cys315 is shown in bold and red color. All TM domains are highlighted by the grey background. A total 26% sequence coverage was observed for KOR 47 kDa, 19% sequence coverage for KOR 52 kDa.

Theoretically RB-64 modification can add about 431.5 Da to the peptide fragment containing C315\textsuperscript{7,38}. Because the modification by RB-64 was not stocked in the database, the modified peptide was likely found in the pool of unmatched peptides after the Mascot searches. One unmatched peptide, m/z 1381.80, was close to the modified peptides predicted by *in silico* digestion as shown in Table 5.4. An ongoing MS/MS analysis of this potential modified peptide (YFCIALGY) preliminarily confirmed the covalent modification of C315\textsuperscript{7,38} (data not shown).
Chymotrypsin

KOR (52 kDa)
Figure 5.5 MALDI mass spectra of KOR after chymotrypsine digestion. (5a) chymotrypsin (5b) KOR 52 kDa (5c) KOR 47 kDa are analyzed by Bruker Ultraflex MALDI TOF/TOF. In (5b) and (5c), x-axis zoom inserts were showing RB-64 modified YFCIAGY (MW 1381.8) as compared to unmodified (MW 949.5) peptides. The strong peak (1523.7) indicates the chymotrypsin autolysis.

Table 5.4 RB-64 modified peptides predicted by in silico chymotrypsin digestion

<table>
<thead>
<tr>
<th>Peptide position Start-End</th>
<th>Unmodified monoisotopic [MH]^+, Da</th>
<th>Modified monoisotopic [MH]^+, Da</th>
<th>Sequence</th>
<th>Missed Cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>315 - 320</td>
<td>639.4</td>
<td>1070.9</td>
<td>CIALGY</td>
<td>0</td>
</tr>
<tr>
<td>314 - 320</td>
<td>786.4</td>
<td>1217.9</td>
<td>FCIALGY</td>
<td>1</td>
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<tr>
<td>313 - 320</td>
<td>949.5</td>
<td>1381.0</td>
<td>YFCIALGY</td>
<td>2</td>
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<tr>
<td>315 - 330</td>
<td>1741.9</td>
<td>2173.4</td>
<td>CIALGYTNSSLNPILY</td>
<td>1</td>
</tr>
</tbody>
</table>
Prepulse inhibition (PPI) means the inhibition of a startle reflex when a prepulse happens before the startling stimulus, such as a loud sound (238-241). Clinical studies have shown that schizophrenic patients have deficient PPI. PPI can be disrupted by psychomimetics, and the reversal of this impaired PPI was used to predict potential antipsychotic drugs. Because the parent compound of RB-64 is salvinorin A, the most potent naturally occurring hallucinogen (1), we predicted that both RB-64 and salvinorin A have effects on PPI. And we observed an extraordinarily high potency of RB-64 as compared to salvinorin A. Experimental details are shown in chapter 2. This part of work was performed in collaboration with Dr. William Wetsel (Duke University).

**Null Activity** — Salvinorin A and RB-64 produced changes in activity during null trails (without any stimuli) compared to vehicle-treated mice (Table 5.5). ANOVA demonstrated significant effects for treatment (dose nested in compound) \[ F_{8,88} = 9.612, p < 0.001 \]. Mice treated with salvinorin A showed significant increases in null activity with 0.5 mg/kg \( (p < 0.001) \) compared to all other doses of the compound and the vehicle controls. Effects of RB-64 were somewhat different. The two lower doses of RB-64 exerted little effects on null activity, whereas the two highest doses resulted in increased activity compared to the vehicle control \( (p < 0.001) \).
Table 5.5 Null activity levels (mAmp displacement) in mice treated with vehicle, salvinorin A, or RB-64.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Null Activity (mAmp displacement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Tween Vehicle</td>
<td>11.16 ± 4.34</td>
</tr>
<tr>
<td>salvinorin A</td>
<td></td>
</tr>
<tr>
<td>0.25 mg/kg</td>
<td>19.44 ± 5.83</td>
</tr>
<tr>
<td>0.5 mg/kg</td>
<td>28.79 ± 3.99*</td>
</tr>
<tr>
<td>1.0 mg/kg</td>
<td>13.63 ± 3.65</td>
</tr>
<tr>
<td>2.0 mg/kg</td>
<td>13.76 ± 3.65</td>
</tr>
<tr>
<td>RB-64</td>
<td></td>
</tr>
<tr>
<td>0.005 mg/kg</td>
<td>8.22 ± 3.73</td>
</tr>
<tr>
<td>0.01 mg/kg</td>
<td>16.81 ± 4.4</td>
</tr>
<tr>
<td>0.05 mg/kg</td>
<td>64.89 ± 10.88*</td>
</tr>
<tr>
<td>0.1 mg/kg</td>
<td>51.6 ± 12.16*</td>
</tr>
</tbody>
</table>

*p < 0.05 compared to vehicle controls.

Baseline Startle Activity — Mice treated with salvinorin A or RB-64 showed dose-dependent parabolic changes in baseline startle activity (Figure 5.6a). ANOVA revealed significant main effects of salvinorin A and RB-64 treatment \( F_{8,88} = 13.197, p < 0.001 \). Bonferroni corrected pair-wise comparisons confirmed the dose-dependent effects of salvinorin A on startle activity. The 0.25 and 0.5 mg/kg doses produced slight changes in baseline startle responses, whereas 1 mg/kg salvinorin A resulted in enhanced startle responses relative to the vehicle control \( p < 0.004 \) and 0.25 and 2 mg/kg salvinorin A\( p < 0.034 \). Mice given RB-64 also showed dose-dependent parabolic changes (Figure
5.6a). Bonferroni comparisons revealed that the lowest dose of RB-64 had no effects on the startle response, whereas the 0.01 mg/kg dose produced augmented startle responses compared to vehicle ($p < 0.001$) and all other doses of RB-64 ($p < 0.002$).

**PPI** — Overall PPI was affected by salvinorin A (Figure 5.6b) and RB-64 (Figure 5.6C). ANOVA yielded significant main effects of treatment for overall PPI [$F_{8,88} = 6.606$, $p < 0.001$]. Bonferroni corrected pair-wise comparisons found that overall inhibition was similar for vehicle and 0.25, 0.5, or 1 mg/kg salvinorin-A, while 2 mg/kg significantly suppressed inhibition ($p < 0.044$). By comparison, 0.01 and 0.05 mg/kg RB-64 resulted in marked increases in overall inhibition compared to the vehicle control ($p < 0.021$). Mice given 0.1 mg/kg RB-64 showed a significant reduction in overall inhibition relative to vehicle-treated mice ($p < 0.046$).

Mice treated with vehicle showed prepulse-dependent PPI. Administration of salvinorin A (Figure 5.6c) or RB-64 (Figure 5.6c) resulted in a loss of prepulse-dependent PPI and the highest dose of each compound decreased overall inhibition. RMANOVA demonstrated significant within-subject effects of prepulse intensity [$F_{3,264} = 30.084$, $p < 0.001$] and a significant prepulse intensity by treatment interaction [$F_{24,264} = 4.602$, $p < 0.001$]. Bonferroni corrected pair-wise comparisons confirmed prepulse-dependent PPI in vehicle controls, with all prepulse intensities statistically different from each other ($p < 0.011$). Salvinorin A or RB-64 disrupted prepulse-dependent PPI at all prepulse intensities. Mice given 0.25, 0.5 or 1 mg/kg salvinorin A had increased PPI to the 4 dB prepulse relative to vehicle ($p < 0.051$) (Figure 5.6b); PPI responses at the 8 and 12 dB
prepulses were similar for animals administered vehicle, or 0.25, 0.5, or 1 mg/kg salvinorin A. However, mice given 2 mg/kg salvinorin A had marked reductions in PPI at the 8, 12, and 16 dB prepulses compared to vehicle \((p > 0.011)\) or all other doses of the compound \((p < 0.047)\); responses to 4 dB were unaffected from the vehicle control.

When animals were given 0.005, 0.01 or 0.05 mg/kg RB-64, PPI to the 4 dB prepulse were enhanced relative to vehicle \((p < 0.047)\) (Figure 5.6c). By comparison, 0.005 RB-64 depressed responses to the 12 and 16 db prepulses \((p < 0.010)\). The 0.01 and 0.05 doses increased PPI to the 8 dB prepulse relative to the vehicle controls \((p < 0.031)\). Similarly, 0.05 mg/kg RB-64 augmented responses to the 12 and 16 db prepulses \((p < 0.052)\). Responses to the highest dose of RB-64 were different than those for the 0.01 and 0.05 doses. Here, responses to the 12 and 16 dB prepulses were significantly depressed compared to the vehicle control \((p < 0.003)\).

Collectively, these data show that PPI responses to salvinorin A and RB-64 show some differences and some similarities. Overall PPI was reduced by the highest dose of salvinorin A, while 0.01 and 0.05 mg/kg RB-64 increased and 0.1 mg/kg RB-64 decreased PPI. By comparison, both salvinorin A and RB-64 disrupted prepulse-dependent PPI.
Figure 5.6 PPI responses to salvinorin A and RB-64 by C57BL/6J mice. (6a) Startle responses to the 120 db stimulus for animals administered different doses of salvinorin A or RB-64. (6b) Percent PPI to the 4, 8, 12, and 16 db prepulses for animals given various doses of salvinorin-A. (6c) Percent PPI to the 4, 8, 12, and 16 db prepulses for animals given various doses of RB-64. N = 10-16 mice/treatment. * p < 0.05, compared to vehicle controls; + p < 0.05, comparisons of prepulse-dependent PPI at the 4, 8, 12, and 16 dB prepulses within a single treatment; # p < 0.05, comparisons of startle responses (panel A) within compound to 0.5 mg/kg salvinorin A or 0.01 mg/kg RB-64.

5.3 Discussion

GPCRs are integral membrane proteins, which are well-known for their hydrophobic nature. GPCRs and their derived peptides tend to aggregate in solution, bind to the container wall and segregate from the matrix. It is still a big challenge to structurally characterize GPCR proteins by MS and only limited cases are reported (242-245). A
previous attempt by Dr. Howells’s group to characterize KOR using trypsin in-gel
digestion and MALDI-TOF MS only yielded 26% coverage (Poster presented in SFN
meeting 2006). Similar low coverage was also seen in other GPCR studies, including
CB1 (35%) and CB2 (29%) (242), MOR (37%) and DOR (28%) (245, 246). The
accessible peptides are usually located on N- and C-terminus, intracellular and
extracellular loops, while the major TM domains are largely invisible. Our primary goal
for the mass spectrometry study here was to identify the labeling site of RB-64. Matrix
assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was
used to in our research due to its high sensitivity and tolerance to contaminants (e.g.
salts). In our study, we also observed a low coverage 19-26% of total KOR sequence;
however, a significant part of the accessible peptides were derived from TM domains
(Figure 5.4), including the RB-64 labeling site on TM7 (YFCIALGY). Our ongoing work
involves using LC-MS to improve the coverage of total KOR sequence. The combination
of high performance liquid chromatography (HPLC) with mass spectrometry could help
separate the complex peptide sample and decrease the suppression of ion signal. In a
recent MS study of cannabinoid receptor (CB2), nanoLC combined with LTQ-FT and the
4000 Q-Trap mass spectrometers achieved ~90% coverage of CB2 sequence (242).

For our sample preparation, we used a tandem affinity purification approach and then
applied an in-gel chymotrypsin digestion of KOR. For complex protein mixtures, gel-
based electrophoresis is often a favorable choice due to its high capacity for protein
separation (247). In-gel digestion usually produces specific peptide fragments that are
detectable by the mass analyzer. Every protein has a unique set of peptides digested by an
enzyme (e.g. trypsin or chymotrypsin), leading to unique peptide mass pattern. Trypsin is the most commonly used protease, cutting the protein at the carboxyl side of R and K residues. However, for our KOR protein, trypsin will yield a very large peptide (MW 7039.7 Da), which falls out of the MS detection range (600~4000 m/z). Chymotrypsin is a better choice for KOR because it cleaves at the C-terminus of phenylalanine (F), tyrosine (Y), tryptophan (W), and leucine (L) when these residues are not followed by proline (P). Moreover, these hydrophobic residues are richly distributed in TM domains. In this approach, we do take the risk that chymotrypsin can cut our target peptide into very small pieces (CIALGY, MW 639.3); however a modified Cys has prevented the cleavage of nearby Y and F residues. So two-missed-cleavage peptide (YFCIALGY) within TM7 was the major product. It was clearly shown that this modified peptide (MW 1381.8) has a MW shift 431.5 as compared to the corresponding unmodified one (MW 949.5) (Table 5.4). Our ongoing research is to further confirm the labeling site by MS/MS sequencing of YFCIAGY.

Besides the mass spectrometric analysis of the labeling site of RB-64, we also applied traditional pharmacological approach to quantify the labeling effects of RB-64 and other compounds (Figure 5.2). Diffusible ligands bind to receptors mainly through non-covalent interactions, such as electrostatic forces, hydrogen bonds, van der Waals’ forces and hydrophobic effects. In contrast, covalently-bound ligands can directly link to the receptor protein, thus having unique properties and pharmacological profiles. As is shown in our research (Table 5.1), RB-64 and RB-48 both have high affinities, potencies and selectivity to KOR. Furthermore, under certain conditions, both compounds can
irreversibly bind to the KOR by direct covalent labeling (Figure 5.2). In solution, these two compounds can undergo different chemical reaction pathways, namely addition and substitution reactions. However, in a biological environment, such as the binding pocket of KOR, multiple unknown factors (e.g. ligand orientation, side chain reactivity, etc.) may not allow all the possible chemical reactions to occur. Our mass spectrometric analysis of the affinity labeling clearly showed that both compounds underwent substitution reaction with only one MW shift (431.5) present. It means both RB-64 and RB-48 underwent the substitution reaction, even though the isothiocyanate group in RB-64 was a favorable group for addition reaction.

Mass spectrometry is a powerful tool to study posttranslational modification of proteins (248). In our case, MS unambiguously identified the labeling site of covalently-bound ligands RB-64 and RB-48. Even though there are only a limited number of GPCRs have been studied by MS approach (242, 249-251), we are optimistic about the future of applying MS to structurally characterize GPCR proteins. The results obtained here validated our previous binding site model (2, 7, 168) and provided us insights into the mechanism for covalent modification of GPCRs. As a covalent binding ligand, RB-64 had shown great potential for being a molecular probe to explore opioid receptors, also for being a therapeutic agent due to its psychoactive nature.
Chapter 6: Future Directions and Conclusions

6.1 Future Directions

6.1.1 Quantifying G protein Expression

For our SCAM study (Chapter 4), we used G protein overexpression (2) to induce the conformational changes of KOR. Although the semi-quantification of G protein by antibody-blotting demonstrated an enhancement of the G protein-expression, the accurate G protein quantity remains elusive. Here I propose to use an MS-based Stable Isotope Labeling by Amino acids in Cell culture (SILAC) method to further quantify the G protein expression in HEK293 T, \( \text{G} \alpha_{16} \) and \( \text{G} \alpha_{i2} \) stably expressed cells as described in chapter 4. For classic SILAC (Figure 6.1), cells are grown in culture medium containing \(^{13}\text{C}\)-labelled frequently occurring amino acids, such as Arg and Lys (252, 253). Generally the isotope labeling requires a +4Da MW shift to avoid the undesirable overlap with the naturally occurring isotopic patterns from individual peptides. The use of \(^{13}\text{C}_6\) -Arg can give rise to a +6 Da MW shift and allow a safer assignment of a labeled peptide as compared with unlabeled one. Recently differential labeled Arg permits the comparison of three different cells in one experiment (254, 255). This new technique uses natural \(^{12}\text{C}_6\) -\(^{14}\text{N}_4\)-Arg, isotope labeled \(^{13}\text{C}_6\) -\(^{14}\text{N}_4\)-Arg (+6 Da), and \(^{13}\text{C}_6\) -\(^{15}\text{N}_4\)-Arg (+10 Da), which will permit our study of G protein expression in three types of cells (HEK293 T, \( \text{G} \alpha_{16} \) and \( \text{G} \alpha_{i2} \) stably expressed cells). During sample preparation, one type of cells is supplied with one kind of differentially labeled Arg. Next, each type of cells is counted and pooled together (1:1:1 ratio) before the lyses. Further purification (SDS-gel or LC) can preserve the quantitative information in the original cell types since the differentially
labeled proteins go through the same purification, separation, and mass spectrometric analysis process. The ratio of G protein is determined by examination of the MH+ ion signal intensities which contain $^{12}\text{C}_6-^{14}\text{N}_4\text{-Arg}$, $^{13}\text{C}_6-^{14}\text{N}_4\text{-Arg}$ (+6 Da), and $^{13}\text{C}_6-^{15}\text{N}_4\text{-Arg}$ (+10 Da) in the full spectra.

Figure 6.1 SILAC method for quantitative proteomics. Proteins are labeled metabolically by culturing cells in media that are isotopically enriched or isotopically depleted. Modified from Figure 3 of ref (252).

6.1.2 Chasing the Active and Global Conformation of KOR

My study of the KOR conformation mainly focused on TM7, the upper part of TM6 and EL2. My colleague, Dr. Timothy A. Vorterms, conducted a similar SCAM study on TM2 (160). These findings revealed a different pattern change for different TMs. TM 6 and 7 both showed a cluster pattern and there was a possible rotation of TM7. TM6 is well accepted being a toggle switch during the activation process of GPCR. TM2 didn’t show clearly the cluster pattern. However, SCAM can only give us an active-like conformation, not the true active conformation of KOR because there is no agonist
bound. One way to get an active conformation is to use our covalently-bound agonist, RB-64, to label KOR, then perform the SCAM. The difference in the SCAM pattern between RB-64 labeled and unlabeled KOR can reveal some subtle changes in KOR conformation. Another way to get a global view of the conformational changes of KOR during the activation process is by NMR or MS. Both methods have been able to obtain high-resolution structural information on proteins and receptor-ligand interactions (256). However, even with high-field magnets, selective labeling methods, and new pulse sequences, many proteins are too large for analysis by NMR. GPCR proteins are also difficult to be enriched to high concentrations required for NMR study. Here I propose to continue this conformation study by applying the MS-based Hydrogen/Deuterium exchange (H/D exchange) method. Amide H/D exchange is useful to study of protein structure and dynamics as well as receptor-ligand interactions (256-258). The degree to which an amide hydrogen is exposed to solvent reveals the details of its environment. Conformational changes induced by ligand binding cause variations in amide exposure, and these variations can be determined by measuring the rate of amide hydrogen exchange with solvent deuterium. Importantly, there is almost no limitation for protein concentrations used for H/D exchange. To compare the conformational changes during the activation process, H/D exchange experiments will be performed on both the free KOR and KOR-RB-64 complex. The difference in exchange kinetics can reveal the precise information in receptor-ligand interactions. Mapping the whole protein by H/D exchange kinetics can thus identify ligand bound (active) and unbound (inactive-like) KOR conformations.
6.1.3 Crystallographic Study of KOR

The crystallographic study of GPCR proteins still face many challenges (259), such as GPCR expression, purification and crystallization. The conformational plasticity of GPCRs makes the situation even worse. Up till now, only two GPCR structures have been solved from high resolution X-ray data (17, 18, 25, 182). Among these available structures, rhodopsin has a high resolution inactive-state and a low resolution active-state structures and the β2AR has a high resolution inactive-like structure. The determination of an active-state structure would fill a large gap in our understandings of GPCR structure, and would provide more insight into GPCR function. Interestingly these two GPCRs (rhodopsin and β2AR) were all co-crystallized with their corresponding ligands. As we have detailed in chapter 5, the covalently-bound ligand RB-64, an agonist of KOR, provides an unprecedented opportunity for stabilizing KOR in active states as well as initiate crystal lattice formation. Our growing knowledge in KOR expression, purification and separation also added more hope for this challenging task.

6.2 General Conclusions

In this dissertation, I have detailed our study of the interactions between salvinorin A and KOR through combined mutagenesis/computer modeling method. I also examined the role that G protein overexpression played on KOR conformational changes. Our findings revealed the molecular mechanism by which a small-molecule, salvinorin A, binds to and activates KOR. Salvinorin A not only uses the majority of its flexible functional groups (C-2, C-4 and C-12 functional groups) to have the optimal interactions with KOR, thus
stabilizing itself in the binding site with about -11 kCal/mol, but also takes advantage of
the conformational changes induced by G protein-coupling (mainly Ga(i2)) which leads to
active state stabilization and activation of a series of downstream signaling events. GPCR
signaling seemingly is not only regulated by the receptor itself and its agonist, but also by
the cellular environment in the case of our data with G proteins. All these observations
point to another level of complexity for GPCR signaling. Meanwhile, our rational design
of covalently-bound ligands based on salvinorin A’s structure further validated our
understanding of KOR and salvinorin A interactions. As a molecular probe, the covalent
ligand (RB-64) provides us with unprecedented opportunities to elucidate the structure
and function of KOR. It is our hope that a better understanding of opioid receptors, and
all other GPCRs, will facilitate advances in drug developments and greatly improve
human life.
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