2002

Signal transduction and G-protein incorporation properties of human serotonin 1A receptors

Harish Venkat Thiagaraj

The University of Montana

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SIGNAL TRANSDUCTION AND G-PROTEIN INCORPORATION PROPERTIES OF HUMAN SEROTONIN 1A RECEPTORS.

HARISH VENKAT THIAGARAJ

B.Pharm, ANNAMALAI UNIVERSITY, INDIA

Presented in partial fulfillment of the requirements for the degree of Master of Science

The University of Montana

February 2002

Approved by

[Signatures]

Chairperson

Dean, Graduate School
The human (H) serotonin (5-hydroxytryptamine; 5HT) 1A receptor (R) is implicated in a number of disorders including migraine headache, depression, and anxiety. H5-HT1AR is a member of the superfamily of G protein coupled, 7 transmembrane domain receptors (GPC7TDR’s) and is closely related to the beta adrenergic receptors. Because of these characteristics and an advanced level of study, the H5-HT1AR is a model system among the GPC7TDR’s and we have utilized the receptor for a series of structurally-based investigations.

The specific goal of the current studies is to better understand structure-activity relationships at the IL3 and IL2/G protein surfaces. Taking advantage of the known primary structure of the receptor, we have synthesized a series of peptides (P’s) from putative intracellular loop (IL) regions of the receptor. We have investigated the ability of these peptides to interfere with receptor-G protein coupling (agonist inhibition assays) and in some cases to directly stimulate G protein (cAMP assays; and [$^{35}$S]y-S-GTP incorporation). In the studies outlined here, the focus is on two new peptides (P8 and P9 from IL3), which will be compared to previous peptides.

Additionally, P11, a partially characterized IL2 peptide, was studied in greater detail. P11 was active in uncoupling receptor from G protein, but inactive in triggering signal transduction. P1, P8, and P9 were active in both the uncoupling and signal transduction realms to various degrees. In a unique finding, P9 decreased basal levels of [$^{35}$S]y-S-GTP incorporation. For comparative purposes, the non-peptide dipropyltryptamine (DPT) was also examined as a competitive ligand-binding site agent at H5-HT1AR. These results supplement the fundamental hypothesis that the N-terminus of IL3 of H5HT1AR is critical to G protein coupling and activation, while the N-terminus of IL2 is responsible for coupling only.
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ABBREVIATIONS

1. 5-HT : 5-Hydroxytryptamine
2. 7TMD: 7 Transmembrane Domain
3. 8-OH-DPAT: 8-hydroxy-di-n-propylamino tetralin
4. cAMP: cyclic AMP
5. CHO: Chinese Hamster Ovary
6. DMT: Dimethyltryptamine
7. DPT : Dipropyltryptamine
8. GABA : Gamma-aminobutyric acid
9. GDP : Guanine Mono-phosphate
10. GMP: Guanine Di-Phosphate
11. GPCRs: G-Protein coupled receptors.
12. GTP : Guanine Tri-Phosphate
13. H-5HT1AR : Human 5-HT 1a Receptor
14. IL2: Intracellular loop 2
15. IL: Intra-cellular loops
16. TM: Transmembrane
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ACKNOWLEDGEMENTS

It is a pleasure to thank the many people who made this thesis possible. The list is never ending and I hope to mention at least a few of them here.

It is impossible to overstate my gratitude to my mentor and Committee Chairman, Dr. Keith Parker, whose enthusiasm, inspiration, and his constant appreciation helped me to realize how much fun Science can be. The opportunities and knowledge provided by you Dr. Parker is immeasurable by any means. Throughout the 2 years I spent in his lab you have provided encouragement, sound advice, excellent teaching, great company, and lots of good ideas. I would have been lost without you. To me you would always be the “Best Boss” I would ever have.

Special thanks to Dr. Charles Eyer, Dr. Steve Lodmell and Dr. Ethan Russo for sitting on my Thesis committee. The contributions from these three scientists to my thesis were infinite. Their honest commentary on the work, made this thesis turn out far better than intended. I would like to acknowledge once again, their excellent ideas and cooperation.

Lucia Muniz, Celine Wishcamper, and all my other fellow graduate students for their constant encouragement and most of all their companionship. I don’t think I would better colleagues to go through Graduate School than the great group here. Carol, Brian Andrea and all the other crew from the Parker lab for being great colleagues and most of all for putting up with me!

Faculty and Staff at the Department of Pharmaceutical Sciences, for their encouragement and invaluable inputs. The School of Pharmacy, Department of Pharmaceutical Sciences, and the Graduate school, for giving me this wonderful opportunity to be here, and make my stay as comfortable as possible.

Ganesh, Navin, Steve, Alex, and the whole gang, I cant thank you enough for “adopting” me the three years I was here. I would cherish my moments with you all for the rest of my life. Most of all I thank you for being “family” to me.

Missoula and Montana for providing me three years of non-stop fun and the great outdoors. The people of Missoula for being the “friendliest people of the world”.

Lastly, and most importantly, I wish to thank Mom Dad, and my brother Girish. thank you for having me, raising me, supporting me, teaching me and most of all loving me. To you I dedicate this thesis.
Functions of Serotonin

Serotonin (Fig. 1) or 5-hydroxytryptamine (5-HT) is an indolealkylamine derivative that was first recognized as a powerful vasoconstrictor occurring in blood serum (Erspamer, 1966). It was partially purified, crystallized, and named in 1948 (Rapport MM, 1948), and its structure was deduced a year later. It has been functionally implicated in a number of disorders including migraine headache, depression and anxiety (Barnes, 1999). 5-HT has apparent implications in Obsessive Compulsive (OCD) and Panic disorders (Klein, 1991). Independent work indicated that serotonin was widely distributed in nature and occurred in tissues other than blood. It has been shown to be in many representatives of the animal kingdom, in wasp stings and scorpion venom, in various fruits, such as pineapples, bananas, and plums, and in various nuts.

It has been estimated that an adult human contains about 5 to 10 mg of serotonin, 90% of which is in the intestine and the rest in blood platelets and the brain (Deliganis, 1999). One role of the compound is as a neurotransmitter, whose participation is being investigated in diverse functions including learning, sleep, and control of mood (Pierce, 1989; Peroutka & S.J., 1995). The structural similarity of serotonin to several drugs known to cause mental aberrations, such as LSD, has prompted much speculation as to the role of serotonin in naturally occurring mental disorders such as schizophrenia or depression (van Zwieten et al., 1990)

5-HT is synthesized, from the amino acid L-tryptophan, in neurons and stored in vesicles. Serotonin is found in three main areas of the body: the intestinal wall; large constricted blood vessels; and the central nervous system. The most widely studied
effects have been those on the central nervous system. The functions of serotonin are numerous and appear to involve control of appetite, sleep, memory and learning, temperature regulation, mood, behavior (including sexual and hallucinogenic behavior), cardiovascular function, muscle contraction, endocrine regulation, and depression (Shih JC, 1995). In some cases, 5-HT’s role is modulatory; however, there are cases such as sleep and in certain types of hallucination, where 5-HT function appears to be the primary determinant.

![5-Hydroxytryptamine](image)

**Figure 1**

**5-HYDROXYTRYPTAMINE**

**5-HT Receptors**

There were initially 2 receptors which were found to mediate the actions of serotonin (Gaddum & Picarelli, 1997). With progress of years of research and the employment of radiolabeled ligands a third class of the 5-HT receptors was identified (Peroutka et al., 1979). The last decade has seen the identification of numerous new subtypes of the 5-HT receptor. This was possible mainly due to the advancement of molecular biological techniques (Wishart, 1999). Currently there are seven types of the receptor which is further classified into various subtypes (Hoyer D, 1994). Most of these subtypes were classified based on their pharmacological activity (Hoyer D, 1994). Some
subtypes were initially identified by sequencing cDNA-deduced primary amino acid sequences (Peroutka & S.J., 1995) and in other cases, similarities in signal transduction assisted in classification.

All the 5-HT receptors except the 5-HT3 receptor belong to the superfamily of 7 transmembrane G-protein coupled receptors (Maricq et al., 1991; Hoyer D, 1994). The 5-HT3 receptor is closely linked to GABA, AMPA/kainate, cholinergic (nicotinic) and glycine receptors, which fall under the category of ion-channel receptors. The 5-HT3 receptor, like all ligand-gated receptors, has a large glycosylated extracellular N-terminus, with 4 subsequent transmembrane segments (Maricq et al., 1991)

![Figure 2](image)

**Figure 2**

2-DIMENSIONAL REPRESENTATION OF A TYPICAL GPCR, SHOWING THE HYDROPHOBIC DOMAINS INSERTED INTO THE PLASMA MEMBRANE

The 5-HT receptors (excepting 5-HT3) belonging to the 7 transmembrane family are coupled to heterotrimeric guanine nucleotide binding G-proteins. (Strader, 1994). The
superfamily of G-protein coupled receptors (GPCRs) constitutes the largest class of cell surface receptors, and thus represents the primary mechanism by which cells sense and respond to their external environment (Fig. 2)(Marchese, 1999). GPCRs transduce their signals across the plasma membrane via an interaction with heterotrimeric G-proteins, which leads to an activation of intracellular effectors such as adenylyl cyclase (AC) or phospholipase C (PLC) and subsequent generation of second messengers such as cAMP (cyclic adenosine-monophosphate) or calcium (Dratz, 1993). These effects are amplified and transmitted down through a cascade of intracellular events leading eventually to the physiological response of the cell to the stimulus (Marinissen & Gutkind, 2001).

The enormous diversity of receptors, G-proteins and effectors, together with the widespread distribution of receptors across many tissues,(Nebigil et al., 1995) reflects the important role that this family of proteins plays in regulating physiological and pathophysiological processes (Martin, 1999). Table I lists the various G-proteins which couple with multiple 5-HT receptor subtypes and their functions. These GPCRs possess a multiple glycosolated extracellular amino terminus (N-terminus) and intracellular carboxy tail. They are comprised of 7 transmembrane (TM) domains (Fig. 3) which form α helices arranged in distorted cylinder like fashion, the extracellular side of this core forming the ligand-binding domain (Baldwin, 1994)

**G Protein Coupled Receptors**

3 intracellular loops (IL) are located between the transmembrane helices (Bourne, 1997). These loops are the putative sites of interaction with the heterotrimeric G-protein. The structure of the GPCR’s have been an challenging to elucidate, because they are
membrane bound and difficult to crystallize. The bacteriorhodopsin model was an excellent first representation of the 7TM/GPCR. Though the bacteriorhodopsin model is not G-protein coupled, it has been a very useful initial evaluation tool of all GPCRs in general. Rhodopsin,(Palczewski et al., 2000) which is G-protein coupled is now the accepted prototype for this class of receptors (Acharya et al., 1997; Bamidge, 1997; Unger, 1997).

![Figure 3](image)

**Figure 3**

**BALDWIN ARRANGEMENT OF 7 A-HELICES IN GPCR**

G-proteins are composed of three subunits (alpha, beta and the smaller gamma subunits)(Bikker, 1998). Beta and gamma are, in turn, tightly associated as a dimer. The
alpha subunit shares homology with the GTPase family, possesses intrinsic GTPase activity and contains sites for myristoylation and palmitoylation (Casey, 1995). All gamma subunits contain sites for isoprenylation. These acetylations target G-proteins to the membrane compartment, the principal site for G-protein signaling. Four G-protein subfamilies have been identified and classified according to the known 23 alpha subunits (Gq/11, Gi/0 Gs and G12/13) (Mulheron, 1994). There are a similar variety of beta and gamma subunits, which are classified according to association with alpha subunits. These G-protein subfamilies can be either stimulatory (Gq/11,Gs) or inhibitory (Gi/0) (Burstein et al., 1996).

Ligand binding results in a conformational change in the receptor cytoplasmic domain (Sheikh SP, 1996) (Farrens, 1996) that promotes association with an inactive GDP-bound heterotrimeric G-protein (Fig 4). This interaction enhances dissociation of GDP from the receptor/G-protein complex, facilitating GTP binding, alpha subunit activation, (Onrust et al., 1997) and dissociation from the receptor. Dissociation of G-protein from the receptor results in alpha subunit dissociation from the trimeric complex, facilitating its interaction with effector molecules such as AC. Intrinsic alpha subunit GTPase activity catalyses GTP hydrolysis causing inactivation, a process often enhanced or activated by effector binding. The GDP-bound alpha subunit re-associates with beta and gamma subunits to form an inactive G-protein heterotrimer, which is once again capable of interacting with the receptor. (Bowler et al., 1998). It is critical to note for the work discussed in this thesis, that the G-protein bound form of the receptor has high agonist affinity, while the uncoupled receptor has a lower affinity state.
SCHEMATIC REPRESENTATION OF A GPCR, THE GTP CYCLE AND LINKAGE TO AN EFFECTOR, ADENYLYL CYCLASE.
The 5-HT1A receptor is one of the most studied receptor types among the 5-HT receptors. Structurally, as mentioned in the previous paragraph, the 5-HT1A receptor is a member of the superfamily of G-protein coupled 7 transmembrane domain receptors and is closely related to β adrenergic receptors (Kobilka, 1987; Fargin, 1988; Raymond, 1999). Human (H) 5-HT1AR is intronless and codes for a core sequence of 422 amino acids, yielding a molar mass of about 46,000. The actual molecular weight, however, is much higher as the extra-cellular N-terminus is heavily glycosylated.

The receptor is negatively coupled to adenylyl cyclase, and principally causes hyperpolarization (Sanders, 1998). In the CHO cells used in this study (Raymond et al., 1993), Gia's 2 and 3 are the primary coupling subunits (Arthur et al., 1993). There is also evidence that some 5-HT1A receptors are positively coupled to adenylate cyclase; this may be accounted for either by the existence of different types of 5-HT1A receptors or the coupling of 5-HT1A receptors to different G-proteins (Zifa & Fillion, 1992). Interestingly, 5-HT1A receptors in the raphe nuclei, act as somatodendritic autoreceptors which inhibit neuronal cell firing and 5-HT release onto postsynaptic sites.

Several agonists show selectivity for the 5-HT1A receptor, particularly 8-hydroxy-di-n-propylamino tetralin (8-OH-DPAT), which acts as a full agonist in most systems, while the non-benzodiazepine anxiolytics, buspirone, ipsapirone and gepirone, and other ligands such as MDL 72832 are partial agonists. The synthesis of selective and silent antagonists at this receptor has proved more difficult. Several apparent antagonists have been characterised, such as NAN 190, BMY 7378, MDL 73005EF, WAY 100,135,
UH 301, spiroxatrine and SDZ 216525. However, all have demonstrated partial agonist properties in studies of somatodendritic autoreceptor function, perhaps due to the much larger receptor reserve associated with these as opposed to postsynaptic receptors. To date, the only selective high affinity silent antagonist at this receptor is WAY 100,635 (Fletcher et al., 1993).

5-HT1A Receptor-G-Protein Interface:

The major aim of this thesis is to better understand the 5-HT1AR-G-Protein Interface. To better understand the dynamics of this system, we have designed putative peptides (Taylor & Neubig, 1994; Palm D, 1995), which mimic various portions of the Intracellular-Loops of the 5-HT1AR. To particular interest to our thesis was Intracellular loop 3 (IL3) and also to a small extent Intracellular loop 2 (IL2). There is very strong evidence that G-Proteins interact at these loops (Merkouris et al., 1996; Thompson, 1998). Our interest was to zoom into these sites of interaction and identify putative regions of both IL2, (Lembo et al., 1997) where the loops are responsible for coupling or activation of G-Protein or both. Fig 5 describes in detail, the various regions of interest of the Human 5-HT1A Receptor-G-Protein Interface.

The putative peptides when administered into the cell, will compete with Intracellular loops to couple to G-Protein. If the peptides are representative of regions on Loop 2 where G-Protein couples, the peptide will uncouple G-Protein from Intracellular loop(s). This uncoupling of G-Protein from the Intracellular loops will result in a decrease in the affinity of the receptor for its ligand (8-OH-DPAT in our case). This in other words is an
indirect Agonist Inhibition effect. Thus by estimating the ‘Agonist Inhibition’ effect of these peptides we will be able to estimate the uncoupling properties of these peptides.

Activation of G-Protein will result in the G-Protein to couple to its effector (Adenylyl cyclase in our case). This will result in cascade of signaling events resulting in producing of a signaling response. In our system, since the 5-HT1AR is negatively coupled to Adenylyl cyclase, the signal would be a lowering of intracellular cAMP. Thus if the putative peptides represent a region of the Intracellular loop(s) responsible for activation of G-protein, the peptide should activate G-Protein by itself. Activation of G-Protein is also estimated by Incorporation of γ-S-GTP. Details of this approach are explained in Methods.
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<th>G-protein</th>
<th>Effector pathway</th>
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<tr>
<td>5-HT1a</td>
<td>Gi</td>
<td>Inhibition of adenylate cyclase</td>
</tr>
<tr>
<td></td>
<td>Gi</td>
<td>Opening of K+ channel</td>
</tr>
<tr>
<td></td>
<td>Go</td>
<td>Closing of Ca2+ channel</td>
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<tr>
<td>5-HT1b</td>
<td>Gi</td>
<td>Inhibition of adenylate cyclase</td>
</tr>
<tr>
<td>5-HT1dα</td>
<td>Gi, other ?</td>
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</tr>
<tr>
<td>5-HT1dβ</td>
<td>Gi (probably same as 5-HT1b)</td>
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<td>Gi</td>
<td>Inhibition of adenylate cyclase</td>
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<td>Gq</td>
<td>Phosphoinositide hydrolysis, ↓ K+ conductance</td>
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<td>Gq</td>
<td>Phosphoinositide hydrolysis, ↑ intracellular Ca2+</td>
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<td>Gq</td>
<td>Phosphoinositide hydrolysis, ↓ K+ conductance</td>
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</table>

SPECIFIC AIMS

Extracellular Space

Figure 5

SCHEMATIC REPRESENTATION OF THE LOCATION OF VARIOUS PEPTIDES OF INTEREST AND THEIR REPRESENTATION OF THEIR RESPECTIVE REGIONS IN INTRACELLULAR LOOPS 2 AND 3 OF THE 5-HT1AR

Peptides P11 represents the N-terminal end of Intracellular loop2. Peptides P1, P8 and P9 represent the native region N-terminal end of Intracellular loop 3. P8 and P9 are substitutes and truncates of Peptide P1.
SPECIFIC AIMS

A. To determine the uncoupling (agonist inhibition) and signal transduction (cAMP) capabilities of peptides P8 and P9.

B. To examine the concentration-dependency of agonist inhibition for P8 and P9 as well as parent peptides P1 and P11.

C. To develop GTP incorporation assays for studying peptide stimulation of G protein (peptides P1, P8, P9, and P11 will be utilized).

<table>
<thead>
<tr>
<th><strong>PEPTIDE</strong></th>
<th><strong>SEQUENCE</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>IFRAARFRIRKTVKK</td>
</tr>
<tr>
<td>P8</td>
<td>YGRIFRAARFRIRKTVKK</td>
</tr>
<tr>
<td>P9</td>
<td>RFRIRKTVKK</td>
</tr>
<tr>
<td>P11</td>
<td>IALDRYWAITD</td>
</tr>
</tbody>
</table>

TABLE II
PRIMARY SEQUENCES OF VARIOUS PEPTIDES INVESTIGATED
MATERIALS AND METHODS

Materials

Culture Media and Reagents:

Hams F-12 Medium (F12); Dulbecos Modified Eagle Medium (DMEM); Earles Balanced Salt Solution (EBSS); Trypsin – GIBCO/Life Technologies, Grand Island, New York. Calf Serum (CS); Fetal Bovine Serum (FBS) – Summit Biotechnology, Fort Collins, CO. Geneticin (G418 Sulphate) – Calbiochem, La Jolla, California

Reagents:

HEPES buffer – U.S. Biochemical, Cleveland, Ohio. EDTA; Tris Buffer; CaCl2; Diethyl ether–J.T. Baker, Phillipsburg, New Jersey. Mianserin; Pargyline; Isobutylmethylxanthine (IBMX); Sodium Deoxycholate; Aprotonin; Leuepeptin Benzamidine; Forskolin – Sigma Chem Co, St. Louis, MO. [3H]Ketanserin; [3H]8-OH-DPAT; [35S]γ-S-GTP–NEN, Boston MA. DMSO– Mallinckrodt, St. Louis, Missouri. EIA Direct cAMP Kit–Assay Designs, Ann Arbor, MI. Serotonin– ICN Pharmaceuticals, Irvine CA.

Other:

All other reagents were standard scientific grade, typically from general scientific supplies such as VWR and Fisher. Glassware, small apparatus, and any additional items were scientific grade from standard suppliers.
Methods

Cell Culture

Chinese Hamster Ovary (CHO) cells (transfected with the H5HT1A receptor gene) were obtained from Dr. John Raymond, Medical University of South Carolina. They were sub-cultured as a monolayer in 80 cm² or 175 cm² flasks. The medium for cell culture for this cell line was Ham’s F-12 medium. Medium was fortified with 10% Fetal Bovine Serum and 200 μg/ml Geneticin for selection of the transfected phenotype. Cultures were incubated at 37°C under a humidified atmosphere of 5% CO₂, 95% air. The cells were plated to reach confluency in approximately 7 days. At confluency, the cells were harvested using trypsin (0.25%) and used for assay or sub-cultured for later experiments.

Receptor Preparation

The first step of the assay is to prepare receptors from transfected CHO cells. Harvested cells were maintained at 4°C until utilized for the assay. The cells were sedimented in a super speed centrifuge @ 2000 RPM following dilution with ice-cold, serum-fortified medium at 4°C for 10 minutes. The pellet resulting from this spin was resuspended in Earle’s Balanced Salt Solution (EBSS) and again centrifuged for 10 minutes at the same speed. Based upon the receptor preparation desired, the cells obtained from the above rinsing procedure were treated as follows:
A: **Whole Cells:** The cells were gently suspended in 30 mls of ice cold binding buffer (50 mM Tris, pH 7.4; 4 mM CaCl2; 10 μM pargyline). The cells were used directly in the assay.

B: **Membranes:** Homogenization of the cells results in a crude membrane preparation of the receptor. This was done by resuspending the rinsed cell pellet in ice-cold binding buffer, homogenization on teflon-glass, and centrifugation at 5000 RPM for a period of 45 minutes at 4°C. This results in lysing of the cells. The final crude membrane fraction was re-suspended in 30 mls binding buffer and subjected to homogenization, first, on teflon-glass followed by brief exposure to a Brinkmann Polytron at setting 5 for a period of 10 seconds.

C: **Solubilized Receptor:** Cells were placed in 1 ml of solubilizing buffer (20 mM Tris, pH 8.0; 0.6% sodium deoxycholate; 1 mM EDTA; 1 mM dithiothreitol; 100 mM NaCl; 1 mM benzamidine; 10 μg/ml leupeptin; 100 mg/ml soybean trypsin inhibitor; and 10 μg/ml aprotinin) on ice for 1 hour (Mulheron et al., 1994). The mixture was then centrifuged for 300,000g-min. The supernatant, which carries the solubilized receptor, was removed, diluted with 30 mls of ice-cold binding buffer, and mixed gently prior to assay.
**Ligand-Receptor Binding Assay (Agonist Inhibition)**

As described in the Introduction section, the basis for Agonist Inhibition assays is that the peptides when uncoupling the G-Protein from the receptor will result in the lowering of the affinity state of the receptor. In our study we have used tritiated 8-hydroxy-2-(di-n-propylamino)tetralin [3H]8-OH-DPAT as the agonist and performed Agonist Inhibition assays to determine the uncoupling effect of the peptide. The displacement of [3H]8-OH-DPAT will indicate the affinity state of the receptor. An important point to note here is that the peptides were expected to uncouple G-Protein and change affinity rather than compete with [3H]8-OH-DPAT at the ligand-binding site.

The displacement of [3H]8-OH-DPAT from the receptors was determined using established protocols (Pierce, 1989; Hayataka, 1998; Ortiz, 2000). The assays were carried out by combining 700 µl receptor preparation, 100 µl binding buffer (total binding) or 100 µl binding buffer containing 10 µM serotonin (to determine non-specific binding), 100 µl of peptide (various concentrations), and 100 µl of [3H]8-OH-DPAT (varying concentrations), yielding a total volume of 1 ml. The mixture was made in triplicates and incubated at 30°C for a period of 30 mins. The assay was then stopped using 4 ml of ice-cold Tris buffer (50 mM, pH 7.4) followed by immediate vacuum filtration over Whatman GF/B filters (GF/F in the case of solubilized receptor). This was followed by 2 washes with 5 ml of buffer. The filters were then counted in 5 mls of Ecoscint liquid scintillation fluid in a Beckman LS 6500 scintillation counting system. To achieve a nominal value of 50 µg/filter, receptor preparation proteins were determined by the Bradford colorimetric method (1976).
cAMP Assay

Confluent, intact CHO cells were used to determine cAMP formation. Medium from cells grown in 24-well plates was removed. After two rinses with serum-free medium, cells were treated with 0.5 ml serum-free medium containing 100 μM isobutylmethylxanthine (IBMX), and one or more of the following agents (final concs.): forskolin (30 μM); 5-HT (0.1-10 μM); peptides (various concentrations). Incubation with agitation proceeded for 20 min. at 37°C. The reaction was stopped by aspiration of the medium, and the cells were incubated with 0.5 mls of 100 mM HCl for 10 min. at room temperature. Well contents were removed and centrifuged at 4000 rpm for 15 min. at room temperature. Aliquots of supernatant were used to determine cAMP in a direct enzyme-linked immunoassay with a kit from Assay Designs. The microplate was read at 405 nm with a 96-well reader.

γ-S-GTP Binding Assays

γ-S-GTP binding assays were designed to verify if the peptides activated G-Protein. Though this was accomplished by cAMP assays to a far extent, these assays are rather a more direct method of measurement of G-Protein estimation than cAMP assays. Normally the G-Protein incorporates some basal GTP as part of their GTP-GDP cycle. Receptor stimulation will result in an increased incorporation of GTP. Thus by measuring the amount of G-Protein will indicate the degree of activation of the receptor. In actual conditions GTP possesses intrinsic GTPase activity hence for our experiments we used a stable analog of GTP - γ-S-GTP. The incorporation of γ-S-GTP is an outcome of the activation of G-Protein by the peptides.
The steps involved in the cell culture/harvesting were identical to the agonist inhibition assay until we reached the point of centrifugation with binding buffer. At this point the cells were centrifuged using HEPES buffer (20 mM HEPES, pH 7.4; 5 mM MgCl2; 1 mM EDTA; 1 mM dithiothreitol; 100 mM NaCl; 10 μM GDP; 10 μM pargyline; 0.2 mM ascorbate) at the same speed and time as in the agonist inhibition assay. After this step, the crude membranes were re-suspended in 15 mls of HEPES buffer, and homogenized with a Teflon-glass homogenizer. In the assay (Wieland, 1994), 350 μl of the crude membranes were combined with 50 μl of peptide solution (various concentrations) and/or 5-HT (various concentrations) and 100 μl of 0.1 nM [35S] γ-S-GTP and incubated for 30 min. at 30 °C. The mixture was filtered on GF/C filters, and washed twice in buffer followed by drying and liquid scintillation counting. Negative control was the above minus the peptide. Non-specific binding was determined in the presence of cold γ-S-GTP (10 μM). Positive control was H5-HT1AR membranes in the same incubation mixture plus 0.1-10 μM 5-HT.

**Peptide Synthesis and Preparation**

Peptides were synthesized at the University of Montana's Murdock Molecular Biology facility utilizing modifications of classical solid phase techniques (Marglin & Merrifield, 1970; Ortiz, 1999) on an ABI 431A automated peptide synthesizer. Following synthesis, peptides were solvent extracted and subjected to chromatography in acetonitrile/2mml/1 HCL gradients with a Waters 625 HPLC System. Peptides were lyophilized and stored desiccated at −20°C. For use, peptides were initially dissolved in de-
ionized water or 5% DMSO. Subsequent dilutions of peptides were in binding buffer. Control experiments revealed no effect of DMSO in the assay systems at the highest final concentration of DMSO (0.5%).

**Data Analysis**

Results of binding experiments are expressed as percentage of controls. IC50s were determined from non-linear regression analysis of dose response curves. Inversion plots were analyzed by best-fit regression analysis, with results expressed as ratios using maximal binding in absence of inhibitor as reference value. For cAMP quantification, standard curves were prepared and subjected to linear regression analysis. Results are expressed as percent of forskolin-stimulated controls. In the case of γ-S-GTP incorporation, results are expressed as percent of basal incorporation (i.e. the control value when 5-HT or other experimental ligands are absent). In all cases both computer assisted and manual data analysis occurred. Statistically, results are expressed as mean plus or minus standard error of the mean. When significance was determined, a two-tailed Student’s t-test was utilized.
RESULTS AND DISCUSSION

Peptide P8

Hypothesis

P8 represents a segment of H5-HT1AR’s native IL3 responsible for both receptor-G protein coupling and G protein activation. Its activity will be superior to P1.

Sequence

YGRIFRAARFRIRKTVKK

Introduction

Peptide P8 is structurally similar to parent peptide P1 from the TM5/IL3 interface, except that P8 has an additional three amino acids at the N-terminal end. The interface between transmembrane segments and intracellular loops is hypothetical. Based upon hydropathy analysis, putative designations between TM segments and IL’s have been assigned. Additionally, there is thought that the boundary is indistinct, perhaps changing slightly depending on environmental circumstances the receptor is exposed to. Therefore, we thought it wise to probe a short distance into the putative membrane region to explore whether this change increased or possibly decreased the activity of P1. P8 is the longest peptide that we have used. The overall expectation of this peptide was that it would very much behave like P1 and if it did or did not, we could further hypothesize on the role of the N-terminal end of the peptide sequence in coupling and/or activation.
Agonist Inhibition/Concentration-Dependency

Similar to results previously determined with P1 (Hayataka et al. 1998), P8 demonstrated concentration-dependent agonist inhibition in membrane preparations of H5-HT1AR (Fig. 5). As with other peptides, agonist inhibition was examined in all three receptor preparations (Table III).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC$_{50}$ (µmol/L)*</th>
<th>% Inhibition Of Forskolin Stimulated cAMP$^*$</th>
<th>% Change in $\gamma$-S-GTP Incorporation $^@$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P8</td>
<td>Whole Cell: 0.8 ± 4</td>
<td>24 ± 7</td>
<td>13 ± 2</td>
</tr>
<tr>
<td></td>
<td>Membrane: 5 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Solubilized: 27#</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE III: SUMMARY OF RESULTS FOR PEPTIDE P8 FROM THE TM5/IL3 REGION OF THE HUMAN 5-HT1A RECEPTOR**

*Whole cell, membrane, and solubilized indicate the preparation of receptor in which the IC50 was determined; n’s = 3-5 unique preparations in each case. $^*$Expressed as percent inhibition relative to controls treated with 30 µmol/L forskolin (FSK). $^@$Expressed as percent of background incorporation. $^#$Maximum Inhibition @ 10$^{-5}$ M

Overall, the range of these results (micromolar IC50’s for whole cell and membrane receptor preparations) is similar to P1 (Hayataka, 1998). However, there is an un-anticipated aspect to these results. Whole cell preparations were the most sensitive followed by membrane preparations with solubilized receptors being the least sensitive. This is arguably, an unusual expectation for P8, as the order of sensitivity is opposite to that seen for the parent, P1. While the result with solubilized receptors is incomplete due
to the difficulty of working with this poorly soluble peptide in the labile solubilized preparations, P8 appears to be less active against solubilized receptors compared to either of the other receptor preparations. It is distinctively possible that the anomalous result for P8 in solubilized preparations is an artifact of the difficulty in working with these labile receptors. Nevertheless, the order of sensitivity for P8 inhibition in membrane and whole cell receptor preparations is still reversed compared to P1.

The inversion plots adapting the Lineweaver-Burke formulation of enzyme kinetics (Fig. 6) provided a similar result to that seen for P9 (later this thesis). However, it has a small similarity to the parallel lines of P11 (later this thesis). From these results, we hypothesize that P8 is a non-competitive inhibitor at the H5-HT1AR. From this conclusion we further postulate that P8 binds to an allosteric site in the receptor complex and not at the ligand binding-site. However, the unusual order of sensitivity between receptor preparations remains unexplained, and the possibility that P8 interactions with the H5-HT1AR/G protein complex are more varied than indicated by non-competitive mechanisms is distinctly possible.

cAMP Determination

Figure 7 indicates the results of cAMP assays with P8 and 5-HT. P8 did not significantly inhibit forskolin-stimulated cAMP. Although the results indicated a trend towards inhibition of cAMP formation, variability is too great to accept the hypothesis that cAMP production is decreased. Thus, P8 may be not be able to activate G-protein by itself, or if it can, its ability is limited. Although the results with P1 were significant
(Hayata, 1998), the trend is very similar to P8 in that P1 was able to decrease cAMP formation very modestly.

γ-S-GTP Incorporation

P8 and 5-HT both stimulated incorporation of $[^{35}\text{S}]\gamma\text{-S-GTP}$ into membrane preparations of H5-HT1AR (Fig. 8). P8, however, was not as effective as 5-HT, but it did incorporate γ-S-GTP on its own. This result reinforced the trend observed with cAMP studies. That is, while the activation of $G_i$ and subsequent decrease in cAMP concentration was not statistically significant, it is possible that a significant result could have been obtained with larger $n$. Thus, it is possible that P8 is weakly active in stimulating $G_i$, much like P1.

Hypothesis Modification

P8 is not more active in triggering signal transduction than P1. Although the results between cAMP determinations and γ-S-GTP incorporation experiments are mixed, the trend seems to be that P8 was modestly active in triggering signal transduction. In the area of agonist inhibition, P8 and P1 seemed to be quite similar, although P8’s order of results between receptor preparations suggests something unusual is happening. The modified hypothesis is that P8 represents a segment of H5-HT1AR responsible for coupling to and activating G protein, and that its activity is similar to P1. Currently, we conclude that adding three amino acids to the N-terminus of P1 made little difference in P1’s biology. As is the case with all of these peptides, final issues regarding the
determinants and site(s) of binding will not be completely resolved until additional experiments with cross-linking and high resolution spectroscopy are performed.
DISPLACEMENT OF $^{3}$H-8-OH DPAT BY PEPTIDE P8 IN MEMBRANE PREPARATIONS OF THE HUMAN 5-HT 1A RECEPTOR.

Results shown are means +/- SEM, with n's of 2-5. Experimental conditions are outlined in Methods.

Summary results for P8 are listed in Table III.

X-axis: Inverse concentration of the ligand, $[^3]$H$8$-OH-DPAT in 1/nmol/L. Thus, 1 corresponds to 1 nmol/L whereas 5 corresponds to 0.2 nmol/L. Y-axis: LR is the concentration of the ligand-receptor complex; LR max determined by Scatchard analysis. The axis was originally plotted as the dimensionless ratio: $1/LR/LR$ max. The lower line (♦-♦) is control $[^3]$H$8$-OH-DPAT binding in the absence of P8. The middle line (□-□) is the $[^3]$H$8$-OH-DPAT binding in the presence of $10^6$ P8. The upper line (Δ-Δ) is $[^3]$H$8$-OH-DPAT binding in the presence of $10^5$ P8. All experiments were completely run at least twice in triplicate. Correlation coefficients for the lines are all above 0.99. Experimental conditions are outlined in Methods.
INHIBITION OF FORSKOLIN-STIMULATED cAMP ACCUMULATION OF 5HT AND PEPTIDE P8 IN WHOLE CELL PREPARATIONS OF THE HUMAN 5HT1A RECEPTOR.

Results are mean +/- SEM from 3 experiments run in duplicate or triplicate. Values are expressed as percent of forskolin (FSK)-stimulated cAMP. All conditions contained forskolin (30 µmol/L). 5-HT concentration was 1 µmol/L. P8 concentration was 10 µmol/L. Other experimental conditions are outlined in Methods. Summary results for P8 are shown in Table III.
Figure 9

$[^35S]\gamma$-S-GTP INCORPORATION INTO H5HT1AR MEMBRANES

BY 5HT AND P8.

Results are mean +/- SEM of 2 experiments, all run in triplicate. Values are expressed as percent of $[^35S]\gamma$-S-GTP incorporated in controls (basal) lacking 5-HT or P8. $[^35S]\gamma$-S-GTP concentration in all conditions was 0.1 nmol/L. 5HT concentration was 0.1 µmol/L. P8 concentration was 10 µmol/L. *P8 significantly different from control (p<0.05). Other experimental conditions are outlined in methods. Summary results for P8 are found in Table III.
Peptide P9

Hypothesis

P9 represents a truncated region of H5-HT1AR’s TM5/IL3 interface that is modestly active in coupling but inactive in activation of cognate G protein.

Sequence

RFRIRKTVKK

Introduction

Peptide P9, along with P8, is one of the primary molecules to be explored in this thesis. P9 is a naturally occurring segment of the TM5/IL3 region. It is a truncated version of the parent peptide from this region, P1. Unlike P1, however, P9 has the first 5 amino acids from the N-terminus of IL3 deleted. The remaining 10 amino acids represent the “hot” spot at positions 6-9, plus the remaining 6 amino acids in the C-terminal portion of P1.

The basic premise behind P9’s synthesis was that the role of the first 5 amino acids in P1 could be tested by measuring activity in their absence. Before testing P9, we postulated that it would be active in uncoupling receptor from G protein, but likely less capable than P1. We further hypothesized that it was unlikely to be active in triggering signal transduction, as all truncated peptides examined at that point (Ortiz et al., 2000) had lacked activity. As the results given below show, this hypothesis needs some modification.
**Agonist Inhibition/Concentration-Dependency**

Figure 9 demonstrated concentration-dependent agonist inhibition by P9 in membrane preparations of H5-HT1AR. As with other peptides studied earlier, agonist inhibition by P9 was also examined in whole cell and solubilized receptor preparations. Table IV indicates the IC50’s of P9 with various preparations of H5-HT1AR. The IC50 of 25 µM for P9 in membrane preparations compares to P1’s IC50 of about 3 µM (Hayataka et al., 1998). Thus, P9 is over 8 times less active than P1 in receptor/G protein uncoupling.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC50 (µmol/L)</th>
<th>% Inhibition of Forskolin Stimulated cAMP</th>
<th>% Change in γ-S-GTP Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>P9</td>
<td>Whole Cell: 60 ± 4</td>
<td>Membrane: 25 ± 2</td>
<td>Solubilized: 30 ± 3</td>
</tr>
</tbody>
</table>

**TABLE IV: SUMMARY OF RESULTS FOR PEPTIDE P9 FROM THE TM5/IL3 REGION OF THE HUMAN 5-HT1A RECEPTOR**

*Whole cell, membrane, and solubilized indicate the preparation of receptor in which the IC50 was determined; n’s = 3-5 unique preparations in each case. ^Expressed as percent inhibition relative to controls treated with 30 µmol/L forskolin (FSK). @Expressed as percent of background incorporation.

Whole cells were the least sensitive of the receptor preparations with respective to agonist inhibition of peptide P9. This may suggest that there is some difficulty in the peptide P9 traversing membranes. The differences among the three forms of receptor
preparation were relatively small when compared against the other peptides studied earlier (Hayataka et al., 1998; Ortiz et al., 2000). This could be due to the fact that P9 is a comparatively shorter peptide. Because of the relative closeness of IC50 values for P9 in different receptor preparations, and the large differences seen with P1, P9 was about 60 times less active than P1 at solubilized receptors.

Figure 10 gives us an idea about the type of inhibition produced by peptide P9 at membrane preparations of H5HT1AR. By comparing the reciprocal plots of [3H]8-OH-DPAT concentration to that of [3H]8-OH-DPAT – 5-HT1AR complex concentration in the adaptation of the Lineweaver-Burke plots of enzyme kinetics, it can be inferred that P9 produced non-competitive inhibition. However, this binding is complex, and there is a chance that further experiments may reveal mixed inhibition characteristics. Our best hypothesis at this time is that P9 is binding to some allosteric site in the receptor complex and hence shows non-competitive interactions.

Scatchard analysis has been a standard method for analyzing equilibrium binding parameters of drugs with receptors. The Scatchard plot is a graph of (on the y axis) the amount of radioligand bound divided by the amount of radioligand free in the solution, versus (on the x axis) the amount of radioligand bound. The Bmax value is equal to the intercept on the x-axis when y = 0, and the absolute value of the slope is equal to the 1/KD value. Linearity of the line indicates that a drug binds to a single site. We performed Scatchard analysis on every binding experiment done with every peptide; thus there would be scores of Scatchard plots if we were to present every one of them. Scatchard analysis was performed mainly to calculate maximal binding (Bmax) information, which was further used in the inversion calculations. It is important to point out that what was
being measured in each case is the binding of the agonist ([H]8-OH-DPAT); changes in the binding parameters of agonist produced by peptide can then be monitored. Figure 11 gives an example of a Scatchard plot for [H]8-OH-DPAT binding in the presence of 10 μM P9.

The Hill formulation is a method for analyzing drug saturation binding curves to determine whether the interaction between ligand and receptor is cooperative. It is important to point out again, that binding of [H]8-OH-DPAT is being measured. In the presence of 100 μM P9, shown here (Fig. 12), the slope of the line is equal to .97, which is very close to 1. [H]8-OH-DPAT binds to H5-HT1AR with a unitary binding site in a non-cooperative manner (Weber, 1997). P9 did not change that relationship. As with Scatchard analysis, Hill plots were conducted in many experiments. The results shown here are an example. Since all Hill plots gave coefficients of about 1, we probably did not gain a tremendous amount of knowledge, other than to conclude that none of the peptides (P1, P8, P9, P11) changed the nature of non-cooperative binding of [H]8-OH-DPAT with the receptor.

cAMP Determination

As seen in Figure 13, P9 did not change forskolin-stimulated cAMP formation. 5-HT in this case inhibits 81% (of forskolin stimulated cAMP). The combination of 5-HT and P9 also was not different from 5-HT alone. This suggests that P9 is unable to activate the G-protein directly. P9's actions on cAMP formation were not easy to interpret. The results were variable and over the series of experiments conducted over two years, the
conclusions changed. The cAMP findings may make more sense following presentation of $\gamma$-S-GTP incorporation experiments in the next section.

**$\gamma$-S-GTP Incorporation**

Experiments with $[^{35}\text{S}]\gamma$-S-GTP incorporation showed almost identical results as in cAMP with respect to 5-HT; that is, 5-HT increased $\gamma$-S-GTP incorporation by 50% over control. P9 alone showed lower incorporation of $\gamma$-S-GTP than the basal incorporation (control), a unique result. In combination with 5-HT, P9 lowered $\gamma$-S-GTP incorporation compared to 5-HT alone. This may well represent the basal $\gamma$-S-GTP lowering seen from P9 alone. This striking result, which was repeated multiple times has not been explained at this point. The result may suggest a direct modulation by P9 of the GTP binding site on the G-protein alpha sub-unit.

Or, P9 may be influencing a vital GTP binding regulatory element. In either case, the result is dramatic and significant. The result could be consistent with the cAMP data. If a peptide was inhibiting G protein function as P9 appeared to, this would probably show up in forskolin-stimulated experiments as a neutral effect. That is, in a negatively coupled system like H5HT1AR, it is difficult, perhaps unlikely, to further stimulate adenylyl cyclase beyond that produced by a high concentration of forskolin. If nothing else, a lesson learned from these studies is that various forms of measuring signal transduction are powerful. Although different tests may be examining similar aspects of signal transduction, they are not examining exactly the same thing. Possible differences that may arise from conducting such tests may be quite useful in learning more about the system.
Hypothesis Modification

The first part of the original hypothesis seems reasonable. P9 does seem to represent a segment of IL3 important to coupling receptor and G protein. Since its activity was less than P1, the missing 5 amino acids in P9 do seem to play a role in forming the optimal conformation of the loop to produce coupling. The second part of the hypothesis needs a change. While in cAMP experiments it appears that P9 is inactive, in γ-S-GTP incorporation, P9 is active, in a negative sense. Whether such activity represents the native receptor region’s actions, is not known. The second part of the hypothesis changes to: P9 represents a segment of the native loop 3 responsible for regulation of GTP binding to the G protein’s alpha subunit.
DISPLACEMENT OF \(^{(3)H}\)-8-OH DPAT BY PEPTIDE P9 IN MEMBRANE PREPARATIONS OF THE H5HT1AR

Results shown are mean +/- SEM, with n’s of 2-5. Experimental conditions are outlined in Methods. Summary results for P9 are shown in Table IV.

X-axis: Inverse concentration of the ligand, $[^3]H$8-OH-DPAT in 1/nmol/l. Thus, 1 corresponds to 1 nmol/l whereas 5 corresponds to 0.2 nmol/l. Y-axis: LR is the concentration of the ligand-receptor complex; LR max determined by Scatchard analysis. The axis was originally plotted as the dimensionless ratio: 1/LR/LR max. The lower line (♦-♦) is control $[^3]H$8-OH-DPAT binding in the absence of P9. The middle line (□ □) is $[^3]H$8-OH-DPAT binding in the presence of $10^{-5}$ P9. The upper line (Δ-Δ) is $[^3]H$8-OH-DPAT binding in the presence of $10^{-4}$ P9. All experiments were completely run at least twice in triplicate. Correlation coefficients for the lines are all above 0.99 Experimental conditions are outlined in Methods.
Figure 12

SCATCHARD ANALYSIS OF $[^3H] 8-OH-DA$ BINDING IN 5HT1AR MEMBRANES IN THE PRESENCE OF $10^{-5}$ M P9 IN H5HT1AR MEMBRANES

The x-axis is picomolar. The $K_d$ for $[^3H] 8-OH-DA$ is about $1.3$ nM. Bmax is about $16$ pM. Other experimental conditions are outlined in Methods.
HILL ANALYSIS OF $[^3]H$ 8-OH-DPAT BINDING IN 5HT1AR MEMBRANES IN THE PRESENCE OF $10^{-4}$ M P9

Results are from a specific experiment in which points were gathered in triplicate (L) in the free concentration of $[^3]H$ 8-OH-DPAT. (LR) is the concentration of the $[^3]H$ 8-OH-DPAT/H5HT1AR complex. $[LR]_{max}$ was determined from Scatchard Analysis. Other experimental conditions are outlined in Methods.
INHIBITION OF FORSKOLIN-STIMULATED cAMP ACCUMULATION OF 5HT AND PEPTIDE P9 IN WHOLE CELL PREPARATIONS OF THE HUMAN 5HT1A RECEPTOR.

Results are mean +/- SEM from 3 experiments run in duplicate or triplicate. Values are expressed as percent of forskolin (FSK)-stimulated cAMP. All conditions contained forskolin (30 μmol/L). 5-HT concentration was 10 μmol/L. P9 concentration was 0.1 mmol/l. Other experimental conditions are outlined in Methods. Summary results for P9 are shown in Table IV.
Figure 15

\[^{35}\text{S}\]\text{-GTP INCORPORATION INTO H5HT1AR MEMBRANES BY 5HT AND P9}

Results are mean +/- SEM of 2 experiments, all run in triplicate. Values are expressed as percent of \[^{35}\text{S}\]\text{-GTP incorporated in controls (basal) lacking 5HT or P9.}\[^{35}\text{S}\]\text{-GTP concentration in all conditions was 0.1 nmol/l. 5HT concentration was 1 \(\mu\text{mol/L. P9 concentration was 0.1 mmol/l. *P9 significantly different from control (p<0.01). Other experimental conditions are outlined in Methods.}}

Peptide P1
Hypothesis

P1 represents a segment of native IL3 responsible for coupling to and activation of receptor. As a synthetic, isolated peptide, P1 shows non-competitive binding and can activate G protein on its own.

Sequence

IFRAARFRIRKTVKK

Introduction

P1 is the parent peptide from which all the other IL3 peptides were derived and compared. Most of the work on the peptide was carried out by other students (Hayataka et al., 1998). Work with P1 constitutes a small but important part of this thesis, necessary because experiments using a range of agonist concentrations large enough to yield inversion analysis as well as γ-S-GTP incorporation had not been conducted in the earlier investigations. Doing these experiments in the current series of studies allowed a more complete understanding of P1 while providing critical comparisons with the principal peptides of the thesis.

Agonist Inhibition/Concentration-Dependency

Figure 15 indicates the concentration-dependency of specific [³H]8-OH-DPAT binding in the presence of two concentrations of P1 (1 and 10 μM) in membrane preparations of H5-HT1AR. The result from the inversion plots is unique from the other peptides and in fact shows dual characteristics of inhibition. From these lines that have
different slopes we speculate that P1 shows “mixed inhibition” with a trend towards uncompetitive inhibition. There is more than a suggestion of competitive binding in this graph. Thus, of all the peptides studied, there is a greater chance with P1 that at least part of its activity is due to competition with agonist at the receptor’s ligand binding-site.

γ-S-GTP Incorporation

Figure 16 presents the results from [35S]γ-S-GTP incorporation assays in membrane preparations of H5-HT1AR. P1, much like P8 incorporated about 30% γ-S-GTP above control. This signal was not as strong as 5-HT’s 60% incorporation over basal levels, but it does show that P1 behaves similarly to P8 (which has three additional amino acids at the N-terminal) with respect to γ-S-GTP incorporation. Most importantly it does point out that P1 has an effect on its own. These results are consistent with those that Hayataka et al. (1998) found with cAMP, in which P1 gave a small but significant direct stimulation of Gi.

Hypothesis Modification

The original hypothesis is accepted with one substantial caveat. P1 may be binding to the receptor’s ligand binding-site. This is a serious uncertainty as perhaps the greatest concern with any of these peptides concerns their site of binding, particularly if one of the sites of binding is the ligand binding-site. P1, although the parent peptide, may not be the most useful experiment tool.
CONCENTRATION-DEPENDENCY OF SPECIFIC $[^3\text{H}]8$-OH-DPAT BINDING IN THE PRESENCE OF P1 IN MEMBRANE PREPARATIONS OF H5HT1AR.

X-axis: Inverse concentration of the ligand, $[^3\text{H}]8$-OH-DPAT in 1/nmol/L. Thus, 1 corresponds to 1 nmol/l whereas 5 corresponds to 0.2 nmol/l. Y-axis: LR is the concentration of the ligand-receptor complex; LR max determined by Scatchard analysis. The axis was originally plotted as the dimensionless ratio: $1/LR/LR$ max. The lower line (♦-♦) is control $[^3\text{H}]8$-OH-DPAT binding in the absence of P1. The middle line (□-□) is $[^3\text{H}]8$-OH-DPAT binding in the presence of P1 (10-6M). The upper line (Δ-Δ) is $[^3\text{H}]8$-OH-DPAT binding in the presence of P1 (4 X 10-6M). Correlation coefficient for upper line 0.97; correlation coefficients for middle and lower lines exceed 0.99. All experiments were completely run at least twice in triplicate. Other experimental conditions are outlined in Methods.
Figure 17

$[^{35}\text{S}]\gamma$-S-GTP INCORPORATION INTO H5HT1AR MEMBRANES BY 5HT AND P1.

Results are mean +/- SEM of 2 experiments, all run in triplicate. Values are expressed as percent of $[^{35}\text{S}]\gamma$-S-GTP incorporated in controls (basal) lacking 5HT or P1. $[^{35}\text{S}]\gamma$-S-GTP concentration in all conditions was 0.1 nmol/l. 5HT concentration was 0.1 µmol/L/l. P1 concentration was 1 µmol/L/l. *P1 significantly different from control (p<0.05). Other experimental conditions are outlined in Methods.
Peptide P11

Sequence

IALDdrywaitd

Hypothesis

P11 represents a region of H5-HT1AR responsible for coupling to and activation of the cognate G protein

Introduction

The work on peptide P11 was actually the continuation of the work done by Tom Ortiz (Ortiz et al., 2002) a graduate student in the Department of Pharmaceutical Sciences. An important point to note here is that P11 is a peptide from the transmembrane 3 (TM3)/intracellular loop 2 (IL2) region of H5-HT1AR. The work on P11 was prompted by a number of considerations. First, the preliminary data from P11 suggested that the peptide uncoupled receptor from G protein (see Table 5, Fig. 17; this thesis) but lacked further activity. Second, we wished to complement existing data from signal transduction experiments by utilizing γ-S-GTP incorporation assays. Third, the existing data with agonist inhibition assays required some completion by varying agonist concentrations such that full-scale inversion analysis could be conducted. Fourth, n’s for the cAMP experiments needed expansion. In the results reported below for P11, P11 is also compared with P7, a weakly active but previously unreported peptide (Ortiz et al., 2002)
Agonist Inhibition/Concentration-Dependency

Peptide P11 produced concentration-dependent effects as measured by agonist inhibition; these changes were seen whether determined in whole cell, crude membrane, or solubilized receptor preparations. The agonist inhibition results are summarized in Table V (Ortiz et al., 2002). These results have been previously reported but are presented here to provide the context for new findings that are to follow. Agonist inhibition is a procedure based upon the receptor’s affinity state; when the receptor is G protein coupled, it is in the high affinity state. When the receptor is uncoupled from the G protein, it is in the low affinity state. Thus, if a peptide uncouples G protein and receptor in a concentration-dependent fashion, then the effect is represented by a dose-response inhibition curve (Maguire et al., 1976; Peroutka et al., 1979; Hayataka, 1998; Ortiz, 2000). By inference, the uncoupling actions of P11 reported here imply the ability of the N-terminal region of the native I2 loop to couple with G protein.

For comparative purposes in membrane preparations, the agonist inhibition effects of P7, a truncated version of P1, a native 15 MER (Hayataka, 1998) from the TM5/IL3 interface, are given in Figure 17 along with the activity of P11. In these membrane preparations, P7 was barely active in comparison to P11 or P7’s parent P1; the same generalization holds with respect to whole cell and solubilized preparations. We have not conducted structure-activity studies with P11 analogues. However, it is important to contrast the biology of any active peptide with an inactive or minimally active “control.” For that reason, P7 is reported at this time.
There is concern with any of these peptides regarding their ability to reach the putative site of action at the receptor/G protein interface. The large difference in IC50's for P11 in different receptor preparations suggest the possibility of reduced access to the interface when the peptide must penetrate the membrane barrier. Peptide P11 has been analyzed in all three receptor preparations in the agonist inhibition format (Table V). Solubilized receptors were most sensitive, followed by whole cells and membranes. Since P11 was about 3-8 times more active against solubilized receptors compared to the membrane and whole cell receptor preparations, it is possible that the peptide has somewhat better access to the receptor/G protein interface in the solubilized setting. This pattern of activity is similar to that seen with P1, the native peptide from the TM5/IL3 interface, studied in earlier work (Ortiz, 2000). On the other hand, P7 had minimal agonist inhibition activity in all receptor preparations (see Table V for P7). These activities were so weak that IC50's could not be determined at the concentrations tested (limited by peptide solubility); thus, maximal inhibitions at 10^{-4} \text{ mol/l} are given in Table V for P7, rather than IC50's. Thus, for a peptide like P7, it is difficult to know if it has trouble reaching the putative intracellular site of action since complete concentration-dependency information is unavailable in this experimental format. Nevertheless, it is important to emphasize the conclusion that active peptides studied by the methods used here show distinctively different quantitative and qualitative concentration-effect relationships relative to inactive peptides.

One of the great concerns for a peptide used in any system is its specificity. We have suggested specificity parameters for P1 and its derivatives at TM5/IL3 using a
variety of controls (Hayataka, 1998; Ortiz, 2000). We believe that the data shown in Figure 18 provides strong evidence for the action of P11 at the TM3/IL2 interface rather than another site such as the ligand binding-site. In this concentration-dependent analysis, if P11 were acting at the ligand binding-site, then it would competitively interfere with $[^3H]-8$-OH-DPAT binding. If P11 were acting at some allosteric site on the receptor complex, then the plot would show non-competitive interactions. Rather the plot is uncompetitive, exactly what would be expected if the site of interaction is between receptor and G protein. Another way of looking at this is that parallel inhibition lines like those seen in this graph represent interaction of inhibitor with the agonist/receptor complex itself rather than competing for agonist at the ligand binding-site or binding to an allosteric site. Final resolution of specificity and site(s) of action will require high-resolution spectral studies as mentioned in the conclusion of the thesis.

**cAMP Determination**

When P11 was analyzed for biological activity in whole cell preparations utilizing measurement of forskolin-stimulated intracellular cAMP concentrations, it was inactive, either alone or in combination with 5-HT (Fig. 19; also see Table V). For reference, this negative result is in contrast to the parent peptide from IL3, peptide P1, which stimulated G protein directly at micromolar concentrations(Weber, 1997). In fact many of P1’s substitution derivatives (Ortiz, 1999) also stimulated G protein, some of them better than P1. The results for P11 from the IL2 loop imply a distinct differentiation between uncoupling actions (agonist inhibition) and lack of G protein activation (decreases in cAMP concentration in this negatively coupled system).
γ-S-GTP Incorporation

P11 was ineffective at stimulating incorporation of [35S]γ-S-GTP above background levels (Fig. 20; also see Table V). These results are consistent with the inactivity of P11 in G protein activation as shown via cAMP formation (Fig. 19). Additionally, P11 did not interfere with 5-HT’s ability to stimulate GTP incorporation, indicating that P11, as previously shown in Figure 18, does not competitively interact with 5-HT at the receptor’s ligand binding site. These results are again consistent, in conjunction with the agonist inhibition results, with a peptide that represents a portion of the native receptor that is responsible for coupling to G protein, but not activating it.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC50 (µmol/L)*</th>
<th>% Inhibition of Forskolin Stimulated cAMP$</th>
<th>% Change in γ-S-GTP Incorporation$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole Cell</td>
<td>Membrane</td>
<td>Solubilized</td>
</tr>
<tr>
<td>P11</td>
<td>16 ± 2</td>
<td>7 ± 1</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>P7</td>
<td>ND</td>
<td>25 ± 1</td>
<td>49 ± 8</td>
</tr>
</tbody>
</table>

TABLE V: SUMMARY OF RESULTS FOR PEPTIDE P11 FROM THE TM3/IL2 REGION AND TRUNCATED PEPTIDE P7 FROM THE TM5/IL3 REGION OF THE HUMAN 5-HT1A RECEPTOR

*Whole cell, membrane, and solubilized indicate the preparation of receptor in which the IC50 was determined; n’s = 3-5 unique preparations in each case. Results for P7 are maximum percent inhibition at 10^-4 M rather than IC50 values. $Expressed as percent inhibition relative to controls treated with 30 µmol/L forskolin (FSK). $Expressed as percent of background incorporation. ND: experiments not done for this condition.
Hypothesis Modification

Since P11 is active as measured by uncoupling but inactive in further bioassays, the hypothesis must be rejected and modified as indicated below. The results presented here for P11 corroborate and extend previous work with 7TMDR (Dohlman et al., 1991; Savarese & Fraser, 1992; Baldwin, 1994; Strader et al., 1994; Varrault, 1994). While many members of this superfamily utilize the I2 loop in G protein interactions, there is much to be known about the specific determinants of coupling and G protein activation. Since P11 is similarly active to the IL3 peptide P1 in the agonist inhibition format, we suggest that P11 uncouples receptor from G protein in an analogous fashion to that previously observed for P1.

P11, however, does not show activity in the signal transduction system as judged by its inability to decrease cAMP concentrations in the forskolin-stimulated format nor in its inability to stimulate G protein incorporation of γ-S-GTP. As P1 is a mimic of the receptor’s IL3 N-terminus, and P11 is a mimic of the receptor’s IL2 N-terminus, we reach the following conclusion: in the H5-HT1A receptor, the N-termini of both intracellular loop 2 and loop 3 are involved in receptor-G protein coupling; only the N-terminus of IL3, though, is further implicated in G protein activation. In previous work with the 5-HT1AR, (Varrault, 1994) demonstrated that the C-terminal section of IL3 is involved in G protein coupling and activation. Further, they showed that the entirety of IL2 produces G protein coupling and activation. The results from our study extend this observation by establishing that the N-terminal region of IL2 is specialized for coupling.
but is not involved in activation; this is our current working hypothesis. Since the IL2 peptide P11 reported here has potential as a probe of the receptor/G protein interface due to its differential effects, it could be a powerful tool in exploring the interface with techniques such as multi-dimensional NMR (Burritt et al., 1998).
DISPLACEMENT OF SPECIFICALLY-BOUND $[^3H]8$-OH-DPAT

in membrane preparations by the TM3/i2 H5HT1AR peptide probe, P11 (Δ-Δ), and the TM5/i3 H5HT1AR peptide probe, P7 (O-O). Experimental conditions are outlined in Methods. Results shown are means +/- SEM, with n’s of 2-5. Summary results for P7 and P11 are found in Table V.

X-axis: Inverse concentration of the ligand, $[^3]$H8-OH-DPAT in 1/nmol/L. Thus, 1 corresponds to 1 nmol/L whereas 5 corresponds to 0.2 nmol/L. Y-axis: LR is the concentration of the ligand-receptor complex; LR max determined by Scatchard analysis. The axis was originally plotted as the dimensionless ratio: 1/LR/LR max. The lower line (♦-♦) is control $[^3]$H8-OH-DPAT binding in the absence of P11. The middle line (●-●) is $[^3]$H8-OH-DPAT binding in the presence of 2 µmol/L P11. The upper line (Δ-Δ) is $[^3]$H8-OH-DPAT binding in the presence of 8 µmol/L P11. All experiments were completely run at least twice in triplicate. Correlation coefficients for the lines, top to bottom are: 0.880; 0.990; 0.994. Experimental conditions are outlined in Methods.
INHIBITION OF FORSKOLIN-STIMULATED cAMP ACCUMULATION BY 5HT AND P11.

Results are mean +/- SEM from 5 experiments run in duplicate or triplicate. Values are expressed as percent of forskolin-stimulated cAMP. All conditions contained forskolin (30 μmol/L). 5-HT concentration was 10 μmol/L. P11 concentration was 0.1 mmol/l. Other experimental conditions are outlined in Methods. Summary results for P11 are shown in Table V.
$[^{35}\text{S}]\gamma\text{-S-GTP}$ INCORPORATION INTO $\text{H}5\text{HT}1\text{AR}$ MEMBRANES BY 5HT AND P11.

Results are mean +/- SEM of 2 experiments, all run in triplicate. Values are expressed as percent of $[^{35}\text{S}]\gamma\text{-S-GTP}$ incorporated in controls (basal) lacking 5HT or P11. $[^{35}\text{S}]\gamma\text{-S-GTP}$ concentration in all conditions was 0.1 nmol/L. 5HT concentration was 1 µmol/L. P11 concentration was 0.1 mmol/L. Other experimental conditions are outlined in methods. Summary results for P11 are shown in Table V.
Dipropyltryptamine (DPT)

Hypothesis

DPT is an agonist at the H5-HT1AR

Introduction

Dipropyltryptamine (DPT) is a synthetic drug (Shulgin, 1969; Soskin, 1975) that was studied briefly in the 1960’s and 70’s and then largely forgotten since identification of multiple serotonin (5-hydroxytryptamine; 5-HT) receptor (R) subtypes (Barnes, 1999). DPT has been virtually unknown in the scientific literature since the mid-1970’s when its use as an adjunct to psychotherapy faded. DPT is structurally related to the better known dimethyltryptamine (DMT), a potent hallucinogen (Barker et al., 1981; Pierce, 1989; Glennon, 1992; Deliganis, 1999). The studies reported here represent the initial characterization of dipropyltryptamine at cloned H5-HT1AR (Arthur et al., 1993; Hayataka, 1998; Ortiz, 1999; Cowen, 2000) and to a lesser degree cloned rat 5-HT2AR (Baldwin, 1994).

Both H5-HT1AR and rat 5-HT2AR is seven transmembrane domain (7TMD), G-protein coupled (GPC) receptors, and both have been linked to biomedical conditions such as depression, anxiety, and migraine headache. Since H5-HT1AR is negatively coupled to the adenylyl cyclase signal transduction system via Gi, functional attributes of ligands that bind to the receptor can be monitored by quantification of intracellular cyclic AMP (cAMP).
Additionally, since both H5-HT1AR and rat 5-HT2AR are GPCR, receptor activity can be followed by GTP incorporation techniques. Results reported here suggest that DPT is a high affinity partial agonist at H5-HT1AR and a lower affinity agonist at rat 5-HT2AR. These conclusions have implications for use of DPT or structural analogues at the ligand binding site of H5-HT1AR (Blair et al., 2000). The primary reason for presenting DPT’s activity in the context of this thesis is to provide comparison and contrast to the effects of peptides P1, P8, P9 and P11 at 5-HT1AR. Whereas the peptides are, in general, not competitive ligands at the H5-HT1AR ligand-binding site, DPT, which is competitive provides a clear contrast.

**Displacement of $[^3H]8$-OH-DPAT from H5-HT1AR**

DPT was synthesized by Dr. Chuck Thompson at the University of Montana (Fig 21). The first series of experiments were designed to determine the affinity of DPT at membrane preparations of 5-HT1AR. Figure 22 shows that DPT produced excellent concentration-dependent effects as measured by inhibition of specific agonist $[^3H]8$-OH-DPAT binding at H5-HT1AR. The displacement of $[^3H]8$-OH-DPAT at H5-HT1AR indicates that DPT has moderately high affinity with an apparent IC$_{50}$ of about 0.1 µM.

In Figure 23 two different concentrations of DPT (40 nM and 200 nM) were compared with a control lacking DPT against various concentrations of $[^3H]8$-OH-DPAT bound to H5-HT1AR. In this adaptation of the Lineweaver-Burke plot of enzyme kinetics, both agonist $[^3H]8$-OH-DPAT concentrations (x-axis) and agonist-receptor complex concentrations (y-axis) are inverted. The resulting straight lines allow estimation
of the nature of DPT/agonist interactions. Interpretation of the experiments analyzed here is consistent with DPT inhibiting agonist binding competitively.

**cAMP Determination**

DPT’s ability to bind at the H5-HT1AR raised the question of its potential to trigger the signal transduction system. Determination of intracellular cAMP concentrations tests the hypothesis that DPT is an agonist. Fig 24 suggests that DPT reduced forskolin-stimulated cAMP concentrations. However, this agonist-like effect is not significant. In combination with 5-HT, DPT diminished the effect observed with 5-HT alone. This is consistent with the conclusion that DPT is an antagonist. These results lead to apparent rejection of the original hypothesis that DPT is an agonist at this receptor.

**γ-S-GTP Incorporation**

Figure 25 summarizes another approach to testing DPT’s capacity to trigger signal transduction at H5-HT1AR. As anticipated, 5-HT by itself incorporated 92% more than the basal incorporation of $[^{35}\text{S}]\gamma$-S-GTP (buffer). Additionally, DPT showed significant concentration-dependent incorporation of $[^{35}\text{S}]\gamma$-S-GTP, though the amount of incorporation is not as much as that of 5-HT itself. When 5-HT and DPT were administered together, the effect of 5-HT alone was reduced by DPT. Together, DPT and 5-HT were not able to exceed the effect of 5-HT alone or to even equal the 5-HT effect alone. This would indicate that DPT acts as an antagonist. With DPT producing weak agonist effects alone, but acting as an antagonist in the presence of agonist (5-HT), it is
proposed that the original hypothesis that DPT is an agonist be rejected. The modified hypothesis is that DPT is a partial agonist at H5-HT1AR. The puzzling results with cAMP may have been heading in this direction but require more experiments for a complete test.

**DPT at the Rat 5-HT2a Receptor**

For the sake of completeness, preliminary results for DPT's interactions at the rat 5-HT2a receptor will be reported. When $[^3H]$Ketanserin is bound to the receptor, DPT displaced the ligand in a concentration-dependent manner. The apparent affinity is lower than that reported at H5-HT1AR, however, as the IC50 is 200 times higher. In signal transduction assays with $[^{35}S]y$-S-GTP incorporation, DPT showed activity consistent with that of an agonist. Thus, the working hypothesis is that DPT is a low affinity agonist at rat 5-HT2AR. Whether the differences reported here for 5-HT1A and 5-HT2A receptors are species related is unknown at this time.
SYNTHESIS OF N,N-DI-N-PROPYLTRYPATMINE

Tryptamine (1.6 g; 10 mmol) and diisopropylethylamine (1.04 ml; 60 mmol) were dissolved in diethyl ether (60 ml) at 0 °C. 1-Iodopropane (1.02 g; 0.6 ml; 60 mmol) was added dropwise with stirring over 1 h. The reaction was stirred 16 h at room temperature, filtered to remove salts, and evaporated to an oil. The crude product, which was contaminated with N-propyltryptamine and some unreacted tryptamine, was chromatographed on silica gel to afford the product, N,N-di-n-propyltryptamine in 46% yield. The purified product had elemental and spectral characteristics consistent with literature values.
INHIBITION OF [³H]8-OH-DPAT BINDING BY DIPROPYLTRYPTAMINE (DPT) IN MEMBRANE PREPARATIONS EXPRESSING THE HUMAN 5-HT1A RECEPTOR (H5-HT1AR).

Results are mean +/- SEM with n's of 2-5. Other experimental conditions are outlined in Methods.
Figure 24

CONCENTRATION-DEPENDENCY OF $[^3\text{H}]8$-OH-DPAT BINDING IN THE PRESENCE OF DIPROPYLTRYPTAMINE (DPT) IN MEMBRANE PREPARATIONS OF THE HUMAN 5HT1A RECEPTOR (H5HT1AR).

Correlation coefficients exceed 0.99 for the lower two lines; correlation coefficient for the upper line is 0.89. Additional experimental conditions are described in Methods. [L] is $[^3\text{H}]8$-OH-DPAT. On the x-axis 1 = 1nM; 5 = 0.2nM, etc. On the y-axis [LR] is the concentration of the $[^3\text{H}]8$-OH-DPAT-H5HT1AR complex.
Figure 25

EFFECTS OF SEROTONIN (5HT) AND DIPROPYLTRYPTAMINE (DPT) ON FORSKOLIN-STIMULATED CYCLIC AMP (cAMP) FORMATION IN WHOLE CELL PREPARATIONS OF THE HUMAN 5-HT1A RECEPTOR (H5-HT1AR).

All conditions contain forskolin (30 μM). Additional experimental conditions are described in Methods. DPT conc. is 0.1 μM in all cases.
EFFECTS OF SEROTONIN (5HT) AND DIPROPYLTRYPTAMINE (DPT) ON INCORPORATION OF $^{35}$S$\gamma$-S-GTP INTO MEMBRANE PREPARATIONS OF THE HUMAN 5-HT1A RECEPTOR (H5-HT1AR).

Basal represents incorporation in the control setting (buffer). *DPT (10$^{-7}$M) significantly different from control (p<0.05); **DPT (10$^{-6}$M) significantly different from control (p<0.02). Additional experimental conditions are described in Methods. 5-HT conc. is 0.1 μM in all cases.
CONCLUSIONS

The various peptides studied during the course of this project narrate a very multifaceted, but subtle story about the H5-HT1AR/G-protein interface. Many of the peptides showed reproducible activity at the receptor. An important point, which should be emphasized, is that P8 and P9 were the focal peptides in this project. Mini-projects performed new types of experiments with P1 and P11 to help better understand knowledge previously learned about these peptides. P7, a largely inactive peptide, has been included to contrast the activities of the other peptides, and finally, dipropyltryptamine (DPT) has been studied and analyzed here to compare an agent, DPT, active at the receptor’s ligand binding-site with agents that putatively act at the receptor/G protein interface.

To put these results in perspective, we shall compare the peptides as we have done with the actual methodology; i.e. Agonist Inhibition/Concentration-Dependency and Signal Transduction (cAMP determinations; and γ-S-GTP incorporation). Finally, the context of these studies with other knowledge about 5-HTR’s and other GPCR’s will be discussed, as well as future projections for this approach beyond the current thesis.

Agonist Inhibition/Concentration-Dependency

Which Positions in the Peptides are Critical to G Protein Coupling?

P8 vs. P1: The IC50’s as well as the concentration-dependency curves of P8 (this thesis) and P1 (Hayataka et al., 1998) indicate that they behave in a similar manner; this
may seem obvious; P8 and P1 are structurally very similar, with P8 having an additional three amino acids at the N-terminal end. This could well suggest that the increased size (of P8), extending into TM5, does not affect agonist inhibition, and by inference, coupling of the native loop and G protein, in any significant manner. While this similarity may seem given at this point, it was by no means understood prior to the experiments, and the structural difference needed to be tested.

**P9 vs. P1:** The results for P9, which is P1 minus the first five N-terminal amino acids, indicated that P9 (this thesis) has lower agonist inhibition activity relative to P1 (Hayataka et al., 1998). Depending on receptor preparation being analyzed, P9 is 8 to 60 times less effective than P1. This corroborates, to an extent, the general observation with the panel of peptides already studied, that truncates of P1 have low activity or are inactive. Previous work by Ortiz et al. (2000) concluded that positions 6-9 of P1 (the first 4 positions of P9's N-terminus) are most critical to receptor/G protein coupling, and that the next 6 positions toward P1's C-terminus were necessary but not as critical. The P9 sequence is these exact 10 amino acids. Thus, studying P9 gave a stiff, if not completely stringent, test of Ortiz' hypothesis. The additional conclusions garnered from studying P9, however, are that the first five N-terminal amino acids of P1 are not irrelevant to coupling activity of IL3. Thus, the entirety of P1 seems to be active in coupling. The role of positions to the C-terminal side of P1 remains to be tested.

**P7 vs. P1:** P7 (this thesis) fits into this picture. It is the first 9 positions of P1. Using the theme of truncating to the N-and C-terminal sides of critical residues 6-9, P7
"completes" the picture started with P9. For all intents and purposes, P7 is inactive. It is not only too small, but it lacks the important residues to the C-terminal side of position 9. Even though P7 retains key residues 6-9 of P1, the loss of positions 10-15 is too great. While truncating positions 1-5 in P9 markedly reduced activity, it did not eliminate activity. Loss of positions 10-15 in P7 virtually eliminated activity. It seems that the amino acids to the C-terminal side of positions 6-9 are more important than the positions to the N-terminal side of positions 6-9.

P11: Though derived from an entirely different loop (I2), P11 has substantial agonist inhibition characteristics. P11 produced inhibition in the micromolar range in all receptor preparations. By inference this action represents the ability of the N-terminal region of the native I2 loop to couple with G-protein. The results corroborate and extend previous work with 7TMDR (Dohlman et al., 1991; Savarese & Fraser, 1992; Baldwin, 1994; Strader et al., 1994; Varrault, 1994). While many members of this superfamily utilize the I2 loop in G-Protein interactions, there is much to be known about the specific determinants of coupling. From the results reported here, it seems that the first 11 positions of I2 adjacent to TM3 are essential for I2/G protein coupling. Although P11 and P1 are from different receptor loops, their activities in agonist inhibition are close (P1 is slightly more active). Seemingly, the N-terminal segments of both I2 and I3 are critical to receptor/G protein coupling.
Dipropyltryptamine (DPT) as a Reference: DPT activity in the adaptation of Lineweaver-Burke analysis (Fig. 23) strongly suggested that $[^3\text{H}]8$-OH-DPAT and DPT compete for the same binding site. Since $[^3\text{H}]8$-OH-DPAT is known to have highly specific binding to the ligand binding-site of 5-HT1AR, DPT must be considered a specific 5-HT1AR-ligand binding-site agent. While this observation is interesting in its own right, it is irrelevant to this thesis except for the assumption that the peptides are not binding to the ligand-binding site. Therefore, DPT’s pharmacology is presented as the contrast to what the peptides should not be.

P11: P11’s activity in the adaptation of Lineweaver-Burke analysis suggested an uncompetitive mechanism. This result starkly contrasts with DPT’s activity. It is difficult to conclude other than that P11 does not interact at the H5-HT1AR ligand-binding site. The site of interaction remains unknown, but the uncompetitive mechanism gives support to the conclusion that the peptide binds between receptor and G protein.

P1, P8, and P9: P8 and P9 show non-competitive inhibition characteristics. P1 also shows uncompetitive properties at higher concentrations; this is interesting because P8, its very close relative, also seems to show such tendencies. Overall, though, P1 has “mixed” interaction properties with an apparent component of competitive inhibition. The purpose of studying these peptides is to better understand their biology. The information summarized in this section contributes to this advancement. Even more
important, though, is the application of this biology in evaluating the long-term potential of these peptides as probes of the H5-HT1AR/G protein interface. P11, P8, and P9, all possessing non-competitive or un-competitive mechanisms, should be useful tools for helping probe the native receptor/G protein structure. P1 is suspect if it is partially or completely active at the ligand binding-site.

**Signal Transduction**

*Do the Peptides Directly Activate G Proteins?*

P1, P8, and P9: A preliminary answer to this question for P1 was available from previous cAMP determinations (Hayataka, 1998). P1 is weakly active in decreasing cAMP in this negatively coupled system. This conclusion is fortified by addition of the \(\gamma\)-S-GTP incorporation experiments of this thesis. P1 increased \(\gamma\)-S-GTP incorporation 30% above basal levels of incorporation. Therefore, the answer is positive to this question. P1 has the capacity to directly stimulate G proteins.

P1 and P8 produce analogous results in signal-transduction experiments. This is especially true with \(\gamma\)-S-GTP assays where the P1 and P8 results were identical. The cAMP assays gave similar results for the two peptides. The conclusion is that P8, like P1, stimulates G-protein on its own. The change in structure (with P8) involving three additional amino acids on the N-terminus of P1 produces little, if any, effect in signaling properties.

One important understanding that we came to, as more and more \(\gamma\)-S-GTP determinations were made, is that even though the cAMP and \(\gamma\)-S-GTP assays often yield
similar results, the certainty of cAMP results was often lower. The principal reason for this is that the signal generated in γ-S-GTP assays is larger and more consistent than the signal generated in cAMP assays. The bottom line is that greater confidence in conclusions is produced by γ-S-GTP results.

The uncertainty of cAMP results is illustrated with P9, where the peptide does not significantly change cAMP levels. This conclusion masks the underlying variability of results, however. In multiple experiments over two years, there were instances where cAMP didn’t change, where it went up, and where it went down. P9 represents the most extreme example of uncertainty with cAMP and these peptides, but a level of unusual uncertainty is there for all the peptides.

Though the results from γ-S-GTP incorporation typically paralleled cAMP results, there was a unique result with P9. The result from P9, which represents the truncated end of the N-terminal end of IL3, suggests that the N-terminal end could not only be responsible for coupling of G-protein but also de-activation.

To keep the signal transduction properties in perspective, it is important to emphasize that none of the peptides or DPT produced effects comparable to that of the neurotransmitter 5-HT. That is, in terms of both potency and effectiveness of the various agents, 5HT was the best, and everything else fell somewhere below its benchmark actions.
P11: could be considered as a peptide that did not have any activity in the signal-transduction system; this held true for P11’s effects on incorporation of γ-S-GTP as well as the effects in the adenylyl cyclase/cAMP system. As P11 is a mimic of the receptor’s IL2 N-terminus and P1 is a mimic of the receptor’s IL3 N-terminus, we can now conclude that though both IL2 and IL3 are involved in coupling with G-Protein, only IL3 is involved in G-protein activation. Our study corroborates the results from (Varrault) where it was shown that IL2 was involved in receptor coupling and activation. Varrault’s study did not distinguish between loop sub-regions, however. We can now add to their observation by stating that the N-terminal of IL2 is involved in coupling only and does not mediate any activation.

Future Work

The studies with P1, P8, P9, and P11 described in this thesis have substantially contributed to the long-range goals of structural determination at the human 5HT1A receptor/G protein interface. Hayataka’s initial work with P1 established the feasibility of working with an IL3 peptide as a biochemical probe of the interface. Ortiz’s studies with substitution (such as P2 and P3) and truncation (such as P6) derivatives of P1 demonstrated potentially useful differential biochemical properties of the new probes. These studies provided the background to develop further hypotheses about peptide length and sequence that were tested with P8 and P9 in this thesis work.

The combined studies of this thesis with the developmental experiments by Ortiz with P11 created a feasible tool for analysis of IL2. As has been discussed earlier, the various biochemical parameters now known for these peptides suggest that some of the
peptides will be more useful than others in future studies. Envisioned future studies include cross-linking selected peptides to purified G proteins; and utilization of selected peptides to interact with purified G proteins in the context of multi-dimensional NMR and other high-resolution analyses. All of these experimental approaches will be designed to give discrete information about the binding of the peptides to G protein sites. By analogy, then, the high-resolution information gained from these experiments will be used to model receptor loop interactions with G proteins in the native state. Since the peptides are incomplete representatives of the native situation, they are imperfect tools. However, it is expected that the encouraging properties discovered in this thesis work indicate that these peptides have the potential to contribute to the developing understanding of this receptor system.

Although not formally a part of this thesis work, one additional piece of experimental data done in conjunction with the planned thesis work, should be mentioned to better anticipate the future. Ortiz discovered that the substitution derivative of P1 known as P3 has perhaps the best potential of any of the studied peptides to activate G protein as determined by cAMP determination. Figure 26 extends this conclusion by exploring P3’s ability to increase \[^{35}\text{S}]\gamma-S\text{-GTP} incorporation into G protein. The results suggest that the cAMP results were correct, as P3 is quite capable of stimulating GTP incorporation. Thus, we conclude that the following peptides will be selected as the probes to be used in cross-linking and NMR studies: at IL3: P3 and P9; at IL2: P11. Some of the inactive peptides such as P7 may also be useful as controls. Future structure-activity work with P11 may also be done to provide differential capabilities such as those available with the P1 derivatives.
ENHANCEMENT OF GAMMA-S-GTP INCORPORATION BY PEPTIDE P3 IN MEMBRANE PREPARATIONS OF THE H5HT1AR.

$[^{35}S] \gamma$-S-GTP was bound to membrane preparations of H5HT1AR at a concentration of 0.1 nM. Non-specific binding was determined in the presence of 10 μM cold $\gamma$-S-GTP. Assays were conducted in triplicate at 30°C for 30 min., and stopped by filtration with GF/C filters. All determinations were with n = 2. Values are expressed as percent of $[^{35}S] \gamma$-S-GTP incorporated in controls (basal) lacking 5HT or P3.
SCHEMATIC DIAGRAM OF LOOP 2 AND 3 ACTIVITIES AT THE 5HT1AR/G PROTEIN INTERFACE.

Intracellular loop 2 (ic2) connecting transmembrane (TM) segments 3 and 4, and intracellular loop 3 (ic3) connecting transmembrane segments 5 and 6 are pictured. Arrows symbolize portions of the loops thought to be involved in coupling of receptor to G protein, producing conformational changes that ultimately result in G protein activation. The bar represents the N-terminal region of ic2, which appears to be involved in coupling only. The inset from the N-terminal region of ic3 gives the sequence for P1, and segments of P1 and the analogous receptor region thought to be responsible for coupling and activation.
Summary

Peptides P1, P7, P8, and P9 from intracellular loop 3 of the human 5-HT1A receptor and P11 from intracellular loop 2 were studied for their receptor/G protein uncoupling and signal transduction activation properties. P7 was inactive. P11 was active in uncoupling receptor and G protein, but inactive in triggering signal transduction. P1, P8, and P9 were active in both the uncoupling and signal transduction realms to various degrees. P9, especially, demonstrated unique activity in the signal transduction system, namely, decreasing basal levels of \([^{35}S]\gamma-S-GTP\) incorporation, suggesting a possible regulatory role with respect to GTP binding to G protein. The non-peptide dipropyltryptamine (DPT) was also examined as a competitive ligand-binding site agent at H5-HT1AR. Overall, these results in combination with previous studies in this laboratory and evidence from other laboratories, suggests a model of the human 5HT1A receptor/G protein interface in which the N-terminal of intracellular loop 2 is responsible for coupling to but not activation of G protein. On the other hand, the N-terminal of intracellular loop 3 is responsible for both coupling to and activation of G protein. While the entire 15 amino acid stretch of loop 3 studied seems to have a role in these activities, there is a four amino acid stretch beginning with position 6 that is particularly vital. Positions 6-15 may be specifically involved in regulation of GTP incorporation into G protein. These ideas about H5-HT1A receptor/G protein interaction are summarized in Figure 27.
REFERENCES


