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Synthetic peptide probes of the human 5-HTb1A receptor/G protein interface

Thomas Christian Ortiz
The University of Montana

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SYNTHETIC PEPTIDE PROBES OF THE HUMAN 5-HT\textsubscript{1A} RECEPTOR/G PROTEIN INTERFACE

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

The University of Montana
1998

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Chairman, Board of Examiners

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Dean of the Graduate School

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Date
Abstract

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Title: Synthetic Peptide Probes of the Human 5-HT\textsubscript{1A} Receptor/G Protein Interface

Advisor: Keith K. Parker, Ph.D.

To study the interactions in G protein/receptor coupling, peptide probes synthesized according to the published sequences of the human 5-HT\textsubscript{1A} receptor were utilized in a model system of human 5-HT\textsubscript{1A} receptors stably expressed in Chinese hamster ovary (CHO) cells. Activity of these peptides was determined by their ability to displace a specifically-bound agonist, $[^3H]$8-OH-DPAT, from cells, as well as through signal transduction assays measuring cAMP formation. Previously, a 15 amino acid peptide (15mer) from the transmembrane 5/intracellular loop 3 region (TM5/i3) of the human receptor has been shown to be biologically active in these assays.

Two 15mers were synthesized to determine effects that non-conservative amino acid substitutions have on the interactions between receptor and G protein. The first, altered at positions 6 and 7 from its N-terminus by substitution of an arginine to glycine (R\textsuperscript{222} to G\textsuperscript{222}) at position 6, and phenylalanine to alanine (F\textsuperscript{223} to A\textsuperscript{223}) at position 7, which is termed 15mer GA. The second peptide included additional substitutions at positions 8 (R\textsuperscript{224} to G\textsuperscript{224}) and 9 (I\textsuperscript{225} to A\textsuperscript{225}) from the N-terminus, and is termed 15mer GAGA. A third synthetic peptide, termed 11mer, from the TM3/i2 region containing a highly conserved D/ERY motif at the N-terminus, was also characterized in the same manner as the altered 15mers.

Each of the synthetic peptide probes of the human 5-HT\textsubscript{1A} receptors tested produced a concentration-dependent effect in displacing the specifically-bound agonist $[^3H]$8-OH-DPAT from the receptors, with IC\textsubscript{50} values in the low micromolar ranges. The rank of potency was 11mer > 15mer GA > 15mer GAGA. This was observed in all preparations of the receptor, including the whole cell preparations, indicating that each peptide was able to gain at least moderate intracellular access. In the signal transduction assays only one of the peptides, 15mer GAGA, was able to inhibit forskolin-stimulated cAMP production greater than 50% of the controls, with the 11mer from the i2 site being the least active.

The findings support the current models of 7TM/GPCR, in that the N and C-termini of i2 and i3 are functional domains of receptor G protein coupling, and that the i3 site is important for effective receptor-G protein coupling and G protein activation.
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Introduction

A. Serotonin Receptors

Serotonin (5-hydroxytryptamine, 5-HT) is an indolealkylamine signaling molecule found in numerous systems throughout the body, and thus is involved in a number of physiological processes. Historically, 5-HT was known initially as both serotonin, due to vasoconstrictive effects during platelet aggregation (1), and enteramine because of its actions on the smooth muscle in the intestinal mucosa (2). 5-HT has been shown to be a neurotransmitter and neuromodulator in both the central and peripheral nervous systems, as well as an autacoid (3).

Dysregulation of serotonergic systems has been implicated in several pathophysiological states. Serotonergic mechanisms are thought to be involved in hallucinations (4-5). There is much clinical evidence supporting 5-HT involvement in migraine headache (6-7), panic disorders (8), and depression (3). Some experimental evidence indicates that other disorders such as Tourette's syndrome, schizophrenia, autism, and multiple sclerosis are in some part due to serotonergic dysfunction (9).

Receptors that mediate the actions of 5-HT were discovered in the late 1950's. Initially, two types of receptors for 5-HT were thought to exist (10) and were classified according to their pharmacological characteristics. Later studies using radiolabeled ligands found two distinct binding sties in the rat
frontal cortex (11) called 5-HT\textsubscript{1}, which displayed a high affinity for [\textsuperscript{3}H] 5-HT and 5-HT\textsubscript{2}, which had a low affinity for [\textsuperscript{3}H] 5-HT, but a high affinity for [\textsuperscript{3}H] spiperone. Soon thereafter, a third class of 5-HT receptor was characterized, the 5-HT\textsubscript{3} receptor (12).

Until recently, all receptors (5-HT or otherwise) were identified and classified through purely pharmacological methods. However, with the advent of molecular biological techniques, the number of receptors and subtypes within a family has grown substantially. At present, the family of receptors for 5-HT can be divided into seven types, and further subdivided into several subtypes (13-14). These relationships are summarized in table 1.

<table>
<thead>
<tr>
<th>Table 1: Current Classification of Receptors for Serotonin. Adapted from Hoyer, et al, 1994 (13) and Kennett, 1997(14).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptor:</td>
</tr>
<tr>
<td>G protein Linkage:</td>
</tr>
<tr>
<td>Effector System:</td>
</tr>
<tr>
<td>Ion channel</td>
</tr>
<tr>
<td>Subtypes:</td>
</tr>
</tbody>
</table>

\textsuperscript{AC = adenylyl cyclase, PLC = Phospholipase C. For a description of G proteins, see table 2.}
\textsuperscript{*The G protein/signal transduction for 5-HT\textsubscript{1B} has yet to be elucidated.}

These receptors have been classified according to a number of criteria; pharmacological, cDNA-deduced primary amino acid sequences, signal transduction and effector molecules (13,15-16). 5-HT\textsubscript{3} receptors are the lone ligand-gated ion channel receptor in the 5-HT family, closely related to other
ion channel receptors (GABA, glycine, AMPA/kainate glutamatergic and nicotinic cholinergic) in that they consist of a large glycosylated extracellular N-terminus, with four subsequent transmembrane segments (17).

The remainder of the known 5-HT receptors are members of a larger superfamily of seven transmembrane receptors (7TMR) which are linked via heterotrimeric guanine nucleotide binding proteins (G proteins) to their effector molecules (18). As previously mentioned in table 1, receptors of the 5-HT_1 type are generally associated with G_i. Receptors of the 5-HT_2 type are coupled with G_q, resulting in phosphoinositide turnover and intracellular Ca^{++} mobilization through the phospholipase C pathway. The others, although less well characterized, appear to be coupled to G_q, with increases in cellular cAMP.

B. 5-HT_1A Receptors

The 5-HT_1 receptors were originally grouped together because of high affinity for 5-HT and 5-carboxyamidotryptamine (5-CT), as well as being antagonized by agents like methysergide, but are now grouped mainly on molecular biological criteria (13). All 5-HT_1 mammalian subtypes are encoded by a single intronless gene sharing greater than 60% identity across the putative transmembrane domains (13). All known subtypes of the 5-HT_1 receptor class have been cloned and are negatively coupled to adenylyl cyclase (AC) through G_i (13).
The 5-HT_{1A} receptors are among the most well studied subtype of this class of receptor. 5-HT_{1A} receptors are primarily neuronal, are found mainly in the central nervous system (CNS) with some examples located peripherally. In the CNS, high receptor densities lie within the dorsal raphe, hippocampus, and throughout the cortex (19). 5-HT_{1A} receptors are believed to play roles in body temperature regulation, feeding and sexual behavior, anxiety, depression, and migraine headache (19). The receptor has been cloned from a number of species, including the rat and human, which share 99% identity in the transmembrane regions (20-21). The human 5-HT_{1A} receptor was discovered in a cDNA library using a β-adrenergic receptor probe (20) and the clone, termed G-21, was subsequently found to contain the 5-HT_{1A} receptor (22).

In most expression systems, the receptor is negatively coupled to AC via G_i (23). However some expression systems display both inhibition of AC and activation of the phospholipase C (PLC) pathway, although PLC activation is not as efficient as the receptor-mediated inhibition of AC. The reason for this is not clear, since this phenomenon has not been detected in tissue preparations (23) and thus has no known physiological relevance.

C. Seven transmembrane/G protein-coupled receptors (7TM/GPCR)

7TM/GPCR are a superfamily of integral membrane proteins that relay information across cellular membranes, responding to such diverse
extracellular stimuli as photons, pheromones, odorants, peptides, and monoamines (18,24). These serpentine membrane proteins possess a multiply glycosylated extracellular amino terminus and intracellular carboxy tail of varying length. There are 7 putative transmembrane (TM) domains forming α-helices, arranged in a distorted cylinder-like fashion (25), the extracellular core of this cylinder forming the hypothesized ligand-binding domain.

Interspersed between the TM domains on the cytoplasmic face are three intracellular (il-3) loops (some receptors may exhibit a fourth intracellular loop by virtue of a palmitoylated residue on the carboxy terminus, which inserts itself into the membrane (26)). These intracellular loops form the contact site for the heterotrimeric G protein. A schematic of a generic 7TM/GPCR is presented in figure 1.

![Figure 1: Schematic of a typical seven transmembrane/G protein coupled receptor. N = sites of N-linked glycosylation, C = sites of cysteine modification, e1-3 = extracellular loops, il-4 = intracellular loops, TM1-7 transmembrane domains. From Spiegel, 1995 (26).](image-url)
Initially, this tertiary structure was deduced from hydrophobicity plots of primary amino acid sequence. Until recently, the basic characteristics and structure of these receptors have been implied from bacteriorhodopsin, a 7 transmembrane protein. Although it is not G protein-coupled, and shares no sequence homology with 7TM/GPCR, the bacteriorhodopsin model nonetheless has proven to be useful in many respects (25). Now, a low-resolution crystal structure of rhodopsin has been elucidated (27-28), providing a true G protein-coupled receptor for modelling purposes.

Upon agonist binding, a series of conformational changes take place in the TM domains, of which movement of TM6 causes a shift in TM3. This alters the cytoplasmic face of the i3 loop region (between TM5 and TM6), thus changing receptor affinity for the G protein, which in turn is activated. Site-directed mutagenesis (29), and receptor chimeras have demonstrated that i2, i3, and the carboxy terminus/i4 are sites for receptor/G protein coupling (30).

D. Heterotrimeric G Proteins

Heterotrimeric G proteins are a family of membrane-associated signal transducers that couple 7TMRs to their effector molecules (31). Containing α, β, and γ subunits, they are classified according to the function of the α subunit (32-33). The α subunits can be divided into roughly 4 classes, based upon their
amino acid sequences and function (33). These relationships are summarized in table 2.

<table>
<thead>
<tr>
<th>Class</th>
<th>Alpha Subunit</th>
<th>Effectors</th>
<th>Intracellular Message</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>s</td>
<td>$\alpha_s$</td>
<td>(+) AC, open Ca$^{++}$ channels</td>
<td>(+) cAMP, (-) MP</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td></td>
<td>$\alpha_{olf}$</td>
<td>(+) AC</td>
<td>(+) cAMP</td>
<td>Olfactory epithelium</td>
</tr>
<tr>
<td>i/o/t</td>
<td>$\alpha_1, \alpha_2, \alpha_3$</td>
<td>(-) AC, open K$^+$ channels</td>
<td>(-) cAMP, (+) MP</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td></td>
<td>$\alpha_0$</td>
<td>close Ca$^{++}$ channels</td>
<td>(-) MP</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>$\alpha_2$</td>
<td>(-) AC</td>
<td>(-) cAMP</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>$\alpha_{11}$</td>
<td>(+) cGMP-PDE</td>
<td>(-) cGMP</td>
<td>Rods</td>
</tr>
<tr>
<td></td>
<td>$\alpha_{12}$</td>
<td>(+) cGMP-PDE</td>
<td>(-) cGMP</td>
<td>Cones</td>
</tr>
<tr>
<td></td>
<td>$\alpha_{gust}$</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Taste buds</td>
</tr>
<tr>
<td>q</td>
<td>$\alpha_q, \alpha_{11}, \alpha_{14}, \alpha_{15}$</td>
<td>(+) PI-PLC, $\beta$ subtypes</td>
<td>(+) IP$_3$, DAG</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>12/13</td>
<td>$\alpha_{12}, \alpha_{13}$</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Ubiquitous</td>
</tr>
</tbody>
</table>

AC = adenyl cyclase, DAG = diacylglycerol, cGMP-PDE = cyclic GMP phosphodiesterase, IP$_3$ = inositol (tris)phosphate, MP = membrane potential, PLC = phospholipase C, PI = phosphatidylinositol.

In its inactive state, the $G_{\alpha\beta\gamma}$ trimer has GDP tightly bound to the guanine nucleotide binding site/GTPase site on the $\alpha$ subunit. In this state, $G_{\alpha}$ has a very high affinity for $G_{\beta\gamma}$ and the receptor. Upon activation of the $G$ protein, the $\alpha$ subunit disassociates from the receptor and the $\beta\gamma$ dimer because of a conformational change due to the exchange of bound GDP for GTP on the $\alpha$ subunit. The $G_{\alpha}$-GTP subunit then activates an appropriate effector molecule and upon GTP hydrolysis this regulation is terminated—thus leaving GDP bound to $G_{\alpha}$. The now inactive $G_{\alpha}$ re-associates with $G_{\beta\gamma}$ and is ready to begin the cycle again. This $G$ protein cycle is presented in figure 2.
Once activated, G proteins can be prevented from cycling in the presence of a nonhydrolyzable substrate such as GTP\textsubscript{γS}, or AlF\textsubscript{4}− which is presumed to resemble the transition state of GTP. This can also be caused by bacterial endotoxins such as pertussis or cholera toxins (31), which ADP-ribosylate the \(\alpha\) subunit, changing the active site and preventing hydrolysis of GTP. In either case, the G protein is unable to hydrolyze its GTP or homologue substrate, and its actions at any effector molecule are essentially irreversible. Once it was believed that only \(G_\alpha\) was involved in intracellular signaling and effector regulation. However, recent evidence reveals a role for the \(G_{βγ}\) dimer in both the activation and regulation of intracellular effector molecules once disassociated from \(G_\alpha\) (33).

Some roles ascribed to \(G_{βγ}\) regulation are modulation of K\textsuperscript{+} and Ca\textsuperscript{2+} ion channel function, control of several proteins in the mitogen activating
protein (MAP) kinase cascade, adenylyl cyclase, phospholipase A₂, and the plasma membrane Ca²⁺ pump (34-35).

It is interesting to note that when coupled to the receptor, the G protein influences the affinity of the receptor with respect to agonist binding (11,36). When inactive Gα-GDPβγ is coupled to the receptor, the receptor displays a high affinity for agonist binding. As the agonist binds, the receptor shifts from high to low affinity, once GTP is exchanged for GDP on Gα.

E. Summary

The scope of this thesis is necessarily limited to what pertains to the biochemistry of human 5-HT₁A receptor. However, as a member of the 7TM/GPCR superfamily, a thorough understanding of all the components involved is necessary to grasp the hypothesis and the methodology used in testing it. Figure 3 displays a schematic of an expressed 5-HT₁A receptor/G protein-effector system to detail the complex relationships involved in these systems.
Figure 3: 5-HT$_{1A}$ receptors, effectors, and second messengers of heterologous expression systems. AA = arachidonic acid, AC = adenylyl cyclase, DAG = diacylglycerol, IP$_3$ = inositol (tris)phosphate, PLA$_2$ = phospholipase A$_2$, PLC = phospholipase C, PIP$_2$ = phosphatidylinositol (bis)phosphate, PKC = protein kinase C. From Raymond (19).
Hypothesis and Specific Aims

A. Hypothesis

The primary purpose in this investigation is to gain a better understanding of the functional domains lying at the interface between G proteins and the human 5-HT_{1A} receptor. Currently, several methods are employed to investigate the interaction between the cytoplasmic intracellular loops of the receptor (i1-i4/carboxyl terminus) and the G protein in 7TM/GPCR. Among these are construction of receptor chimeras, site-directed mutagenesis of amino acid residues, deletion of functional domains, and peptide probes from both natural and synthetic sources.

It is hypothesized that synthetic peptide probes designed to mimic the intracellular loops of the human 5-HT_{1A} receptor will compete with the receptor for binding to the G protein. It is further hypothesized that these peptides will alter the function of the receptor in a manner that can be quantifiable. Results from experiments utilizing these peptides will be interpretable in such a way as to provide useful information on the molecular determinants and functional domains that comprise the human 5-HT_{1A} receptor/G protein interface, and thus can be utilized to study receptor-G protein coupling.

This approach has been used in a number of 7TM/GPCR systems, including various hormone receptors (37), opioid (38), and most relevant
here; the 5-HT system (39). Previously, our laboratory has established that a 15 amino acid peptide (15mer) from the transmembrane 5/intracellular loop 3 region (TM5/i3) of the human 5-HT$_{1A}$ receptor is biologically active with respect to agonist inhibition and signal transduction (40). Two modified 15mers have been synthesized to determine effects that non-conservative amino acid substitutions have on the interactions between receptor and G protein. The sites of alteration for 15mer were selected by interpretation of experimental observations from other groups working with 7TM/GPCR (41). The first alterations to the 15mer is at positions 6 (R$^{22}$ to G$^{22}$) and 7 (F$^{23}$ to A$^{23}$) from its N-terminus. This new peptide is termed 15mer GA. The second, termed 15mer GAGA, includes further substitutions to the 15mer GA at positions 8 (R$^{24}$ to G$^{24}$) and 9 (I$^{25}$ to A$^{25}$) from the N-terminus.

A third peptide, an 11mer, will be tested in the same paradigm to determine if similar interactions between the receptor and G protein occur and can be observed using similar techniques at a site distinct (the TM3/i2 region) from the 15mers. This region was selected as it contains a highly conserved motif near its N-terminus. Most 7TM/GPCR contain a D/ERY motif near the TM3/i2 junction, and as such it has also been implicated as a functional domain.

These peptide probes have been synthesized according to the published human 5-HT$_{1A}$ receptor sequences (20). Figure 4 displays the sequences and domains of the receptor that the peptides are presumed to mimic.
The size requirement of these peptides uses previous work with receptomimetic peptides of various 7TM/GPCR as a guideline. Most of these studies utilize peptides in the range of ten to twenty amino acids, but further work in this area is needed to understand the size and structural requirements. The structure of these peptides has only been estimated by comparing structures from similar peptide probes and through energy minimization during molecular modelling. Three-dimensional energy minimized structures of the peptides are presented in appendix 3.
B. Specific aims

To provide the data necessary to evaluate the hypothesis stated on page 11, the specific aims are to:

1. Characterize the actions of the 15mer GA with the G protein by measuring agonist inhibition in whole cells, as well as in membrane and solubilized preparations of the receptor. Characterization will also entail measuring cAMP formation, thus determining if G protein/receptor interaction is uncoupled, or if GA can activate the G protein directly.

2. Test the ability of 15mer GAGA to interfere with the receptor/G protein interface. Again, agonist inhibition in whole cells, membrane, and solubilized preparations of the receptor will be measured. cAMP formation will also be measured to determine if 15mer GAGA uncouples the receptor from signal transduction.

3. Determine if the 11mer is biologically active in agonist inhibition assays. If so, this will indicate a possible site of the G protein-receptor interaction. If the 11mer is determined to be active, further characterization will entail measuring cAMP formation.
Bioactivity of these peptides are assessed by their ability to displace a specifically-bound agonist, as well as effects on the formation of second messengers through signal transduction assays. The agonist inhibition assays will be based upon the well-known alterations observed in agonist binding upon addition of GTP or stable analogues like GTPγS (11,36). As GTP (or analogue) binds to the agonist-receptor-G protein complex, it disassociates the complex. This disassociation shifts the affinity state of the receptor from a high (coupled with G protein/GDP) to a low affinity state, with respect to agonist binding. This is occurs in a concentration-dependent manner. Thus, if these peptide probes do in fact compete for binding sites on the G protein with receptor-agonist complex, a concentration-dependent displacement of the specifically bound agonist will be observed. This will provide evidence that peptides compete with the receptor for the G protein and effectively uncouple it from the receptor. This will not only be tested in whole cells expressing the receptor and crude membrane preparations of these cells, but also in solubilized receptors, to examine the effects of lipid membranes on the peptides' activities. Since all barriers to the peptides gaining intracellular access have been removed and high-affinity G protein-receptor coupling has been observed in solubilized receptor preparations (43), one would expect an increase of peptide-G protein interaction if lipid membranes pose a barrier to these peptides.

If the peptides interact with the G protein, as determined by agonist inhibition, then there should also be modulatory effects by the peptides on
events downstream from the receptor. This will be tested through examination of the peptides' effects on second messenger formation. The human 5-HT\textsubscript{1A} receptor most effectively couples with G\textsubscript{iα} specifically G\textsubscript{iα2} when expressed in CHO cell cultures (44). In a model system treated with forskolin, an activator of adenylyl cyclase, treatment with an agonist should cause a decrease in the second messenger cAMP through receptor activation and subsequent G\textsubscript{i}-mediated inhibition of adenylyl cyclase. However, if the G protein is uncoupled from the receptor by one of the peptide probes, there should be no decrease in cAMP production in the presence of a 5-HT\textsubscript{1A} agonist, as G\textsubscript{i} is not able to interact with agonist-activated receptor. However, there is a possibility that a receptomimetic peptide could retain enough structural characteristics of the agonist-bound receptor. If this were the scenario, then the peptide might also be able to inhibit cAMP formation in our paradigm, by direct activation of G\textsubscript{i}.
Materials and Methods

A. Chemicals

Chemicals were obtained from the following sources: Ham’s F-12 medium, Dulbecco’s Modified Eagle medium (DMEM), Earle’s Balanced Salt solution (EBBS), trypsin, and Geneticin® (GIBCO/BRL; Gaithersburg, MD). Fetal calf serum (FCS) and Calf serum (CS) (Summit Biotechnology; Fort Collins, CO). Tris(hydroxymethyl)aminomethane (Tris base), N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]) (HEPES), disodium ethylamninie(diamine) tetraaceetic acid (EDTA), and calcium chloride (Baker Chemical Co.; Phillipsburg, PA). Mianserin, pargyline, dithiothreitol (DTT), 3-isobutyl-1-methyl-xanthine (IBMX), sodium cholate, aprotonin, and soybean trypsin inhibitor (Sigma Chemical Co.; St. Louis, MO). [3H]ketanserin and [3H]8-hydroxy-2-(di-N-propylamino)tetrailn ([3H]8-OH-DPAT) (New England Nuclear; Boston, MA). Diethyl ether (Fisher Scientific; Pittsburg, PA). Dimethysulfoxide (DMSO) (Mallinckrot-Baker; Paris, KY). Leupeptin, benzamidine, and G 418 sulfate (Calbiochem; LaJolla, CA). Forskolin (ICN; Costa Mesa, CA). Biotrak® [125I]cAMP radioimmunoassay (RIA) kit (Amersham Lifesciences; Arlington Hights, IL). Enzyme immunoassay (EIA) kit for cAMP was obtained from either Cayman Chemical (Ann Arbor, MI) or Assay Designs (Ann Arbor, MI). Serotonin was obtained from either ICN or
Calbiochem. Other chemicals and reagents were obtained from additional commercial suppliers.

B. Cell Culture

CHO cells expressing the human 5-HT\textsubscript{1A} receptor were cultured in monolayer using Ham's F-12 medium fortified with 10% FCS and 200 μg/mL Geneticin\textsuperscript{®} (G 418 sulfate). Cultures were maintained at 37° C in a humidified atmosphere of 5% CO\textsubscript{2}, 95% air. Upon confluency (5-7 days), cells were either used in assays or subcultured.

C. Agonist Inhibition Assays

When confluent, cells from either 80 cm\textsuperscript{2} or 175 cm\textsuperscript{2} flasks were harvested with 0.25% trypsin in physiological saline with subsequent low-speed centrifugation in ice-cold media. The resulting pellet was resuspended in ice-cold EBBS, followed by centrifugation. Collected cells received one of the following treatments, according to preparation of receptor desired:

(1) Whole cell preparations: The collected cells were suspended in 30 volumes of ice-cold binding buffer (50 mM Tris, 4 mM, CaCl\textsubscript{2}, 10 μM pargyline, pH 7.4). This suspension was used directly in the assay.
(2) Membrane preparations: To obtain crude membrane preparations of the receptor, the collected cells were suspended in ice-cold binding buffer with subsequent centrifugation at 40,000 x g for 45 minutes at 4°C to lyse the cells. This crude membrane fraction was then re-suspended in 30 mL ice-cold binding buffer and subjected to homogenization, first on glass, then with a Brinkmann Polytron.

(3) Solubilized receptor preparations: Collected cells were placed in 1 mL of solubilizing buffer (20 mM Tris, 0.6% sodium cholate, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1 mM benzamidine, 10 µg/mL leupeptin, 100 mg/mL soybean trypsin inhibitor, and 10 µg/mL aprotinin, pH 8.0) on ice for 1 hour. After centrifugation at 40,000 x g for 40 minutes at 4°C, the supernatant was removed and diluted to 30 mL in ice-cold binding buffer to be used in the assay.

All preparations were kept on ice throughout the assay. Protein content was determined using the Bradford method (45). Displacement of \[^{3}H\]8-OH-DPAT (specific activity of 132.8 Ci/mmol) from the receptors was determined using modifications of well-established protocols (46-48). Assays were run in triplicate, incubated in a shaker/bath at 30°C for 30 min. Each reaction mixture consisted of 700 µl receptor preparation, 100 µL of either
binding buffer or 10 μM serotonin in binding buffer to determine non-specific binding, 100 μL 0.50 nM [³H]8-OH-DPAT and 100 μL of peptide, yielding a total volume of 1 mL. Incubations were stopped with 4 mL of ice-cold 50 mM Tris buffer and rapid filtration over Whatman GF/B or, in the case of solubilized receptor preparations, Whatman GF/F glass fiber filters. This is followed with two subsequent 5 mL washes, for a total wash volume of 14 mL. Filters were counted in 5 mL of Ecoscint® liquid scintillation fluid in a Beckman LS 6500 liquid scintillation counting system.

D. cAMP Determination

To determine cAMP formation, an intact cell culture method was used (46,49). When confluent, the media from CHO cells grown either in 12 or 24 well plates was removed. After two washes with serum-free media, cells were treated in 0.5 mL serum-free media with 100 μM IBMX with the final concentrations of 30 μM forskolin, 10 μM serotonin, and either 114 μM (15mer GA), 124 μM (15mer GAGA), or 131 μM (11mer) of peptide and then incubated for 20 minutes at 37° C, with agitation after 10 min. Wells were then aspirated and 0.5 mL of 100 mM HCl was added, and the plate was incubated for 10 minutes, whereupon well contents were removed and centrifuged at 4000 rpm for 15 minutes at 4° C. Supernatants were extracted four times with H₂O-saturated diethyl ether. The aqueous extracts were desiccated overnight
at 60° C. cAMP was quantified with a RIA kit using gamma counting, or an EIA kit using a plate reader at λ = 405 nm.

E. Peptide Synthesis and Preparation

Peptides were synthesized utilizing solid-phase synthesis on an ABI 431A automated peptide synthesizer, using amino acids from ABI. Following synthesis, peptides were purified with a Waters 625 HPLC System using acetonitrile and aqueous 2 mM HCl gradients, and lyophilized. Peptides were initially dissolved in deionized water, or in the case of 11mer, 5% DMSO in deionized water. At highest concentration of 11mer run, the final concentration of DMSO was 0.5%. Control experiments showed that concentrations of 0.5% DMSO had no effect on our system. Subsequent dilutions of the peptides were in binding buffer, or media when used in the cAMP assays.

F. Data Treatment

Graphing and non-linear regression, as well as statistical analysis was with computerized procedures (CA Cricket Graph III®, Prism Graph Pad®, Microsoft Excel®, StatView 512+®).
(1) **Agonist inhibition assays**: Results are expressed as percent of controls. IC$_{50}$ values were determined from non-linear regression analysis of dose-response curves. Apparent K$_i$ values were determined from IC$_{50}$'s according to the Cheng-Prusoff relationship (50):

\[
K_i = \frac{IC_{50}}{1 + [L]/K_d}
\]

Where [L] is the concentration of the radioligand and K$_d$ is its respective equilibrium disassociation constant.

(2) **cAMP assays**: Standard curves were prepared and subjected to linear regression analysis. From the standard curve, unknown quantities of cAMP could be calculated. Data expressed as percent of forskolin-stimulated controls.
Results

A. 15mer GA Peptide

15mer GA, the first of the altered 15mer peptides to be characterized, produced concentration-dependent effects as determined to inhibition of \[^3H\]8-OH-DPAT binding in whole cell (figure 5), membrane (figure 6), and solubilized (figure 7) preparations of the receptor. IC\textsubscript{50} and K\textsubscript{i} values obtained for the 15mer GA were similar in each receptor preparation (table 3), with an overall IC\textsubscript{50} value of 59.7 ± 6.09 μM, and resulting apparent K\textsubscript{i} of 32.5 ± 3.33 μM.

<table>
<thead>
<tr>
<th>Receptor Preparation</th>
<th>IC\textsubscript{50}</th>
<th>K\textsubscript{i}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell</td>
<td>58</td>
<td>31.6</td>
</tr>
<tr>
<td>Membrane</td>
<td>69</td>
<td>37.6</td>
</tr>
<tr>
<td>Solubilized</td>
<td>52</td>
<td>28.4</td>
</tr>
<tr>
<td>Average ± SEM</td>
<td>59.7 ± 6.09</td>
<td>32.5 ± 3.33</td>
</tr>
</tbody>
</table>

In second messenger assays, the 15mer GA was able to produce a modest, but significant (p<0.01) decrease in cAMP formation, when compared to the forskolin controls. This decrease was not significantly different than the 5-HT treatment alone or when 5-HT was combined with the peptide, as shown in figure 8. This suggests that in addition to the 15mer GA competing with the receptor for the G protein, it apparently can assume the
conformation of the agonist-activated receptor. This would then activate $G_{i}$, subsequently inhibiting forskolin-stimulated cAMP production in a similar manner as an agonist activated receptor.

The region of i3 from which the original 15mer was based upon is highly conserved across several species, and the original 15mer was shown to be active in rabbit cortex membranes (40). Therefore, a brief investigation was conducted to determine if the 15mer GA could display cross-species activity by utilizing membrane preparations of rat cortex. The rat 5-HT$_{1A}$ receptor differs from the human receptor only by one amino acid in the region of the 15mer GA. In position two from the C-terminus there is a substitution for lysine with arginine (21) in the rat receptor. This substitution is not contained within the region altered in the 15mer GA. Figure 9 shows the results of this experiment, with similar levels of displacement of bound agonist observed.

![Graph](image.png)

**Figure 5:** Inhibition of $[^3H]8$-OH-DPAT binding by 15mer GA in whole cells expressing the human 5-HT$_{1A}$ receptor. Results are expressed as the mean ± SEM of at least 3 experiments run in triplicate. Conditions are as described in the Materials and Methods section.
Figure 6: Agonist inhibition by 15mer GA in membrane preparations of the human 5-HT$_{1A}$ receptor. Results are expressed as the mean ± SEM of at least 3 experiments run in triplicate. For experimental conditions refer to the Materials and Methods section.

Figure 7: Inhibition of [$^3$H]8-OH-DPAT binding by 15mer GA in solubilized human 5-HT$_{1A}$ receptors. Results are expressed as the mean ± SEM of at least 3 experiments run in triplicate. Conditions are as described in the Materials and Methods section.
Figure 8: Effects of 15mer GA on forskolin-stimulated cAMP. Peptide concentration was 114 μM. Results are means ± SEM of 3 experiments run with n = 9. Values are expressed as percent of forskolin-stimulated cAMP, which was 568.83 ± 42.63 fmol Statistical significance was defined with a repeated measures ANOVA using Fisher PLSD (\( ^* = p < 0.01 \)). Conditions are as described in the Materials and Methods section.

Figure 9: Ability of 15mer GA to inhibit \([H]8-OH-DPAT\) binding in membrane preparations of the rat cortex. Results from membrane preparations of CHO cells expressing the human 5-HT_{1A} receptor are shown for comparison. Results are expressed as the mean ± SEM of 2 experiments run in triplicate. Conditions are as described in the Materials and Methods section.
B. 15mer GAGA Peptide

The second of the altered 15mer peptides based upon the i3 region of the human 5-HT\textsubscript{1A} receptor, 15mer GAGA, also gave a concentration-dependent effect in the agonist inhibition assays. Displacement of [\textsuperscript{3}H]8-OH-DPAT from whole cell (figure 10), membrane (figure 11), and solubilized (figure 12) preparations of the receptor revealed a lower potency than that of the 15mer GA, with resultant higher IC\textsubscript{50} and K\textsubscript{i} values (table 4). An overall IC\textsubscript{50} value of 103.7 ± 8.04 μM, and resulting apparent K\textsubscript{i} of 56.5 ± 9.39 μM were calculated. These values are about twice those of 15mer GA.

<table>
<thead>
<tr>
<th>Receptor Preparation</th>
<th>IC\textsubscript{50}</th>
<th>K\textsubscript{i}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell</td>
<td>107</td>
<td>58.4</td>
</tr>
<tr>
<td>Membrane</td>
<td>113</td>
<td>61.6</td>
</tr>
<tr>
<td>Solubilized</td>
<td>91.0</td>
<td>49.5</td>
</tr>
<tr>
<td>Average ± SEM</td>
<td>103.7 ± 8.04</td>
<td>56.5 ± 9.39</td>
</tr>
</tbody>
</table>

When assayed for effects on second messenger production, 15mer GAGA strongly inhibited forskolin-stimulated cAMP production, and this result was highly significant (p<0.001), although when combined with 5-HT, the resultant inhibition is no more than 5-HT alone, as shown in figure 13. This is different than with the 15mer GA, as there seems to be no effect of the peptide on 5-HT-mediated inhibition of forskolin-stimulated cAMP formation.
Figure 10: Inhibition of [\textsuperscript{3}H]8-OH-DPAT binding by 15mer GAGA in whole cells expressing human 5-HT\textsubscript{1A} receptors. Results are expressed as the mean ± SEM of at least 3 experiments run in triplicate. Conditions are as described in the Materials and Methods section.

Figure 11: Concentration-dependent inhibition of [\textsuperscript{3}H]8-OH-DPAT by 15mer GAGA in membrane preparations of the human 5-HT\textsubscript{1A} receptor. Results are expressed as the mean ± SEM of at least 3 experiments run in triplicate. Conditions are as described in the Materials and Methods section.
Figure 12: Displacement of specifically-bound \[^{3}H\]8-OH-DPAT from solubilized human 5-HT\textsubscript{1A} receptors by 15mer GAGA. Results are expressed as the mean ± SEM of at least 3 experiments run in triplicate. Conditions are as described in the Materials and Methods section.

\[ K_i = 49.6 \mu M \]
\[ IC_{50} = 91 \mu M \]

Figure 13: Inhibition of forskolin-stimulated cAMP accumulation by 5-HT and 15mer GAGA. Peptide concentration was 124 μM. Results are means ± SEM of 4 (forskolin), 5 (5-HT), 4 (5-HT + peptide), and 7 (peptide) experiments with n = 9. Values are expressed as percent of forskolin-stimulated cAMP, which was 675.85 ± 67.14 fmol. Statistical significance was defined with a repeated measures ANOVA using Fisher PLSD (\(* * = p < 0.001\)). Conditions are as described in the Materials and Methods section.
C. 11mer Peptide

The 11mer peptide is from a site distinct from the peptides of the 15mer family, designed to mimic the TM3/i2 region of the human 5-HT_{1A} receptor. The 11mer also gave a concentration-dependent displacement of [^3H]8-OH-DPAT in whole cells expressing the human 5-HT_{1A} receptor (figure 14) as well as membrane (figure 15), and solubilized (figure 16) preparations of the receptor. Analysis revealed IC_{50} and K_i values in the low micromolar range. Unlike the i3 loop peptides, there was a marked difference in the potencies of the peptides between the various receptor preparations. The whole cell parameters were about threefold times that of the membrane and eightfold times that of the solubilized receptor values (table 5). This may indicate that the 11mer, unlike 15mer GA and GAGA peptides, has difficulties in crossing cellular membranes.

<table>
<thead>
<tr>
<th>Receptor Preparation</th>
<th>IC_{50}</th>
<th>K_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell</td>
<td>15.7</td>
<td>8.57</td>
</tr>
<tr>
<td>Membrane</td>
<td>6.71</td>
<td>3.66</td>
</tr>
<tr>
<td>Solubilized</td>
<td>2.11</td>
<td>1.15</td>
</tr>
<tr>
<td><strong>Average ± SEM</strong></td>
<td><strong>8.2 ± 4.89</strong></td>
<td><strong>4.5 ± 2.67</strong></td>
</tr>
</tbody>
</table>
Figure 14: Inhibition of agonist binding in whole cells expressing the human 5-HT$_{1A}$ receptor by 11mer peptide. Results are expressed as the mean ± SEM of at least 3 experiments run in triplicate. Conditions are as described in the Materials and Methods section.

Figure 15: Ability of 11mer to inhibit [$^3$H]8-OH-DPAT binding in membrane preparations of the human 5-HT$_{1A}$ receptor. Results are expressed as the mean ± SEM of at least 3 experiments run in triplicate. Conditions are as described in the Materials and Methods section.
Figure 16: Displacement of $[^3]$H8-OH-DPAT in solubilized human 5-HT$_{1A}$ receptors by 11mer. Results are expressed as the mean ± SEM of at least 3 experiments run in triplicate. Conditions are as described in the Materials and Methods section.

Figure 17: Effects of 11mer on forskolin-stimulated cAMP accumulation in whole cells expressing the human 5-HT$_{1A}$ receptor. Peptide concentration was 131 μM. Results are means ± SEM of 4 experiments run with n = 4. Values are expressed as percent of forskolin-stimulated cAMP, which was 675.85 ± 67.14 fmol. Statistical significance was defined with a repeated measures ANOVA using Fisher PLSD (** = p<0.001, * = p<0.01). Conditions are as described in the Materials and Methods section.
When the 11mer was tested in signal transduction assays, the peptide displayed modest ability to inhibit forskolin-stimulated cAMP formation, but was not statistically significant (p<0.01) from the controls. The peptide had no effect on 5-HT mediated inhibition of cAMP (figure 17), being not statistically different from 5-HT alone (p<0.01).
Discussion

A. Summary of Results

Each of the synthetic peptide probes of the human 5-HT₁A receptors tested produced a concentration-dependent displacement of the specifically-bound agonist [³H]8-OH-DPAT from the receptors. This was observed in all preparations of the receptor, including the whole cell preparations, suggesting that each peptide was able to gain at least moderate intracellular access.

In the second messenger assays only one of the peptides, 15mer GAGA, was able to inhibit forskolin-stimulated cAMP greater than 50% of the controls, with the 11mer from the i2 site exerting no significant effect on cAMP production. Table 6 summarizes these results.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Apparent Kᵢ (µM) †</th>
<th>% Inhibition of Forskolin Stimulated cAMP ± SEM ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole Cell</td>
<td>Membrane</td>
</tr>
<tr>
<td>15mer GA</td>
<td>31.6</td>
<td>37.6</td>
</tr>
<tr>
<td>15mer GAGA</td>
<td>58.4</td>
<td>61.6</td>
</tr>
<tr>
<td>11mer</td>
<td>8.6</td>
<td>3.7</td>
</tr>
</tbody>
</table>

†Whole cell, membrane, and solubilized indicate the preparation of the receptor in which the Kᵢ was determined.
‡Expressed as percent of controls treated with 30 µM forskolin. Statistical significance from controls was defined with a repeated measures ANOVA using Fisher PLSD (* = p < 0.01, ** = p < 0.001).
B. Inhibition of $[^3\text{H}]\text{8-OH-DPAT}$ Binding by the Peptide Probes

15mer GA was observed to displace bound agonist over a very narrow concentration range, and maximal inhibition obtained was without exception less than 100%. The degree of inhibition was highest in the solubilized receptor preparations where 114 $\mu$M 15mer GA resulted in approximately 85% inhibition of $[^3\text{H}]\text{8-OH-DPAT}$ binding, whereas in the other preparations this concentration produced approximately 70% inhibition. Concentrations of 15mer GA higher than 114 $\mu$M were not investigated due to solubility limits of the peptide.

Due to its lack of potency, combined with the narrow concentration-effect range observed, the 15mer GAGA proved to be problematic. These factors combined to allow only three concentrations to be investigated in the agonist inhibition studies. At the highest concentration run, 124 $\mu$M, the peptide was able to produce inhibition only slightly greater than 50% in all three preparations of the receptor. Concentrations greater than 124 $\mu$M were not investigated, again because of solubility limits, while concentrations of GAGA less than 10 $\mu$M had no effect on specifically-bound agonist. This suggests that the alterations to the GA peptide in GAGA were effective in further reducing the ability of the peptide to uncouple the G protein from the receptor, as evidenced by $K_i$ and $IC_{50}$ values approximately twice that for the GA peptide in each assay format.
The fact that both 15mer GA and GAGA were slightly more active in whole cell preparations than that of membrane preparations is surprising, as both these peptides are highly charged and displays generally hydrophilic characteristics. Upon closer examination, the overall secondary structure of the peptide may play a role in its access to the cytoplasm. There is evidence that highly cationic peptides like mellitin, a 27 amino acid residue peptide from bee venom, can gain intracellular access (51). This peptide assumes an \(\alpha\)-helical like secondary structure with its highly charged N-terminus, and can bore holes thorough cellular membranes—thus exerting one of bee venom's cytotoxic effects. The mode of action may be similar with the altered 15mers, as they have a highly cationic C-terminus and can probably assume an \(\alpha\)-helical like structure. This, combined with a more physiologically-intact system than that of the receptors in the membrane preparations, may have allowed more efficient receptor or peptide coupling to the G protein.

The 11mer also displayed a concentration-dependent inhibition of agonist binding, but this effect was observable over a much larger concentration range than with the altered 15mer peptides. Perhaps more noteworthy however, is that the 11mer achieved similar levels of inhibition of \(^{3}\text{H}\)8-OH-DPAT binding at approximately tenfold less concentration than 15mer GA. As expected, the 11mer was most potent with respect to agonist inhibition in solubilized receptors. However, unlike the altered 15mer peptides, the 11mer from i2 was not more active in the intact cells containing the receptor than in membrane preparations. In fact, the ability of the 11mer
to displace specifically-bound [³H]8-OH-DPAT seemed to be proportional to how physiologically intact the system was. Maximal effect was approximately 55% inhibition of binding in the whole cell assays, where 65% and 75% was observed in membrane and solubilized receptors respectively. Furthermore, the concentration of peptide needed to achieve a maximal inhibition of [³H]8-OH-DPAT binding in whole cells, 210 μM, was more than twice that needed in membrane and solubilized receptors.

There was more than a doubling in Kᵢ and IC₅₀ values from the solubilized to the membrane preparations, and a further doubling again from membrane to the whole cell preparations. This suggests that lipid membranes pose a substantial barrier to the 11mer peptide, certainly more than the altered 15mers. This is somewhat surprising as the 11mer displays a more hydrophobic character than the 15mers. The 11mer carries net positive charge of one, while the 15mers are highly cationic peptides with net charges in the +5 to +6 range.

It should be mentioned at this point that interaction between G protein and all of the peptide probes has not been observed directly, and it is entirely possible that the peptides may be acting at a site distinct from the proposed binding between the G protein and receptor. Another possibility is that these peptides are active at sites in addition to the proposed binding sites: due to the experimental observations from the increasingly modified i3 site, the similarity of the IC₅₀ values for each peptide regardless of receptor
preparation, and the smooth sigmoidal shape of the agonist-inhibition curves.

The 11mer potently displaced bound agonist in a concentration-dependent manner, providing some evidence that it can effectively compete with the receptor for G protein binding. However, it displayed multiphasic concentration-effect curves in the membrane and solubilized receptors (see figures 15 and 16). This type of character in binding experiments may indicate additional site(s) of interaction, so it is possible that there are other mechanisms through which the 11mer is able to displace bound agonist, rather than uncoupling the G protein only at the proposed i2 site.

C. Cyclic AMP Assays

The fact that both 15mer GA and GAGA were able to significantly inhibit forskolin-stimulated cAMP lends support to the idea that these peptides are able to not only bind to the G protein, but also can assume a conformation similar to the activated receptor. This would then activate the G protein, and in our system subsequently inhibit cAMP formation. That a peptide could regulate G protein function is not unheard of, as several amphipathic, cationic peptides have been shown to interact with G proteins (51). Mastoparan, a amphipathic tetradecapeptide isolated from wasp venom, has been shown to regulate some G proteins, including G_i, by stimulating GTPase activity (52). Although there is some dispute over the exact
mechanism, one possibility appears to be a direct interaction with the carboxy tail of $G_{\alpha}$ (53). Mastoparan shares some characteristics with 15mer GA and GAGA: it is similarly sized and similar in amino acid composition, with lysine residues near the termini of the peptide.

That 15mer GAGA was more effective than 15mer GA in inhibiting cAMP formation is surprising. It is a possibility that the charged cationic and large hydrophobic side chains present in the original sequence motif -RFRI- favors coupling to the G protein, but not necessarily the full activation of $G_{\alpha}$. As the sequences deviate from those that are found in the native receptor sequence, the loss of the side chains in that motif which makes the receptor progressively less able to couple with the G protein, could increase the receptor's ability to activate the G protein. This could serve as a compensatory mechanism that has evolved between the G protein and receptor to preserve an overall coupling constant.

Perhaps the loss of the bulky side chains from -GARI- to -GAGA- allows the peptide to assume a conformation of binding that is more conducive to G protein activation. As G protein binding and G protein activation are two distinct processes (54), this idea is plausible. Despite the 15mer GA's apparently more potent actions on binding to the G protein, as evidenced by agonist inhibition, it may be that the steric hindrance does not allow the largely cationic tail of the peptides to interact with the activation domains on the G protein in 15mer GA, but not in 15mer GAGA. These
cationic residues have been implicated in G protein activation in 7TM/GPCR systems (41, 54-55).

The 11mer from i2 produced a modest decrease in cAMP formation, but it was not statistically significant when compared to the forskolin-stimulated controls. That the i2 peptide could not inhibit cAMP formation raises the possibilities that this peptide cannot mimic the activated receptor conformation, or that the i2 site does not convey the agonist-mediated message to the G protein. This fits within the current model of 7TM/GPCRs: although all cytoplasmic loops are thought to play a role in receptor-G protein coupling and activation, i3 is thought to be the main site for communication of receptor signaling to the G protein as well as binding (56-57). This then positions the G protein where further conformational changes allow other cytoplasmic loops to interact with the G protein including i2. It is the action of all the intracellular contact sites coalescing on the G protein that is thought to elicit full agonist activation of the G protein.

However, there is the possibility that the 11mer may not be reaching a high enough intracellular concentration. As shown in the binding data, the 11mer at the maximum concentration examined was able to displace only slightly more than 50% of bound agonist in whole cells expressing the receptor. Comparison to its activity in the other preparations suggests that it may be that the lack of intracellular access, not necessarily the peptide itself, that limits the 11mers efficacy in activating the G protein.
D. Comparison of the i3 Loop Peptide Family

The bioactive peptide known as the 15mer from the i3 region of the human 5-HT$_{1A}$ receptor was characterized in our lab in 1996 (40). Binding parameters are summarized in table 7. It is this 15mer upon which the altered 15mers GA and GAGA are based.

<table>
<thead>
<tr>
<th>Receptor Preparation</th>
<th>IC$_{50}$</th>
<th>K$_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell</td>
<td>5.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Membrane</td>
<td>3.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Solubilized</td>
<td>0.5</td>
<td>0.3</td>
</tr>
</tbody>
</table>

When the actions of 15mer GA and 15mer GAGA are compared to the original 15mer peptide (native sequence) from N-terminus of the i3 loop, an interesting pattern emerges. Designating the binding parameters for 15mer as unity, whole number ratios can be calculated and compared for the 15mer GA and GAGA peptides, presented in table 8.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Whole Number Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole cell</td>
</tr>
<tr>
<td>15mer</td>
<td>1</td>
</tr>
<tr>
<td>15mer GA</td>
<td>11</td>
</tr>
<tr>
<td>15mer GAGA</td>
<td>19</td>
</tr>
<tr>
<td>GA/GAGA ratio</td>
<td>0.54</td>
</tr>
</tbody>
</table>
In the whole cell preparation, GA peptide displayed a ten-fold lower potency than the 15mer, while the GAGA alteration was just less than two-fold lower in potency than 15mer GA. This trend continues in both membrane preparations and solubilized receptors, where the greatest disparity exists between the 15mer and the altered 15mers, where the difference was about 100- and 200-fold for 15mer GA and 15mer GAGA respectively. This difference could be due to 15mer having difficulty gaining intracellular access, much the same as the 11mer. Although the 15mer is from a different site than the 11mer, the $K_i$ values are much more similar to the i2 peptide, being only approximately twice those obtained for the 15mer in their respective preparations, and show a similar decreasing trend as the preparation deviates from a physiologically-intact system (see table 5 for 11mer binding parameters).

When the altered 15mer peptides are compared to the parent 15mer in their effects on cAMP formation, all significantly inhibit cAMP formation, when compared to the forskolin-stimulated controls. When the degree of cAMP inhibition is ranked, the 15mer GAGA was by far the most effective (54.8%), followed by 15mer GA (31.4%), with the least effective inhibitor being the 15mer (10%). The 15mer however, was run at a ten-fold lower concentration than its altered siblings. There is an inverse relationship with these peptides; the better the peptide uncouples the receptor from the G protein, the less able the peptide is able to activate the G protein. The reason for this is not clear, but as previously stated it could be that an evolutionary
flexibility has developed in G protein-receptor communication, where a
decrease in the ability of a G protein to couple could be compensated for by an
increase in the receptor's ability to activate the G protein. Again, less steric
hindrance in the coupling region could provide more conformational
freedom with respect to the activating region.

Recently, some data has been generated to address requirements for the
length of the peptide needed for specificity in G protein-peptide interactions.
Preliminary experiments using a peptide with the sequence of the original
15mer, truncated to a 6mer (sequence: ARFRIR), retains the -RFIR- motif
which was altered in 15mer GA and GAGA. At the time of writing, the 6mer
has been tested in whole cells expressing the human 5-HT\textsubscript{1A} receptor and
membrane preparations of the receptor, as well in the cAMP assays. Like the
other members of the 15mer family, it too displays a concentration-dependent
effect in displacing \textsuperscript{3}H8-OH-DPAT from receptors, as shown in figure 18.

However, not all the 15mer's activity was retained, as it was virtually
equipotent to 15mer GAGA in whole cells. This may be due to limited
intracellular access, and further experiments in solubilized receptors will be
needed to determine if lipid membranes pose obstacles to the truncated
15mer.
Figure 18: Inhibition of agonist binding in whole cells and membrane preparations expressing the human 5-HT1A receptor by truncated 15mer peptide (6mer). Results are expressed as the mean ± SEM of at least 3 experiments run in triplicate. Conditions are as described in the Materials and Methods section.

Figure 19: Effects of 6mer on forskolin-stimulated cAMP accumulation in whole cells expressing the human 5-HT1A receptor. 6mer concentration was 1 mM. Results are means ± SEM of 2 experiments run with n = 6. Values are expressed as percent of forskolin-stimulated cAMP, which was 2.59 ± 0.2 nmol. Statistical significance was defined with a repeated measures ANOVA using Fisher PLSD (** = p<0.001, * = p<0.01). Conditions are as described in the Materials and Methods section. FSK = forskolin.
When examined in the cAMP assays, the 6mer peptide at 1 mM concentration was unable to significantly inhibit cAMP accumulation, when compared to the forskolin-stimulated controls, as shown in figure 19. Again, it has not been determined if lipid membranes pose obstacles to intracellular access to the 6mer, so there is a possibility that the peptide may not reach a high enough intracellular concentration to activate G, even if were able to do so.

The results from the truncated 6mer contribute to an emerging picture of the structural and conformational requirements for a peptide-G protein coupling and/or activation at the i3 site. Further experiments detailing peptide interaction directly with the G protein using three-dimensional nuclear magnetic resonance spectroscopy (3D-NMR) would help to understand more clearly the peptide-G protein interactions. Circular dichroism (CD) spectra, when combined with molecular modelling techniques could produce binding and coupling conformations of the peptides, allowing further understanding of the structural and conformational requirements involved in receptor-G protein communication. Expression of cloned G proteins can provide the necessary amounts of material for these studies. Other future directions involving synthetic peptide probes, other than stated above, are determining in a more direct fashion the peptides G protein binding and activating properties.
E. Conclusions

Based upon the information presented for this thesis, several conclusions can be drawn:

1. The synthetic peptide probes of the human 5-HT$_{1A}$ receptor/G protein interface studied here were able to displace a specifically-bound agonist, [$^3$H]8-OH-DPAT, in a concentration-dependent manner. This observation is consistent with competition between the peptides and the receptor for binding site(s) on the G protein. This effectively uncouples the receptor from the G protein, shifting the receptor from a high to low affinity state for bound agonist.

2. The two peptide probes from i3 region investigated significantly decreased cAMP formation, when compared to the forskolin-stimulated controls, suggesting that the i3 peptides may be able mimic the activated receptor, thus activating $G_i$. The peptide from the intracellular loop 2 region, did not significantly decrease the formation of cAMP, when compared to the controls. These results suggest that the i2 peptide may not be able to activate the G protein, or that i3 may be involved to a greater degree than i2, with respect to G protein activation.
3. When comparing the activity of the altered i3 peptides to that of the parent peptide probe, non-conservative amino acid substitutions in a conserved -RFRI- motif drastically reduced the ability of the peptide to displace specifically-bound agonist, while the substitutions improved the peptides ability to decrease cAMP formation. These observations raise the possibility that the -RFRI- motif in i3 may be necessary for efficient receptor-G protein coupling, but is not essential for receptor mediated G protein activation in the human 5-HT\textsubscript{1A} receptor.
Appendix I: Structures of the Peptide Probes

A. Methods

Three-dimensional solution structures of the synthetic peptide probes were generated using Sybyl® molecular modelling software (Tripos, Inc. St. Louis, MO) running on a Silicon Graphics Indigo 2® workstation. Primary sequences of the peptides were entered, and assigned formal charges at pH 7.4. Dielectric constant was 80.0. Structures were then energy-minimized through at least 300 iterations.
15mer GA (N-terminus i3)
15mer GAGA (N-terminus i3)

I217 (N-terminus)

K231 (C-terminus)

R222G

F223A

R224G

I225A
15mer truncated to 6mer
(N-terminus i3)
11mer (N-terminus i2)
Appendix II: Trimethyltin/Protein Kinase C§

§This research was presented as a poster entitled "Differential Activation of C6 Glioma Protein Kinase C Isozymes by a Phorbol Ester and Trimethyltin" at the Western Pharmacology Society's 40th annual meeting in Banff, Alberta, Canada and was published in the Society's proceedings that year (1997) as an abstract:


A. Background

The research presented in this appendix was completed within my initial year at The University of Montana and was a laboratory rotation for Dr. Charles Eyer over the span of June 1996 to December 1996. The goals of this project are to contribute information to his ongoing research into the biochemical mechanisms of trimethyltin (TMT) neurotoxicity.

B. Introduction

The potent neurotoxin trimethyltin (TMT) causes a delayed, irreversible damage characterized by gliosis and neuronal cell death, and is highly specific to the hippocampus, especially in the pyramidal cells (58). The biochemical mechanisms by which this damage occurs is not well understood, therefore TMT's role(s) in the neuronal cell death is as of yet eludicated. Due to the delayed nature of its damage, metabotropic processes have been implicated in the pathogenesis of TMT.
Protein Kinase C (PKC), an enzyme involved in such cellular processes as transcription, growth, differentiation, and regulation of other signaling pathways, has been implicated in the damage caused by TMT (59). Excessive or sustained activation of PKC has been implicated in several neurodegenerative processes, such as excitotoxic or ischemic damage, as well as TMT toxicity. This has been previously reported in a cell line of neuronal origin, the PC12 pheochromacytoma (60). Treatment with TMT, caused a translocation of PKC from the cytosol to the membrane, which correlated to cellular death as measured by lactate dehydrogenase (LDH) release. This was prevented in this model by treatment with a PKC inhibitor, or previous down regulation of PKC with phorbol 12-myristrate 13-acetate (PMA), a PKC activator.

PKCs are a family a serine/threonine kinases that are abundant in a variety of tissues, including the CNS (61). Existing in at least 12 different isoforms, PKCs use the phosphatidylinositol (PI) signal transduction pathway and upon activation, the enzyme translocates from the cytosol to the membrane (62-63). PKC isozymes are classified as either conventional (cPKC), new (nPKC), or atypical (aPKC) according to their various requirements for activation (64). A summary of PKC classification and requirements for activation is presented in table 1A.

This study therefore, was designed to examine the effects of TMT treatment on specific PKC isozyme classes in an immortalized glial cell line. Many known isoforms of PKC have been reported in a C6 glioma cell line.
(65), thereby providing a good model of TMT's effects on PKC subclasses. Ultimately, this information will be used to establish the roles of glial cells in TMT toxicity.

Table 1(AII): PKC Isoforms. Adapted from Nishizuka, 1992 (64).

<table>
<thead>
<tr>
<th>PKC Subclass</th>
<th>Species</th>
<th>Activators</th>
<th>Phorbol Ester Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cPKC</td>
<td>α, βI*, βII*, γ</td>
<td>PS, Ca**, DAG, FFA, LPC</td>
<td>yes</td>
</tr>
<tr>
<td>nPKC</td>
<td>δ, ε, η, θ, μ</td>
<td>PS, DAG, FFA, Cholesterol sulfate (δ)</td>
<td>yes</td>
</tr>
<tr>
<td>aPKC</td>
<td>1, ζ, λ,</td>
<td>PS, FFA, PIP₃, Cer</td>
<td>no</td>
</tr>
</tbody>
</table>

Cer = ceramide, DAG = diacylglycerol, FFA = cis unsaturated fatty acids, LPC = lysophosphatidylcholine, PIP₃ = phosphatidylinositol(tris)phosphate, PS = phosphatidylserine.

* The β isoform has two identified splice variants.

C. Methods

(1) Cell culture and treatment: Rat C6 glioma cells were cultured in 70 cm² flasks using Ham's F-12 media plus 10% fetal calf serum (FCS). Cultures were maintained at 37°C and 5% CO₂. Upon confluency (7-10 days) media was substituted with either Krebs-Ringer buffer (KRB); (125 mM NaCl, 5 mM KCl, 25 mM HEPES, 6 mM glucose, 5 mM NaHCO₃, 1.2 mM K₂HPO₄, 1 mM CaCl₂) or F-12/10% FCS containing appropriate concentrations of either TMT or PMA. KRB treatments were used for 1 hour or less, while the F-12/10% FCS was substituted for the 24 hour treatments. Treatments were stopped by
aspirating media and washing three times with ice-cold KRB. Cells were collected by scraping from the flask into 10 mL of ice-cold KRB and by centrifugation at 4000 rpm for 10 minutes, decanting the supernatant.

(2) Preparation of cellular extracts: Collected cells were resuspended in lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 5 mM EGTA, 2 mM DTT, 1 mM PMSF, 1 mg/mL bacitracin, 10 μg/mL each of leupeptin and aprotin) and homogenized on glass. Cytosolic and membrane fractions were obtained by centrifugation 40000 X g for 40 min. at 4°C. Cytosolic (supernatant) fraction was removed and the pellet (membrane fraction) was resuspended in lysis buffer plus 1% Triton X-100. From each sample, 10 μL aliquots were removed to determine protein concentration (45), while the remainder was stored at -80°C until analysis.

(3) Sample preparation and Western blotting: Samples were analyzed based upon the methods described by Pavlakovic (60) and Schneppheim (66). Samples (20 μg total protein) were mixed with an equal volume of 2X solubilizing buffer (50 mM Tris-HCl, 2% SDS, 4% glycerol). They were then placed in a boiling water bath for 90 seconds and further sonicated 10 seconds at 10% cycle. Separation was on 10% Laemmli polyacrylamide gels under reducing conditions and the proteins transferred to PVDF membranes. Blots were then immediately placed into a blocking buffer (5% non-fat milk in Tris-buffered saline plus 0.1% Tween-20 (TTBS)) for 1 hour. After blocking, the
membranes were incubated for 1 hour in solutions of monoclonal PKC antibodies (Transduction Laboratories, Lexington, KY) directed at specific isoforms (1:1000 dilution in blocking buffer). Membranes were washed in TTBS, then subsequent incubation in anti-mouse IgG conjugated to horseradish peroxidase (HRP) (1:1000 dilution in blocking buffer) for 1 hour. Blots were visualized with enhanced chemiluminesence (ECL) and recorded on X-ray film.

D. Results

The presence of the α, β, γ, δ, ε, θ, ζ, λ, and μ isozymes was established in our C6 cell line by blotting cellular extracts with monoclonal antibodies. These isozymes of PKC were then evaluated on their responses to PMA and TMT exposure. Some results from these experiments are presented in figure 1A. Translocation to the cell membrane by cPKC and nPKC isoforms occurred with the application of PMA. Predictably, the aPKC isozymes were not translocated by this treatment. TMT exposure for up to 1 hour did not translocate any of the PKC isoforms.
Figure 1(AII): Response to of PKC to PMA and TMT at 1 hour. **Top:** Differential translocation between PKC α (MW=82 kDa, upper arrow) and PKC ζ (MW=72 kDa, lower arrow). α can be seen to translocate from cytosolic to membrane fractions in the presence of PMA, but not the ζ isoform (lanes 3 and 4). No translocation of either isoform is observed with 10 μM or 50 μM TMT (lanes 5-8). **Bottom:** Shows PKC δ under the same conditions. Again, no translocation is observed in response to TMT. **Lanes 1 and 2:** untreated controls; **lanes 3 and 4:** 100 nM PMA; **lanes 5 and 6:** 10 μM TMT; **lanes 7 and 8:** 50 μM TMT.

PKCs α, δ, ζ were also evaluated after 24 hours of treatment with PMA and TMT. PKCs α and δ had down-regulated with the PMA treatment, while no translocation was detected with TMT treatment on any isoform. This leads to the conclusion that TMT is not able to translocate any isoform of PKC in a glial cell model, and lends to the possibility that TMT-mediated PKC activation in the neuronal cell line is not a direct effect, but is due to an indirect pathway.
Appendix III: Natural Product Screening

A. Background

This appendix presents data from experiments running in parallel with my thesis research in Dr. Keith Parker's laboratory from January 1997 to the present, and involves screening natural products from several different sources for activity at cloned rat 5-HT$_{2A}$ receptors, stably expressed in NIH 3T3 fibroblasts (a gift from Dr. David Julius, UCSF).

Among the natural products being investigated are Peruvian and Ecuadorian ethnobotanicals reported to be used in migraine prophylaxis, obtained by Dr. Ethan B. Russo of the Western Montana Clinic, and crude venom from several species of marine cone snails (*Conus* sp.), kindly supplied by Dr. Doug Steele, of the University of Utah. Initially, the work was primarily done with the ethnobotanicals, data from these experiments are presented in figure 2A.

As more *Conus* venom samples became available from our collaborators however, the focus of the project shifted in that direction, as did my involvement. Thus the work presented in this appendix will be primarily that of the various *Conus* venom experiments.
Figure 1(AIII): Activity of ethnobotanical extracts of the genus Gesneriad in membrane preparations of the rat 5-HT$_{2A}$ receptor. Plants are preserved and extracted in 70% ethanol, diluted 1:10 in binding buffer, giving a final dilution of 1:100 of the crude extract in the competitive binding assay. Values are of one experiment run in triplicate. Conditions are as described in the Methods section of this appendix.

B. Introduction

The predatory marine snails of the genus Conus, more commonly known as cone snails, utilize a toxic venom containing an array of small peptides, termed conotoxins, to immobilize and capture their prey. These peptides are usually 10-30 amino acids in length and conformationally constrained either by multiple disulfide bonding, or having a stable tertiary structure consisting of γ-carboxyglutamate helices (67). Each species has a characteristic venom composition of conotoxins, with a distinct pharmacological profile (67). Peptides that have been isolated from the crude
venom interact very specifically at a number of biological targets. Sites of biological activity that have been thus identified are summarized in table 2A.

Certain peptides from these classes can distinguish between subtypes of ion channels (68), as well as differential binding sites on the nicotinic acetylcholine receptors (69). Combined with this kind of specificity are generally high affinities, with most $K_d$'s in the low micromolar/high nanomolar range (70). Due to the combination of these characteristics, these peptides have been the subject of much investigation for both pharmacological research tools, and as possible therapeutic agents.

<table>
<thead>
<tr>
<th>Conotoxin Class</th>
<th>Receptor Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Conotoxin</td>
<td>Nicotinic acetylcholine receptors</td>
</tr>
<tr>
<td>$\mu$-Conotoxin</td>
<td>Voltage-gated $Na^+$ channels</td>
</tr>
<tr>
<td>$\omega$-Conotoxin</td>
<td>Voltage-gated $Ca^{2+}$ channels</td>
</tr>
<tr>
<td>Conopressins</td>
<td>Vasopressin receptors</td>
</tr>
<tr>
<td>Conantokins</td>
<td>N-methyl-D-aspartate receptors</td>
</tr>
</tbody>
</table>

For most conotoxins, the specific sites of interaction have yet to be determined (67). To this end, the goals of this project are to first identify species of snails whose venom is active at serotonin 2A receptors ($5-HT_{2A}$) by screening of crude venom extracts. Secondly, the screening of successive high performance liquid chromatography (HPLC)-purified venom extracts from
species that display activity are analyzed to determine the 5-HT$_{2A}$ receptor active component(s) of the venom.

C. Methods

The methods used in the competitive binding assays are the same as stated for membrane preparations in of receptor for agonist inhibition assays in the body of the thesis (see pages 22-24) with the following modifications:

(1) **Cell culture:** NIH 3T3 cells expressing the rat 5-HT$_{2A}$ receptor (gift from Dr. David Julius, UCSF) are cultured in DMEM fortified with 200 µg/mL Geneticin® or G 418 sulfate and 10% CS, instead of the fortified Ham's F-12 media used for the CHO cultures.

(2) **Competitive binding assays:** To obtain membrane preparations of the rat 5-HT$_{2A}$ receptor, no modifications are needed from the membrane preparations of the human 5-HT$_{1A}$ receptor. Conditions for the binding assays are also identical, however non-specific binding is determined in the presence of 10 µM mianserin, and the conotoxins compete for the specific binding sites with 0.25 nM [³H]ketanserin (specific activity of 250 Ci/mmol).
D. Results

The first and only species from the initial screen to be subject to further study is the venom from *Conus striatus* (figure 3A). Despite its average ability to displace \[^3\text{H}\]ketanserin in the initial screen, it was currently being fractionated by Dr. Steele, thus making samples readily available. The entire chromatograph (data not shown) from a crude HPLC fractionation produced several fractions that were pooled to be screened for activity. Figure 4A shows results from these experiments. Almost all of the activity in these samples was contained within pool seven (approximately 50% displacement).

Subsequently, as pooled fraction seven was further fractionated, several peaks were identified and subject to further scrutiny. The results from these experiments are presented in figure 5A. Again, most of the original activity was contained within a single fraction, in this case number thirty-seven displaying greater than 50% displacement of specifically-bound \[^3\text{H}\]ketanserin. This being the case, fraction thirty-seven has been subjected to further HPLC purification, and again screened for activity at the rat 5-HT\textsubscript{2A} receptor.

When further purifications become available, hopefully the 5-HT\textsubscript{2A} receptor active conotoxin(s) can be soon be elucidated. It is possible then, that a heretofore unidentified group of biological targets, receptors of the 5-HT family, will have been identified as a site of action for conotoxins. The implications are that of an entirely new class of conotoxins (see table 2A).
Figure 2(AIII): Activity of crude venom extracts from various species of *Conus* in membrane preparations of the rat 5-HT$_{2A}$ receptor. Extracts are at a final dilution of 1:100. Values are of one experiment run in triplicate. Conditions are as described in the Methods section of this appendix. Tex = textile, fig = figulinus, alb = albidicus, mag = magus, bet = betulinus, rad = radius, str = striatus, geo = geographus, mar = marmoremus.

Figure 3(AIII): Activity of pooled HPLC fractions from *C. striatus* venom extracts in membrane preparations of the rat 5-HT$_{2A}$ receptor. Venom extract concentration was 0.5 mg per assay. Results shown for pools 1-4 are of one experiment run in triplicate, 5-8 are the results of three independent experiments. Conditions are as described in the Methods section of this appendix.
Figure 4(AIII): Activity of HPLC fractions from *C. striatus* venom extracts in membrane preparations of the rat 5-HT$_{2A}$ receptor. Fractions were diluted in binding buffer to yield a final concentration of ~0.5 mg per assay. Results shown are of at least 2 experiments run in triplicate ± SEM. Conditions are as described in the *Methods* section of this appendix.
References


Receptor Binding Sites Affected Differentially by Guanine Nucleotides. 

(12) B.P. Richardson, G. Engel, P. Donatsch and P.A. Stadler (1985) 
Identification of Serotonin M-Receptor Subtypes and Their Specific 

(13) D. Hoyer, D.E. Clarke, J.R. Fozard, P.R. Hartig, G.R. Martin, E.W. 
Mylecharane, P.R. Saxena and P.P.A. Humphrey (1994) International 
Union of Pharmacology Classification of Receptors for 5- 

Product Literature. Ballwin, MO. USA.

Neurochem. 60, 408-416.

Synapse 18, 241-260.

Primary Structure and Functional Expression of the 5HT3 Receptor, a 


Receptor: From Molecular Characteristics to Clinical Correlates.

(20) B.K. Kobilka, T. Frielle, S. Collins, T. Yang-Feng, T.S. Kobilka, U. 
Francke, R.J. Lefkowitz and M.C. Caron (1987) An Intronless Gene 
Encoding a Potential Member of the Family of Receptors Coupled to 

I. Yamamura (1990) The Cloning and Sequence Analysis of the Rat 

(22) A. Fargin, J.R. Raymond, M.J. Lohse, B.K. Kobilka, M.C. Caron and R. 
J. Lefkowitz (1988) The Genomic Clone G-21 Which Resembles the β- 
adrenergic Receptor Sequence Encodes the 5-HT1A Receptor. Nature 335, 
358-360.


(50) Y.-C. Cheng and W. H. Prusoff (1973) Relationship Between the Inhibition Constant (K) and the Concentration of Inhibitor Which Causes 50 Per Cent Inhibition (IC50) of an Enzymatic Reaction. *Biochem. Pharmacol.* **33**, 692-695.


(65) A.L. Gott, B.S. Mallon, A. Paxton, N. Groome and M.G. Rumsby (1994) Rat Brain Glial Cells in Primary Culture and Subculture Contain the $\delta$, $\epsilon$, $\zeta$ Subspecies of Protein Kinase C as well as Conventional Subspecies. Neurosci. Lett. 171, 117-120.


