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MODULATION OF SEROTONIN RECEPTOR SIGNALLING BY PROTEIN KINASE ACTIVATION.

By

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ABSTRACT

I investigated the desensitization mechanisms of the serotonin 1A (5-HT1A) and 5-HT1B receptors at the molecular level. Receptor phosphorylation by second messenger kinases and G-protein coupled receptor kinases (GRK) is an initial step in the functional desensitization of G-protein coupled receptor receptors (GPCRs). Evidence suggested that PKC preactivation desensitizes the 5-HT1A receptor. In Ltk-cells, PKC preactivation is pathway-selective, blocks receptor-mediated calcium mobilization but not cAMP inhibition. My first objective was to identify if the prime phosphorylation target of PKC was the receptor itself. Four PKC phosphorylation sites on the 5-HT1A receptor were eliminated using site-directed mutagenesis. The signalling profile of single mutants, and third loop double and triple mutants were examined in Ltk- cells. I determined that the prime target of PKC was the receptor since multiple PKC phosphorylation sites in the third loop mediated pathway-selective uncoupling of the receptor from the calcium mobilization pathway and not the adenylyl cyclase. The PKC site in the second loop of the 5-HT1A receptor was shown to be a critical G-protein-contact site in both neuroendocrine and mesenchymal cells.

My second objective was to investigate if GRK-mediated phosphorylation could enhance homologous desensitization of the endogenously expressed 5-HT1B receptor in the opossum kidney (OK) cell line. To define the role of endogenous GRK in desensitization, I cloned the OK-GRK2 from the OK cell line and generated a kinase inactive mutant. The GRK2 was shown to phosphorylate an epitope tagged 5-HT1B receptor *in vitro* . However, in intact cells the OK-GRK2 did not enhance agonist-induced desensitization of the 5-HT1B receptor, but enhanced the desensitization of the α2C receptor. The kinase-inactive mutant or reduction in OK-GRK2 protein levels using antisense GRK2 cDNA both attenuated the desensitization of the α2C receptor but not the 5-HT1B receptor. These results suggest that phosphorylation mediated by GRKs *in vitro* may not mimic *in vivo* receptor desensitization. Processes other than those mediated by GRKs may be more important for the desensitization of the 5-HT1B receptor in the OK cells.

In conclusion, I have identified two possible mechanisms by which two related receptors,

5-HT1A and 5-HT1B receptors, are regulated by protein kinases: receptor phosphorylation by PKC and GRK. My results suggest that receptor phosphorylation by PKC plays a role in pathway selective desensitization of the 5-HT1A receptor, while phosphorylation the 5-HT1B receptor by GRK, observed *in vitro*, does not play an important role in the homologous desensitization of the 5-HT1B receptor.

RÉSUMÉ

J'ai étudié les mécanismes de désensibilisation des récepteurs 5-HT1A et 5-HT1B au niveau moléculaire. La phosphorylation des récepteurs par des protéines kinases régulées par un second messager et par des récepteurs kinases (GRK) a été observée à un niveau initial dans la désensibilisation fonctionnelle de plusieurs récepteurs couplés à des protéines G. Il semble de plus en plus certain que la préactivation de la PKC, activée par le diacylglycérol du second messager ou par des esters de phorbol, permet de désensibiliser le récepteur 5-HT1A. Dans des cellules Ltk, l'action de la PKC dépend de la voie puisque l'activation de PKC bloque la mobilisation du calcium à médiation réceptrice mais n'a aucun effet sur l'inhibition de l'AMPc. Mon premier objectif était d'identifier si la principale cible de phosphorylation de la PKC s'agissait du récepteur. Quatre sites de phosphorylation ont été identifiés sur le récepteur 5-HT1A : un dans la deuxième boucle intracellulaire et trois dans la troisième boucle intracellulaire. Ces sites ont été éliminés par mutagénèse ponctuelle dirigée afin de déterminer si le récepteur 5-HT1A était bien la principale cible du découplage induit par la PKC. Le profil des signaux des mutants à point unique et des mutants doubles et triples de la troisième boucle a été examiné dans des cellules Ltk transfectées, en présence et en l'absence de la PKC. J'ai déterminé que la principale cible de la PKC était bien le récepteur puisqu'il a fallu plusieurs sites de phosphorylation de la PKC dans la troisième boucle pour moduler le découplage sélectif de la voie du récepteur à partir de la voie de mobilisation du calcium et non de l'adénylyl cyclase. J'ai toutefois eu la surprise de constater que le site de phosphorylation dans la deuxième boucle du récepteur 5-HT1A était un site de contact des protéines G essentiel pour les cellules neuroendocriniennes et les cellules mésenchymateuses.

J'avais pour deuxième objectif de chercher à savoir si la phosphorylation régulée par des récepteurs kinases (GRK) pouvait promouvoir la désensibilisation induite par un agoniste d'un récepteur 5-HT1B exprimé de manière endogène. Les lignées cellulaires OK expriment un récepteur 5-HT1B de manière endogène, lequel subit une désensibilisation homologue induite par un agoniste. Pour définir le rôle du GRK endogène dans cette désensibilisation, j'ai cloné l'OK-GRK2 d'une lignée cellulaire OK et généré un mutant dont les kinases étaient inactives par

mutagénèse ponctuelle dirigée. Le GRK2 a le pouvoir de phosphoryler un récepteur 5-HT1B étiqueté par un épitope, in vitro. Toutefois, OK-GRK2 ne semble pas moduler la désensibilisation du récepteur 5-HT1B induite par un agoniste dans les cellules intactes mais agit par contre dans la désensibilisation du récepteur α2C. La transfection d'OK-GRK2 de type sauvage a respectivement accentué et atténué l'inhibition de l'accumulation d'AMPc modulée par le récepteur α2C alors qu'aucun effet n'a été observé avec le récepteur 5-HT1B. La transfection du mutant aux kinases inactives ou la réduction des concentrations de protéines GRK2 par l' ADNc antisens de GRK2 ont eu pour effet d'atténuer la désensibilisation du récepteur α2C sans toutefois intervenir sur la désensibilisation du récepteur 5-HT1B. Ces résultats amenent à penser que la phosphorylation régulée par le GRK in vitro n'imite pas nécessairement la désensibilisation du récepteur fonctionnel in vivo. Par ailleurs, des mécanismes autres que ceux modulés par le GRK peuvent jouer un rôle important dans la désensibilisation du récepteur 5-HT1B, au moins dans les cellules OK.

En conclusion, j'ai identifié deux mécanismes possibles selon lesquels deux récepteurs apparentés, les récepteurs 5-HT1A et 5-HT1B, sont régulés par des protéines kinases : la phosphorylation des récepteurs par la PKC et par le GRK. Mes résultats indiquent que la phosphorylation des récepteurs par la PKC joue un rôle important dans la désensibilisation du récepteur 5-HT1A sélective de la voie alors que la phosphorylation du récepteur 5-HT1B par le GRK, même si elle a été observée in vitro, ne joue pas un rôle important dans la désensibilisation régulée par un agoniste de ce récepteur.

Dedicated to my parents and husband for their love and support

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I would like to first thank Dr. P. R. Albert for supervising my graduate studies throughout these years and also countless hours dedicated to helping me in the preparation of my manuscripts, presentations, my doctoral thesis and finalizing experiments in Ottawa.

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My final thank-you is dedicated with lots of love and gratitude to my husband, Marc.

Statement of Contributions

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The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and or summary.

Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

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This thesis is written in manuscript format and is composed of three manuscripts. The contribution of each author is described below.

Chapter 1: Multiple phosphorylation sites are required for pathway-selective uncoupling of the 5-HT1A receptor by protein kinase C. P.M.C. Lembo and P.R. Albert. ***Mol. Pharmacol.*** **48:1024-1029 (1995).**

I was responsible for all the work in this chapter. The manuscript was written by myself and extensively revised by Dr. P.R. Albert.

Chapter 2: A conserved threonine residue in the second intracellular loop of the 5-HT1A receptor directs signaling specificity P. M. C. Lembo, M. H. Ghahremani, and P. R. Albert (***under revision***).

I was responsible for most of the work in this chapter including the construction of the mutant receptor. Mohammad Ghahremani transfected the T149A 5-HT1A receptor in GH4C1 cells only and selected clones. I screened the clones by northern analysis and fully characterized the positive clones including the binding and functional assays. Dr. P.R. Albert performed final cAMP radioimmunoassays on one of the T149A 5-HT1A receptor clones in Ltk- cells. The manuscript was written by myself and extensively revised by Dr. P.R. Albert.

Chapter 3: Cloning and characterization of opossum GRK2. Desensitization of Gi-linked inhibition of cAMP accumulation by α2C-adrenergic but not 5-HT1B receptors in intact OK cells. P.M.C. Lembo and P.R. Albert. (**submitted for publication**).

I was responsible for all the work concerning the cDNA library construction, the cloning, RT-PCR subcloning, the northern analysis, construction of the kinase inactive mutant, the generation of clones stably expressing a variety of kinases, the western analysis, the *in vitro* phosphorylation experiments and the preliminary functional cAMP experiments. Dr. P.R. Albert finalized the cAMP radioimmunoassays in Ottawa. The manuscript was written by myself and revised by Dr. P.R. Albert.

Contribution to Original Knowledge

1) I have demonstrated that all the i3 loop 5-HT1A receptor mutants were functional at inhibiting forskolin-stimulated cAMP accumulation and 5-HT-mediated calcium mobilization responses.

2) I have shown that the pretreatment of single point mutants with 100 nM TPA, completely blocks the 5-HT-mediated calcium mobilization response without affecting the adenylyl cyclase pathway.

3) I have demostrated that receptor phosphorylation uncouples the receptor selectively from activation of PLC but still allows the coupling of adenylyl cyclase inhibition.

4) I have also shown that the 5-HT-induced calcium response is present following the acute pretreatment of the double and triple 5-HT1A receptor mutants with TPA.

5) I have demonstrated that the T149A 5-HT1A receptor mutant in GH4C1 cells inhibits the basal and Gs-stimulated cAMP formation but fails to couple to the inhibition of calcium channel activation.

6) I have also shown that the T149A 5-HT1A receptor mutant in Ltk- cells is completely uncoupled from the calcium mobilization pathway but still retains the ability to inhibit cAMP accumulation.

7) I have isolated two GRKs by RT-PCR amplification of OK cell RNA and cloned a full-length cDNA sharing 92% homology at the amino acid level with the rat GRK2.

8) I have demonstrated by northern analysis the presence of two mRNA transcripts of OK-GRK2 corresponding to 5.0 and 3.0 kb.

9) I have generated a kinase-inactive mutant, OK-GRK2-K220R and demonstrated that both the

wild-type and inactive OK-GRK2 mutant are functional and non functional, respectively based on the *in vitro* phosphorylation of the His-tagged 5-HT1B receptor.

10) I have demonstrated that the OK-GRK2 and the kinase inactive mutant OK-GRK2-K220R display receptor specificity in desensitizing the α2C-receptor mediated inhibition of cAMP accumulation whereas no significant effect was observed on the 5-HT1B receptor.

Abbreviations

[3H]-DPAT
8-hydroxy-(2-(N,N-di-[2,3,3H]propylamino) 1, 2, 3, 4-tetrahydronaphthalene

DAG, FA, PA
diacylglycerol, fatty acid, phosphatidic acid

PI, PIP3, PS,
phosphatidylinositol, phosphatidylinositol 3,4,5 triphosphate, phosphatidylserine

PKC, PLC
protein kinase C, Phospholipase C

TPA, PMA
12-O-tetradecanoylphorbol 13-acetate, phorbol 12-myristate 13-acetate

5-HT, 2M-HT, 5-CT, $[Ca^{2+}]_i$
serotonin, 2-Methyl-serotonin, 5-carboxamidotryptamine, intracellular free calcium concentration

BCIP, IBMX, PTX, NBT
5-bromo-4-chloro-3-indoyl phosphate, 3-isobutyl-1-methylxanthine, pertussis-toxin, nitro blue tetrazolium chloride

PGE1, PNS, VIP
prostaglandin E1, peripheral nervous system, vasoactive intestinal peptide

GPCR, GRK, OK
G-protein coupled receptor, G-protein receptor kinase, opossum kidney

i1, i2, i3,
first, second, third intracellular

h, r, m, d
human, rat, mouse, drosophila

BG, Cb, CCo, CPl, GP, Hc, MdB, MHa, OT, Rn, Se, SM, SpC, SN, St, Th
basal ganglia, cerebellum, cerebral cortex, choroid plexus, globus pallidus, hippocampus, midbrain, medial habenula, olfactory tubercle, raphe nuclei, septum, smooth muscle, spinal cord, substantia nigra, striatum, thalamus.

CLOZA, CYP, KETAN, MESUL, METHE, ZACO
clozapine, cyanopindolol, ketanserin, mesulergine, methiothepin, zacopride.

Table of Contents

1. Introduction and Literature Review

The serotonin receptors including the 5-HT1A and 5-HT1B receptors are key determinants of serotonergic neurotransmission in the brain. The somatodendritic 5-HT1A autoreceptors located on the raphe neurons act as inhibitory receptors while the 5-HT1B receptors function as autoterminal presynaptic inhibitory receptors at serotonergic nerve terminals. These receptors have been shown to negatively feedback and regulate the tone of the serotonergic system.

Longterm treatment with selective serotonin reuptake inhibitors (SSRIs) and antidepressants have been shown to be potently effective in alleviating generalized anxiety disorder and depression. Interestingly, the therapeutic efficacy of these compounds is only observed following a sustained treatment. This delayed onset of action, which is a common feature shared by these drugs has been postulated to result from the progressive desensitization of somatodendritic and autoterminal receptors. This clinical phenomenon can be explained as follows: the sustained administration of serotonergic drugs essentially causes the somatodendritic 5-HT1A receptors (and the presynaptic 5-HT1B receptors) to desensitize resulting in an increase in serotonergic firing and an enhancement in serotonergic neurotransmission. This enhancement in serotonergic activity correlates well with the therapeutic effects of antidepressants and antianxiety drugs.

To fully understand the *in vivo* regulation of the 5-HT1A and 5-HT1B receptors it is imperative to initially characterize the functions and regulation of these receptors at a molecular level. The human brain is a complex organ with many intricate interconnections between neuronal and glial cells imposing enormous limitations to study the regulatory functions of serotonin receptors. Hence, expressing cloned receptors in heterologous cell lines has greatly facilitated the functional characterization of several neurotransmitter receptors. **Our major goal is to understand and characterize the initial steps involved in short-term desensitization of the 5-HT1A and 5-HT1B receptors at the molecular level.**

The serotonin receptors belong to the superfamily of G-protein coupled receptors. These receptors are known to undergo short-term functional desensitization which is generally subdivided

into two types a) agonist-induced (homologous) usually mediated by GRKs (G-protein coupled receptor kinases) or b) heterologous mediated by second messenger kinases (PKC or PKA). To elucidate the molecular mechanisms of short-term desensitization of the 5-HT receptors we addressed the following questions:

A) It was shown that acute activation of PKC by phorbol esters phosphorylated and induced the heterologous desensitization of the 5-HT1A receptor in a variety of mammalian cells. How does PKC mediate the functional desensitization of the 5-HT1A receptor?

B) The opossum kidney cell line endogenously expresses the 5-HT1B receptor which has been shown to undergo agonist-induced desensitization. What molecular factors expressed in this cell system are capable of promoting homologous desensitization of the 5-HT1B receptor?

To fully appreciate the rationale of these experiments an extensive literature review follows which summarizes the following topics: serotonin, sertonin receptors, elements of signal transduction pertaining to G-protein coupled receptors and the mechanisms of functional desensitization of G-protein coupled receptors.

1.1 SEROTONIN

Historical Perspective.

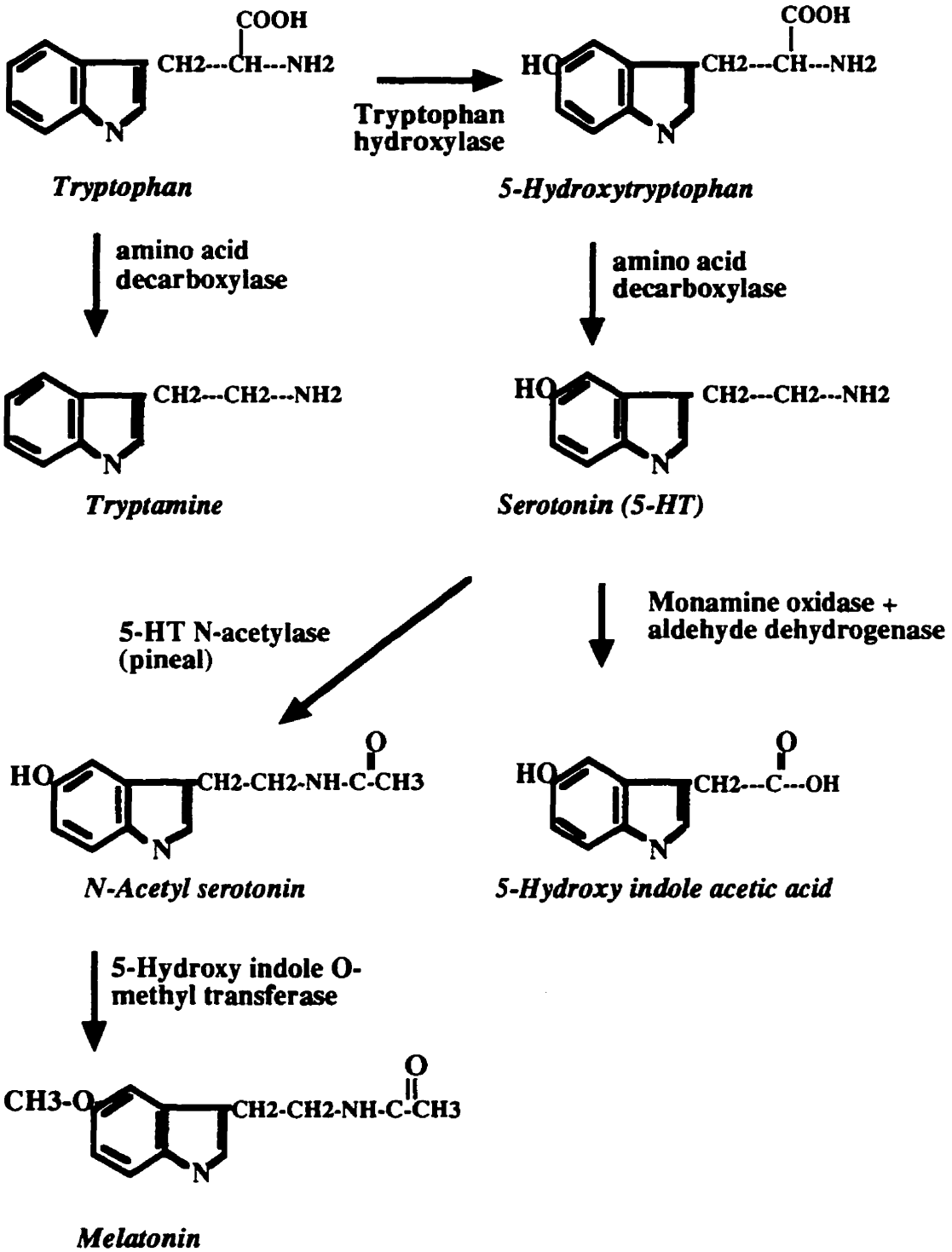
In the 1940's an unknown substance isolated from the serum was found to induce vasoconstriction. This factor was isolated and identified by Page's group (Rapport and Green) and found to be released by platelets in the serum during the clotting of blood. Hence, the common name "serotonin" originated from "serum tonic". This group succeeded in isolating a crystalline complex of serotonin and identified the active moiety as 5-HT (1). In Italy, Erspamer and colleagues identified a smooth muscle contracting substance in enterochromaffin cells of the gastrointestinal mucosa which they named "enteramine". It was later reported in 1952 that enteramine and serotonin were identical compounds (Erspamer and Asero). The following year, Twarog and Page discovered serotonin in brain extracts and suggested that it may function as a neurotransmitter (2).

Interest in the field of serotonin exploded (1953-1970) when serotonin sulphate produced by Upjohn and Abbott became available. The first clinical application for serotonin was the discovery of its potential involvement in the pathophysiology of the carcinoid syndrome (tumors derived from the enterochromaffin cells). Since these cells produced a large amount of serotonin, a diagnostic test was established in the early fifties for this condition (a colour test for 5-hydroxyindoles present in the urine). Many independent investigators began to hypothesize a role for 5-HT in mental illness since serotonergic agonists such as LSD had a psychomimetic activity (1). In addition, investigators working at NIH found profound behavioural effects associated with the use of reserpine and monoamine oxidase inhibitors known to deplete or augment (respectively) the level of serotonin (and in some cases catecholamines) in the brain (3). This led to the hypothesis that the serotonergic and the catecholaminergic systems might affect the mental state of man. Thus, this period was marked by the discovery of serotonin-related drugs and their potential actions and uses. From there on, interest in the physiological role of 5-HT in the central nervous system has preoccupied scientists.

1.2. Biosynthesis and Metabolism of Serotonin

In the adult human body about 90% of serotonin is distributed in the enterochromaffin cells of the gastrointestinal mucosa and the remainder is present in platelets and central nervous system (1 to 2 % in the CNS). Human mast cells do not contain 5-HT but mast cells of other species do (4). Brain cells must synthesize serotonin since it is unable to cross the blood brain barrier. The first step in this pathway is the active uptake of tryptophan via a nonspecific large neutral amino acid carrier. Plasma tryptophan comes from the diet and omission of this amino acid profoundly affects the level of 5-HT in the brain (2). As depicted in **Figure 1** tryptophan is first hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan-5-hydroxylase which is the rate-limiting step in the synthesis of 5-HT. This step can be blocked by *p*-chlorophenylalanine which directly competes with tryptophan and binds directly to the enzyme in an irreversible manner. This is followed by a decarboxylation to 5-HT by the nonspecific aromatic L-amino acid decarboxylase. Serotonin is then taken up into secretory vesicles and stored or rapidly inactivated by oxidation catalyzed by the enzyme monoamine oxidase into 5-hydroxyindoleacetaldehyde which is then metabolized by aldehyde dehydrogenase to 5-hydroxy indole acetic acid (5-HIAA) and excreted via an acid excretion system. The 5-HIAA compound is measured to determine the synthesis of 5-HT in man. In the pineal gland, 5-HT serves as a precursor for melatonin which is a melanocyte-stimulating hormone (4).

Figure 1. Biosynthesis of serotonin



1.3 Serotonergic System

The serotonergic system consists of a group of neurons whose cell bodies are morphologically distinct and are located in the brain stem raphe nuclei and in some regions of the reticular formation. This complex of axonal systems innervates all regions of the CNS with a high density of serotonergic neurons present in the cerebral cortex, limbic structures, basal ganglia, many regions of the brain stem and the grey matter of the spinal cord. The mammalian brain consists of two distinct serotonergic subdivisions: A) a rostral region with cell bodies localized in the midbrain and in the rostral pons which project to the forebrain and B) a caudal region which is principally located in the medulla oblongata where major descending projections are directed to the spinal cord. Serotonin detection in the nervous and non nervous systems was facilitated by a histochemical technique developed by Falck-Hillarp. This is a technique where sections of tissue are freeze-dried and exposed to formaldehyde vapour which causes the indoleamines to emit a yellow fluorescence. Using this technique Dahlstrom and Fuxe were able to demonstrate that the highest level of 5-HT in the brain is in the raphe nuclei. The cell bodies in this area project ascendingly to the forebrain via the medial forebrain bundle whereas descending fibers in this area were shown to project to the dorsal and lateral horns and the intermediolateral column of the spinal cord (5). Detailed distribution of the serotonergic system in the brain became evident and possible with the development of specific antibodies to the amine as well as the introduction of receptor binding and *in situ* autoradiographic techniques.

1.4 Serotonin Receptors

This amine has been implicated in a variety of behaviours such as sleep, pain perception, mood, locomotion, thermoregulation and sexual activity (6). The multiple actions of serotonin are mediated by the specific interaction of this amine with several distinct receptors located in different regions of the brain which have been implicated in these diverse behavioural effects.

In order for 5-HT to be considered a neurotransmitter it was essential to establish that it

could produce physiological effects by activating specific receptors located on the intestinal wall, platelet membrane or on nerve cells. A crucial step towards characterizing serotonin receptors was undertaken in 1957 when Gaddum and Picarelli demonstrated that the serotonin-mediated guinea-pig contraction of smooth muscle was partially blocked by either morphine or dibenzyline (phenoxybenzamine). These investigators concluded that serotonin activated two different types of receptors on the intestinal wall: the "D"(dibenzyline) serotonin receptors located on smooth muscles involved in contracting the muscle and the "M"(morphine) serotonin receptor located on the parasympathetic ganglion facilitating the release of acetylcholine (7). The "D" receptors were found to be equivalent to the 5-HT2 subtype and the "M" receptors were found to be identical to the 5-HT3 subtype (8). Even though, the "M" and "D" serotonin receptor classification became widely accepted, some responses mediated by serotonin could not be labelled within this context.

During the last 20 years, the knowledge of serotonin receptors has undergone dramatic changes. This can be mainly attributed to the advances made in medicinal chemistry from the synthesis of selective ligands and radioligand binding techniques to the advent of molecular biology. The first successful radioligand binding study was reported by Bennett and Aghajanian in 1974 using D-[^{3}H]-lysergide (9). Further studies using tritiated serotonin, spiperone and lysergide enabled Peroutka and Snyder to demonstrate the existence of two distinct receptor subtypes in 1979: A) a high nanomolar affinity receptor site for serotonin- " 5-HT1" receptor and B) a low micromolar affinity receptor for serotonin and high affinity for spiperone - "5-HT2" receptor (10). Subsequently, it was shown that the "5-HT1" binding site could be further subdivided into 5-HT1A or 5-HT1B receptor subtypes based on the affinity for spiperone being high and low, respectively, followed by the recognition of 8-OH-DPAT being a selective ligand for the 5-HT1A receptor subtype. Eventually, the 5-HT1 binding site was subdivided into 5-HT1A, 5-HT1B, 5-HT1C, 5-HT1D 5-HT1E and 5-HT1F receptor subtypes (10).

Since the original scheme of serotonin receptor classification was proposed, an enormous amount of knowledge accumulating from molecular biological work has compelled scientists to restructure the organization of serotonin receptor classification. In recent years, cloning has revealed the existence of more than 14 distinct mammalian 5-HT receptors as well as isoforms of the 5-HT4 and 5-HT7 receptors generated by alternative splicing (11). As depicted in **Table 1,**

serotonin receptors can be subdivided into four main classes based on their pharmacological profiles, their coupling properties and their amino acid sequences.

Except for the 5-HT3 receptor which is a ligand gated channel, all the 5-HT receptor subtypes belong to the large family of G-protein coupled receptors. These receptors interact with heterotrimeric G-proteins and share a putative seven transmembrane structure.

MAMMALIAN 5-HT RECEPTORS

PROPERTIES	5-HT1					5-HT2			5-HT3	5-HT4, 6/7			5-HT5	
	1A	1B	1Dα,β	1E	1F	2A	2B	2C		4	6	7	5A	5B
BIOCHEMISTRY:														
COUPLING	Gi/Go						Gq		channel	Gs			?	
MESSENGER	⇓cAMP, ⇑gK, ⇓gCa (⇑PI)						⇑PI		⇑gNa/K	⇑cAMP, ⇓gK			?	
PHARMACOLOGY:														
LIGANDS:														
AGONIST	DPAT	5-HT	5-HT	5-HT	5-HT	5-HT		5-HT	2M5-HT	5M-HT	5-HT	5-HT	LSD	LSD
ANTAGONIST	CYP	CYP	METHE	–	–	KETAN		MESUL	ZACO	–	CLOZA	CLOZA	–	–
Ki's (-log)														
5-HT	8.3	7.4	7.3	8.2	6.9	6.2		8.4	6.3	6.5	7.2	7.2	6.6	6.6
5-CT	9.5	8.0	7.9	5.1	5.5	5.1		(<6)	(<5)	(<5)	6.6	6.6	7.8	7.4
MOLECULAR:														
SPECIES CLONED	h,r,m	h,r,m	h,r,m,d	h	m	h,r,m		h,r,m	(m-h)	–	r	r	m	m
SIZE (AA)	421	390	377	365	367	497		460	487	?	437	437	357	370
HUMAN CHROMOSOME	5.q12	6.q13	1	?	?	?		?	?	?	?	?	7.q36	2.q13
mRNA DISTRIBUTION	Hc/Rn Se	BG/Hc Rn/Cb	BG/Rn	?	CA1-3 DRn	CCo SpC/ SM		CPl/Hc Th/Mdb	PNS CCo/Hc	Hc/GP SN,OT St	St/OT CCo/Hc	St/OT CCo/Hc	MHa/Cb Hc	MHa

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*page 23, refer to page 12 for abbreviations.

1.4.1 The 5-HT1 Family.

The members of the 5-HT1 receptor family are characterized by their common effect of inhibiting the adenylyl cyclase pathway as well as their high similarity within the amino acid sequences. This family of receptors is comprised of the 5-HT1A, 5-HT1B/1Dβ, 5-HT1Dα , 5-HT1E and 5-HT1F receptors. Interestingly, these receptors do not possess any introns in the organizational structure of their genes. The 5-HTdro2A and 5-HTdro2B, from *Drosophila* also belong to this family.

A) The 5-HT1A receptor.

Distribution.

The 5-HT1A receptor has been cloned from the human, the rat and the mouse. The mRNA for this receptor was detected with highest levels in the hippocampal formation (CA1-CA3), the raphe nuclei, the dentate gyrus, entorhinal cortex, amygdala as well as the lateral septum (12). Autoradiographic studies of the human and rat brains using labelled 8-OH-DPAT and immunohistochemistry using anti-receptor antibodies have confirmed these observations. Moreover, the mRNA and protein have been found to be co-localized which suggests that the 5-HT1A receptors are expressed at the somatodentritic level rather than on axonal terminals. This hypothesis was confirmed by Sotelo *et al.*, demonstrating for the first time the existence of the 5-HT1A autoreceptors on the perikarya and dendrites of serotonergic neurons in the raphe neurons using double immunostaining to detect receptor and 5-HT (13).

Functional Coupling.

To elucidate the pharmacological properties of the 5-HT1A receptor, this receptor subtype has been transfected into a variety of mammalian cells. In 1989, Fargin *et al.*, transfected the human 5-HT1A receptor into Cos-7 cells and demonstrated the following pharmacological binding profile 5-CT > 8-OH-DPAT > NAN-190 = ipsapirone = 5-HT > buspirone > spiperone > mesulergine > ketanserin (14). A similar profile was confirmed by Albert *et al.*, when the rat 5-HT1A receptor was expressed in rat Ltk- fibroblast cells (15). Both groups showed that the 5-

HT1A receptor was a pertussis toxin-sensitive G-protein coupled receptor.

In the mid eighties, it was reported for the first time *in vivo* that the 5-HT1A receptor coupled to the inhibition of adenylyl cyclase in hippocampal membranes derived from the rat and guinea pig (16). This observation was confirmed when the cloned 5-HT1A receptor, transfected in Cos-7, Hela, Sf9 and GH4C1 cells, mediated an inhibition of cAMP accumulation, elicited an increase in intracellular calcium via activation of phospholipase C in certain cell lines and closed voltage sensitive calcium channels to inhibit the influx of calcium (17-19). This receptor has also been shown *in vivo* to open K+ channels in hippocampal neurons via a pertussis toxin-sensitive G protein (20). The opening of potassium channels causes the membrane to hyperpolarize and as a result 5-HT neuronal firing and transmitter release is inhibited (21). This is in excellent agreement with Karschin's report where the human 5-HT1A receptor can open K+ channels when heterologously expressed in cardiac atrial cells (22). In addition, other functional couplings of the 5-HT1A receptor have been reported such as the stimulation of the sodium dependent phosphate uptake via PKC and activation of the Na+/K+ ATPase (23, 24).

A mitogenic activity has been reported with the 5-HT1A receptor. Independent groups have demonstrated that when expressed in NIH-3T3 or Balb C-3T3 cells, the 5-HT1A receptor induced foci formation but also potentiated the effect of EGF on DNA synthesis in a pertussis-toxin sensitive fashion (25, 26). Interestingly, the 5-HT1A receptors expressed on astroglial cells induced the release of the neurotrophic factor S-100β which is known to promote growth of serotonergic neurons in culture. These receptors are also expressed on astrocytes of the mature brain and may play a role in the synaptic plasticity necessary for memory or learning (27).

To conclude, the 5-HT1A receptor can couple to multiple effector systems depending on the cell line expressing the receptor and also which G proteins are inherently present. To date, the native 5-HT1A in brain tissues has never been shown to couple to phospholipase C in a positive fashion (28). Evidence for a PLC coupling effect has been reported only in lymphoid cells where the 5-HT1A receptor is endogenously expressed and agonist-stimulation not only inhibits cAMP accumulation but also enhances PI turnover to mobilize intracellular calcium stores (29).

B) The 5-HT1B and 5-HT1Dβ receptors.

Distribution.

The 5-HT1B receptor is found in the rat, mouse, hamster and opossum but is absent from all other species while the 5-HT1Dβ receptor is absent in rodents but present in humans and dogs with a similar distribution to the 5-HT1B (30). Northern blot analysis and *in situ* hybridization revealed the expression of 5-HT1B and 5-HT1Dβ receptors in the caudate putamen (striatum), frontal cortex, Purkinje cell layer of the cerebellum, CA1 pyramidal neurons of the hippocampus, amydala, and the medulla (31, 32). Binding studies showed a different distribution pattern. Using the 5-HT1B specific radioligand cyanopindolol and the 5-HT1Dβ specific radioligand S-CM-G-TNH2 binding sites were found in the globus pallidus, deep cerebellar nuclei, subiculum and in the superior colliculi (33, 34). The localization patterns of the mRNA and of the receptor protein are quite different, the 5-HT1B receptor appears to be mostly expressed on axonal terminals where it is thought to function as a presynaptic receptor (35).

Functional Expression.

Molecular cloning confirmed that the 5-HT1B was the rodent homologue of the 5-HT1Dβ receptor and a single amino acid substitution in the seventh transmembrane domain of the 5-HT1B, Asn355, was responsible for the different pharmacological profiles between the two subtypes (36). This was confirmed by site-directed mutagenesis whereby replacing threonine 355 of the human 5-HT1Dβ receptor by an asparagine conferred the same binding properties with respect to pindolol as the 5-HT1B receptor (37).

The rat, mouse, and human 5-HT1B receptors have been heterologously expressed in many mammalian cell lines. The pharmacological profile displayed by this transfected receptor is as follows; cyanopindolol > 5-CT = RU24969 > 5-HT > sumatriptan and yohimbine, ketanserin, mianserin, spiperone, and 8-OH-DPAT have a low affinity for this receptor. Whereas when transfected into Hek 293 cells as well as other mammalian cells the 5-HT1Dβ receptor had the following pharmacological profile CT > 5-HT > sumatriptan > yohimbine > RU24969 > cyanopindolol > 8-OH-DPAT (30).

Both receptor subtypes when expressed in different cell lines inhibit the adenylyl cyclase

effector system when activated by agonists (12). *In vitro* evidence now exists demonstrating that the human 5-HT1B receptor when transfected into Ltk- and BALB/c-3T3 cells can couple to the phospholipase C in a positive manner (38). Furthermore, the opossum kidney cell line (OK) endogenously expresses the 5-HT1B receptor and when activated by agonists, mediates an increase in intracellular calcium via activation of phospholipase C enzyme (39). This is the first *"in vivo"* demonstration where a cell line endogenously expressing the 5-HT1B receptor couples to the PLC pathway. These responses were all blocked by pretreatment with pertussis-toxin suggesting the involvement of inhibitory G-proteins (38, 39). Moreover, a mitotic effect mediated by the 5-HT1B receptor was reported in CCL39 and CHL cells where the receptor potentiated the effect of FGF on the synthesis of DNA via a pertussis toxin sensitive G-protein (40, 41). Ample evidence exists demonstrating that the 5-HT1B receptors not only function as autoreceptors on serotonergic neurons but also function as terminal receptors to control the release of several neurotransmitters including serotonin, glutamate and acetylcholine (12).

The availability of genes encoding these receptors and the development of molecular pharmacology such as gene knockout technology has unravelled a possible behavioural role for the 5-HT1B receptor. Mutant mice lacking the 5-HT1B receptor were generated and these mice did not exhibit any obvious developmental defects but were found to be more aggressive as compared to normal mice (42). This is in agreement with previous pharmacological data where serenics or 5-HT1 agonists were shown to have anti-aggressive effects in rodents (43). These observations suggest the involvement of the 5-HT1B receptor in the modulation of aggressive behaviour.

C) The 5-HT1Dα receptor.

In 1989, the 5-HT1Dα receptor was cloned from a canine thyroid cDNA library. It displayed a high homology (75%) in the transmembrane domains to the 5-HT1Dβ receptor and thus named 5-HT1Dα receptor (the human homologue has now been cloned (30)). *In situ* hybridization experiments have revealed the presence of the 5-HT1Dα mRNA in pyramidal cells of the olfactory tubercle. The distribution pattern of the 5-HT1Dα receptor is similar to the 5-HT1B receptor except for the cerebellum area where no 5-HT1Dα receptor sites exist (44). Furthermore, the 5-HT1Dα receptor binding sites display a lower density than the 5-HT1B receptor sites. Studies have suggested that the 5-HT1Dα receptors may be localized on axonal terminals similarly to the 5-

HT1B receptors due to the differential pattern of mRNA and protein distributions (30).

The human and canine 5-HT1Dα receptors have been heterologously transfected into several cell lines and display a pharmacological profile similar to the 5-HT1D receptor (12). Activation of this pertussis-toxin sensitive receptor in fibroblast cells inhibits adenylyl cyclase and stimulates phospholipase C (45).

D) The 5-HT1E receptor.

The 5-HT1E receptor was cloned from a human genomic library using oligonucleotides derived from the 5-HT1A and 5-HT2C receptors. *In situ* hybridization experiments using human and primate brains revealed the presence of 5-HT1E mRNA transcripts in cortical areas and the caudate putamen. Autoradiographic studies also revealed binding sites in these regions (12).

The human 5-HT1E receptor was expressed in murine Ltk- fibroblast cells and shown to couple to G-proteins by the addition of a non-hydrolysable GTP analogue. Being part of the 5-HT1 family, the 5-HT1E receptor couples negatively to adenylyl cyclase in Y1 and Hek 293 cells. But the percent inhibition of cAMP accumulation mediated by this receptor (30-35%) in these cell lines is weak compared to other members of this subfamily (50-60%). This may indicate that the receptor interacts with multiple G-proteins. In agreement with this observation, it was recently shown that the 5-HT1E receptor can couple to either Gi or Gs in African green monkey kidney cells (BS-C-1) depending on receptor density (46). Coupling to the phospholipase C pathway has not yet been reported.

E) The 5-HT1F receptor.

The mouse 5-HT1F receptor was cloned by low stringency screening using the murine 5-HT1B receptor gene (the human and rat homologues have now been cloned (10, 12)). It shares the highest sequence homology with the 5-HT1E receptor, 61% and 54% homology with the 5-HT1B and 5-HT1D receptors. Using quantitative PCR and *in situ* hybridization, mRNA transcripts of the 5-HT1F receptor were detected in the spinal cord, in the uterus, the CA1-CA3 layers of the hippocampus and in the dorsal raphe nuclei (30).

When transfected into NIH-3T3, Hela cells and Ltk- cells, the 5-HT1F receptor mediates a decrease in cAMP accumulation whereas activation of phospholipase C and mobilization of calcium has only been observed in the Ltk-cells (12, 47). It has been suggested that the 5-HT1F receptor

may play a role as an autoreceptor due to the pattern of distribution but *in vivo* data regarding this receptor subtype is still lacking.

1.4.2 The 5-HT2 Family

This serotonin receptor subfamily is comprised of three members, the 5-HT2A, the 5-HT2B and the 5-HT2C receptors. Interestingly, all these receptor subtypes possess two introns within their gene with highly conserved positions. In addition, the receptors all mediate their effects via activation of phosphoinositide metabolism.

A) The 5-HT2A receptor.

The 5-HT2A receptor was cloned from the rat by homology to the 5-HT2C receptor. Northern analyses and *in situ* hybridization experiments have shown mRNA transcripts in the frontal cortex and the spinal cord. This distribution pattern of mRNA correlates well with receptor binding sites suggesting that the receptor protein has a somatodendritic localization. The 5-HT2A has also been detected in the periphery such as in uterine smooth muscle and aorta (30).

Activation of this cloned receptor in various cell lines and *in vivo* leads to an increase in intracellular calcium via IP3-sensitive calcium stores (12, 48). Transfection of this receptor into *Xenopus* oocyte stimulates the opening of calcium-sensitive chloride channel following the release of intracellular calcium. These receptor-mediated responses are pertussis-toxin insensitive suggesting a Gq-protein linked receptor (12).

Being widely distributed in the peripheral system the 5-HT2A receptors mediate several effects including contractile responses seen in the aorta and uterine muscle and platelet aggregation (30). The receptors have also been implicated in many behavioural effects and neurological disorders such as suicidal behaviour, depression, anxiety and schizophrenia but conclusive evidence is still lacking (10).

B) The 5-HT2B receptor.

This receptor was initially reported in 1959 to mediate a contractile activity in the rat stomach fundus (49). The eventual cloning of the mouse and rat 5-HT2B receptors revealed a striking homology to the 5-HT2A and 5-HT2C receptors (45% and 51% respectively). Quantitative PCR and Northern blot experiments demonstrated the 5-HT2B receptor in the rat stomach fundus, heart, brain and small intestine (12).

Similar to the 5-HT2A receptor, activation of the 5-HT2B receptor results in the stimulation of the phospholipase C pathway and the opening of calcium-sensitive chloride channels in *Xenopus* oocytes (50). Recently, the 5-HT2B was reported to not only stimulate the release of intracellular calcium via a novel pathway involving the activation of ryanodine receptors but also capable of activating the protooncogene product p21(ras) in Ltk- cells when stimulated (51, 52). There are many conflicting reports suggesting that the 5-HT2B receptor may have anxiolytic properties but it remains to be established.

C) The 5-HT2C receptor.

This receptor originally named the 5-HT1C receptor, was first described in 1984 in the choroid plexus using the 5-HT1 receptor ligand and cloned from the rat in the late eighties (53). Northern analysis and *in situ* hybridization experiments have shown the presence of the 5-HT2C receptor transcripts in the choroid plexus. These observations have been confirmed by autoradiographic studies (12, 30).

Activation of this receptor leads to the hydrolysis of phosphadityl inositol and an increase in intracellular calcium. This increase in calcium leads to the opening of calcium-sensitive chloride and K+channels and activation of the α -isoform of protein kinase C (30). This receptor may also have a mitogenic activity: NIH-3T3 cells transfected with 5-HT2C receptors injected in nude mice induced tumor formation indicative of cellular transformation but not in CCL39 cells (54).

Mice lacking the 5-HT2C receptor have been generated and shown to be overweight due to an abnormal feeding habit. The data generated from the knockout experiment is in good agreement with the observation that the nonselective 5-HT2C receptor agonist, mCPP, was demonstrated to suppress appetite. In addition, these mice were also prone to spontaneous death resulting from

seizures. The 5-HT2C receptor-deficient mice have not only established a role for this receptor in the serotonergic control of appetite but also in the control of neuronal excitability (55).

1.4.3 5-HT-Gated Ion Channel- The 5-HT3 Receptor.

The existence of 5-HT3 receptors previously known as the "M" receptors was first reported in 1957 by Gaddum and Picarelli (7). In contrast to other 5-HT receptors which are G-protein coupled, the 5-HT3 receptor is a ligand-gated ion channel. When activated it mediates a rapid excitatory response by depolarizing neurons.

The 5-HT3 receptor was isolated by injecting size-fractionated poly A RNA in *Xenopus* oocytes and testing for 5-HT induced currents (56). This receptor shares a high homology with other members of the ligand-gated ion channel superfamily. The receptor contains typical characteristics of an ion channel receptor, the four hydrophobic transmembrane regions (M1-M4) and a large N-terminal extracellular domain containing the Cys-Cys loop postulated to be responsible for formation of a disulfide bond.

The 5-HT3 receptors is expressed in the cortex and hippocampus (30). Expression of this receptor in *Xenopus* and Cos-7 cells has revealed that its a cation-specific ion channel, but poorly selective among cations (56). The 5-HT3 receptor functions as a homomultimer since a single clone generated a current. This is surprising since other members of this family are usually heteropentameric proteins composed of two to four different subunits.

1.4.4 5-HT Receptors Positively Coupled to Adenylyl Cyclase.

A) *The 5-HT4 Receptor.*

Two distinct mRNAs exist for this receptor due to alternative splicing differing only at the C-terminal where a putative PKC phosphorylation site is present in the long isoform (57). The long isoform of this receptor has been detected in the striatum and the olfactory bulb whereas the short isoform has been detected in the striatum only. In the peripheral system such as the ileum, colon and bladder both isoforms are expressed but the atrium of the heart expresses only the short

form (30).

The isoform variants of the receptor respond differently to the non-hydrolyzable analogue GTPγS, this might reflect distinct G-protein coupling. Both subtypes stimulated adenylyl cyclase when transiently transfected into Cos-7 cells but behaved differently with respect to drug potency (57). The resulting increase in cAMP production has been shown to activate PKA. In colliculi neurons, 5-HT4 mediates the closure of K+ channels causing a prolonged depolarization, increases the opening of voltage-sensitive Ca+ channels and neurotransmitter release (58). There is speculation that this receptor may be involved in learning and memory.

B) The 5-HT5A and 5-HT5B receptors.

The 5-HT5A receptor has been cloned from the mouse, the human and the rat whereas the 5-HT5B receptor was cloned from the rat and the mouse only. The 5-HT5B receptor is highly homologous to the 5-HT5A receptor (77%) while homology with other known 5-HT receptors is low. The genomic fragments encoding the 5-HT5 receptors have been cloned and an intron is present in the middle portion of the third intracellular loop at the same position (11).

The 5-HT5A receptor is expressed in the CNS whereas the 5-HT5B mRNA transcript is predominantly expressed in limbic areas. These receptors have been transfected into NIH-3T3 and Cos-7 cells with no effect on either the phospholipase C or the adenylyl cyclase signalling pathways (59). It may be that the 5-HT5 receptors are interacting with ion channels rather than with G-proteins.

The gene encoding the 5-HT5A receptor has been localized to human chromosome 7. This locus is closely linked to the holoprosencephaly type III mutation which results in abnormal brain development. This raises the possibility that the 5-HT5A receptor may play a role in brain development (59). Interestingly, the 5-HT5 receptor has a high affinity for LSD and ergot derivatives and may mediate the effects of these drugs (11).

C) The 5-HT6 receptor.

The 5-HT6 receptor was cloned from the rat and displays very little homology with other 5-HT receptors. The most remarkable characteristic of this receptor is its high affinity to antipsychotic drugs such as clozapine and tricyclic antidepressant drugs (60). The third cytoplasmic loop of this receptor is short while the C-terminal tail is long similar to receptors

coupling positively to the phospholipase C signalling pathway.

Northern analysis revealed that this receptor is predominantly expressed in the brain in the caudate putamen and cerebral cortex. A low expression level of this receptor can be detected in the periphery especially in the rat stomach and the guinea-pig adrenal glands (11).

When transfected in Cos-7 and Hek 293 cells activation of 5-HT6 resulted in the stimulation of adenylyl cyclase (11). The first *in vivo* evidence of the 5-HT6 receptor has recently been reported in the pig caudate putamen membranes where 5-HT receptor agonists induced a concentration-dependent increase in the activity of adenylyl cyclase with a pharmacological profile characteristic of the 5-HT6 receptor (61). Additional pharmacological features such as antagonism by clozapine and the localization in the striatum suggested that this receptor corresponded to the cloned 5-HT6 receptor (62). The high affinity of the 5-HT6 receptor for the typical and atypical antipsychotic drugs implies that this receptor might be a target for these psychotropic drugs.

D) The 5-HT7 receptor.

The 5-HT7 receptor has been cloned from the rat, mouse, human, guinea-pig and *Xenopus laevis* and a homologue in the *Drosophila* system has also been reported (11). There is evidence of an alternative splicing event for this receptor since two independent groups have reported cDNAs that differ only in the C-terminal tail (63, 64). Like the 5-HT6 receptor, this receptor possess a long C-terminal tail .

The 5-HT7 receptor is expressed mainly in the CNS including the pyramidal cells of the CA2-CA3 layers of the hippocampus with a low expression in the periphery. The pharmacological profile of this receptor corresponds to some of the 5-CT-sensitive sites reported in the mammalian brain and to the previously described 5-HT1-like receptors positively coupled to the adenylyl cyclase pathway in the cardiovascular and gastrointestinal systems (30).

The 5-HT7 receptor mediates an increase in cAMP accumulation when expressed heterologously (11). This receptor displays high affinity for 8-OH-DPAT and might actually correspond to the 5-HT1A-like receptors reported to be positively coupled to adenylyl cyclase. These non-defined receptors have been postulated to play a role in circadian rhythms (65).

An *in vivo* effect of this receptor was recently published where a population of [^{3}H]5-HT binding sites corresponding to the 5-HT7 receptors in the rat hypothalamus were shown to be

down-regulated following chronic treatment with fluoxetine, a 5-HT reuptake inhibitor (66). This suggests the that the 5-HT7 receptor might be involved in mediating the therapeutic effects of antidepressant drugs. In addition, the receptor displays high affinity for certain atypical neuroleptics such as clozapine and butaclamol and may also play a role in certain neuropsychiatric disorders as similarly ascribed to the 5-HT6 receptor (62).

1.5 Conclusion

Thus, in a span of two decades, the distribution of 5-HT in both nervous and non nervous tissues has been determined and many of its physiological properties have been investigated. The subtypes of receptors upon which it acts to produce its diverse physiological effects have also been thoroughly studied.

Why are there so many 5-HT receptors? This may, in part, be explained by the various effects mediated by 5-HT in different regions of the brain. Most of these complex behavioural effects not only require the modulation of several physiological functions but also require that these responses become part of the neuronal synaptic plasticity and the existence of a large number of receptors with distinct signalling and expression patterns may enable this biogenic amine to exert its diverse effects.

1.6 The Seven-Transmembrane Receptors and G-Proteins: Structure and Function.

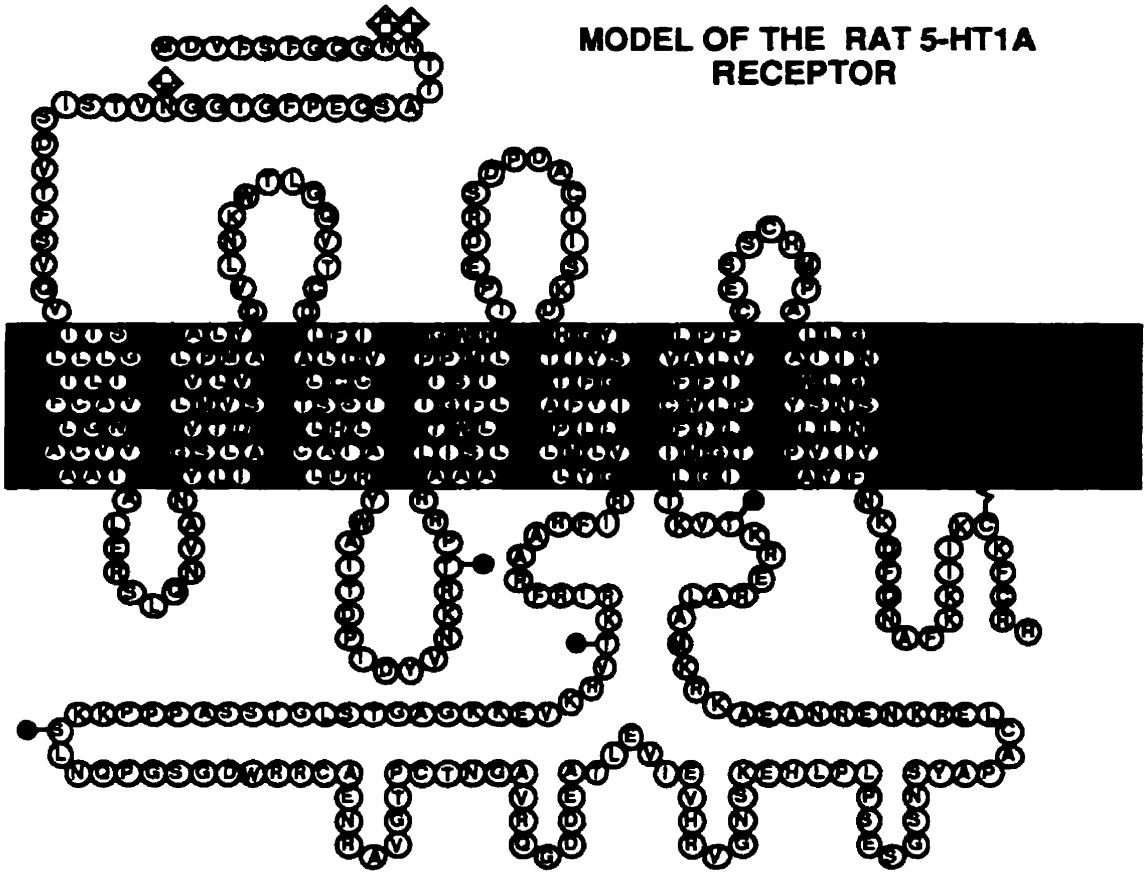
Many functions of organs are controlled by processes which involve the conversion of a primary extracellular stimulus into a secondary intracellular signal which gives rise to a messenger whose activity is to modulate effectors. This paradigm is exemplary of G-protein coupled receptors. This common pathway involves the binding of the first stimulus which encompasses a variety of messages such as hormones, chemokines, autocoids, neurotransmitters, paracrine, and visual, gustatory and olfactory signals to a serpentine receptor. Stimulation of these receptors activates a variety of coupling proteins, termed GTP-binding proteins, that in turn modulate a

number of cellular effectors. These effectors constitute enzymes or ion channels whose activity causes changes in the levels of intracellular second messengers, such as cAMP, inositol phosphates, or calcium ions, ultimately leading to cellular responses often via activation of a variety of protein kinases such as PKA or PKC.

1.6.1. G-Protein Coupled Receptors: Structure and Function

These receptors form a large and functionally diverse family of receptors. Members in this superfamily respond to a variety of diverse extracellular stimuli ranging from light, odorants, eicosanoids, nucleotides, hormones, peptides and neurotransmitters. These receptors are also evolutionarily conserved and have been identified in organisms as evolutionarily distant from man as yeast. More than 300 G-protein-coupled receptors have been cloned and it is generally assumed that the total number exceeds 1000 receptors. Approximately 50-60 % of all clinically relevant drugs exert their actions on G-protein-coupled receptors, hence, understanding the properties and regulation of these receptors is crucial to the advancement of medical research. The potential for the development of novel therapeutic agents targeted to these receptors is enormous (68).

Figure 2: Model of the rat 5-HT1A receptor



Structure . The primary structure of these receptors is based largely on analogies to bacteriorhodopsin, a proton pump having the prototypic heptahelical structure of G protein coupled receptors, a structural motif of seven segments composed of 20-24 hydrophobic amino acids predicted to form alpha helical transmembrane domains. The transmembrane domains are connected by alternating extracellular and intracellular loops. This is shown schematically in **Figure 2** which depicts a typical G-protein coupled receptor, the 5-HT1A receptor. The N-terminus of these heptahelical receptors is located extracellularly whereas the C-terminus is intracellular. This predicted structural arrangement is conserved based on the cloning and sequence determination of more than 300 members of this protein family. It has therefore been proposed that the G-protein-coupled receptors belong to a superfamily of integral membrane proteins.

Amino acid sequence alignment between cloned receptors exhibit the strongest identity in the transmembrane domains and this similarity falls dramatically in the intra- and extracellular loops. All known G-protein coupled receptors are glycoproteins having a putative consensus site Asn-X-Ser/Thr for N-linked glycosylation located in the extracellular domain(s) (69). This N-linked oligosaccharide chain is critical in determining the level of receptor expression at the cell surface but is not required for ligand-binding nor the functional activity of most receptors. This has been shown with the β2-adrenergic receptor, neurokinin receptor 1 (NK1), gonadotropin releasing hormone receptor and for many glycoprotein hormone receptors including the LH, hCG, FSH, TSH and lutropin receptors (68).

Most of these receptors also contain a number of highly conserved Cys residues which play critical roles in the overall structural integrity of the G-protein-coupled receptors. Cys residues 106 and Cys 184 located in the first and second extracellular loops respectively, have been shown to stabilize the tertiary structure of both the β2-adrenergic receptor and the rhodopsin receptor as assessed by mutagenesis experiments (69). These two Cys residues are predicted to form an intramolecular disulfide bridge which links the first and second extracellular loop and presumably compacts the conformation of the receptors. These two Cys residues are found in most G-protein-coupled receptors and it suggests that the intramolecular disulfide bridge is a structural characteristic common to most of these receptors. Another highly conserved Cys is located in the C-terminal region. The function of this Cys residue is still not very clear.

Biochemical studies have demonstrated that this residue is palmitoylated in several G-protein coupled receptors including the α2A and β2-adrenergic receptors, the rhodopsin receptor, the dopamine D1 receptor and the 5-HT1B receptor (70). This palmitoylation reaction is believed to serve as an additional anchor for the cytoplasmic tail of some receptors possibly forming a fourth intracellular loop. Mutational studies have shown that this Cys residue when mutated in the β2-adrenergic receptor (to Leu), the α2-adrenergic receptor (to Ala) and the rhodopsin (to Ser) did not affect the overall tertiary structure of the receptors nor the function (69). But when Cys 341 in the β2-adrenergic receptor was mutated to a Gly residue the receptor lost its ability to stimulate the adenylyl cyclase effector system (71). This suggested that this post-translational modification and the nature of the side chain is critical for the β2-adrenergic receptor's functionality. However, the additional rotational flexibility of the Gly residue compared to Leu, rather than change in palmitoylation, may account for the functional difference. Studies done on the LH receptor which contains two Cys in this region have shown that truncation of the C-terminal upstream of Cys residue interferes with receptor maturation and leads to the intracellular accumulation of the LH receptor and truncation downstream results in functional receptors. This indicated that this Cys plays an essential functional role for the LH receptor (72). Site-directed mutagenesis of the 5-HT1A receptor and the muscarinic m1 and m2 receptors which also possess this Cys residue in the C-terminal region revealed no loss of functionality suggesting that Cys 341 is not crucial for their signalling activity (73, 74). However, replacement of this Cys residue in the 5-HT2A receptor (Cys 397) resulted in a complete loss of activity for the receptor (75).

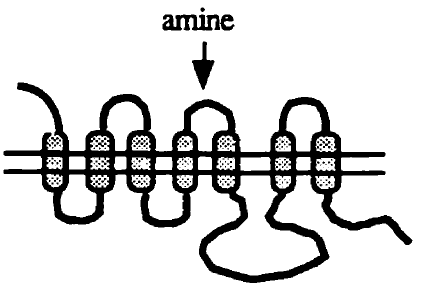
1.6.2 Ligand-Binding Domains

This superfamily of receptors interacts with a wide variety of stimuli ranging in size, from small molecules to large glycoproteins. Information regarding the regions of G-protein coupled receptors critical for binding has been obtained from studies using either the chimeric approach or the single amino acid substitution method. A classical α2/β2-adrenergic receptor chimeric study published in 1988 by Kobilka *et al* ., led to the discovery that transmembrane domain 7 of the α and β-adrenergic receptors was critical for selective antagonist binding and that several

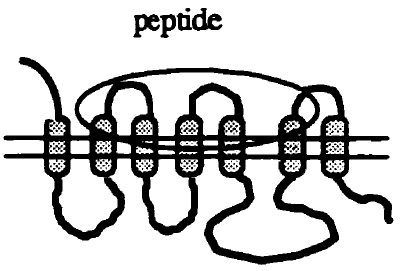
transmembrane domains played a pivotal role in selective agonist binding (76). **Figure 3** is a schematic representation on the diverse modes of activation by ligands of various G-protein coupled receptors.

Figure 3 : Activation of GPCRs by various ligands.

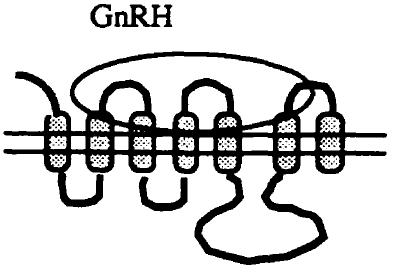
A) Biogenic amine



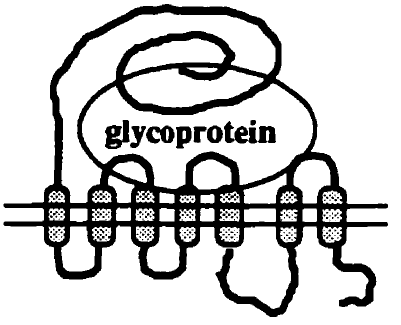
B) Peptide



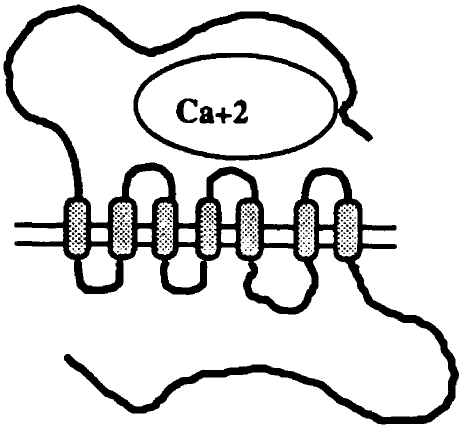
C) GnRH



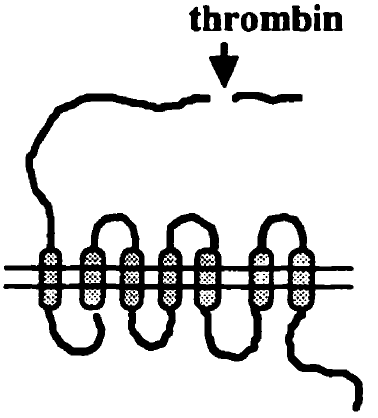
D) Glycoprotein



E) Calcium-sensing receptor



F) Thrombin receptor



Biogenic Amines. G-protein coupled receptors that bind neurotransmitters or biogenic amines such as serotonin have a common ligand-binding site in the central core of the receptor (Fig.3A). Biogenic amines have an amine moiety that is usually charged. On that basis it was reasonable to assume that an acidic amino acid counterpart in the binding core of the receptor would serve as a counter ion for the protonated amine of the ligand.

All known sequences of G-protein coupled receptors which bind biogenic amines have a highly conserved Asp residue in the third transmembrane region (69). The side chain of this residue was hypothesized to function as a counter ion for the amine group of biogenics. Site-directed mutagenesis of the β2-adrenergic receptor provided insights as to the role of this amino acid. It was shown that substitution of the Asp113 to either a Glu/Asn/or a Ser residue resulted in a decrease in agonist and antagonist affinity indicating that Asp113 in the β2-adrenergic receptor plays a strategic role in ligand binding and not receptor activation since these mutant receptors could be fully activated by epinephrine and other agents (76). Analogous observations have been reported with the equivalent Asp mutation in the 5-HT1A and 5-HT2A receptors, muscarinic m1 acetylcholine receptor, dopamine D2 receptors and α-2A adrenergic receptors (77-81).

One of the most highly conserved residues among G-protein coupled receptors is the Asp residue located in the second transmembrane domain. This residue was also shown by site-directed mutagenesis to moderately decrease the agonist affinity and severely impair the efficacy of agonist without altering the interactions with antagonists (68, 69, 76). This was shown for the α2A- and β2-adrenergic receptors, the Angiotensin receptor II, the LH receptor, 5-HT1A and 5-HT2 receptors, muscarinic M1 acetylcholine receptor, LH/CG, human PAF receptor, dopamine D2 receptor and the NK1 receptor (68, 69, 77-81, 82). The respective Asp residue in SSTR-1 and SSTR-2 had no effect on G-protein coupling but precluded the regulation of Na+ on agonist binding (83). Hence, this Asp residue in the second transmembrane domain of most G-protein-coupled receptors plays a vital role in mediating or transducing the conformational change associated with receptor activation.

There are other amino acids which play important roles in the binding of biogenic amines and antagonists. For example, structure-activity relationship studies have revealed that the Asn

312 in transmembrane domain 7 in the β2-adrenergic receptor appears to interact with the phenoxy oxygen of many antagonists (69). This Asn residue is conserved among biogenic amine G-protein coupled receptors that bind phenoxypropanolamine with high affinity including the β2-adrenergic receptor and the 5-HT1A receptor. This Asn residue is replaced with a Phe or Val residue in other G-protein coupled receptors. Mutagenesis studies with the α2-adrenergic receptor where the Phe was mutated to an Asn have shown that the affinity for cyanopindolol, a phenoxypropanolamine antagonist increased (84). Analogously, substitution of the Asn in the 5-HT1A receptor to a Val decreased the affinity for this class of compounds (85). Thus, Asn in transmembrane 7 participates in binding the phenoxypropanolamine antagonists.

To conclude, residues involved in ligand binding of biogenic amine receptors appear to form a hydrophilic pocket between membrane spanning domains 3, 5, 6 and 7. These residues have been implicated in binding the charged end of the ligand and are highly conserved whereas amino acids in transmembrane domains 5 and 6 which bind the opposite end of the amine are more variable.

Other members of the G-protein coupled receptor family bind their ligands in different ways as represented in **Figure 3.** Ligands for peptide hormone receptors such as TRH, angiotensin, endothelin and neurokinin receptors interact with the receptors by associating with the extracellular loops presumably with the hydrophobic pocket provided by the membrane-spanning domains. Mutagenesis studies have shown that the agonist and antagonist binding-sites overlap for this type of receptors but these sites are not identical (68, 69). Interestingly, the pituitary GnRH receptor which lacks a cytoplasmic C-terminal has a similar mechanism of receptor-ligand interaction as the peptide-hormone receptors. The involvement of the extracellular loops in peptide or protein binding has also been investigated with the glycoprotein hormone receptors such as the LH, TSH and FSH receptors. These receptors are characterized by a very large N-terminal extracellular domain which dictates the specificity of high-affinity hormone binding. Additional amino acids on the transmembrane domains not only serve as contact sites for hormone binding but also play a central role in receptor activation (68, 69). The metabotropic glutamate receptors are characterized by the distinctive structural features of possessing very large extracellular N- and intracellular C-terminal regions. This subfamily of receptors includes the recently discovered

parathyroid calcium-sensing receptor which has a similar structural organization (86, 87). When Ca+2 or glutamate binds to the large N-terminal region of these receptors, it causes a conformational change within the N-terminal region which exposes certain amino acids creating a tethered ligand leading to receptor-activation. In contrast, the thrombin receptor exhibits a unique proteolytic mechanism for activation of the receptor. Thrombin recognizes and cleaves the end of the N-terminal region releasing a nonactive fragment. The newly unmasked N-terminal tail serves as the tethered ligand which binds and activates the receptor (88).

1.6.3 G-Protein Coupling Domains.

G-protein coupled receptors transmit their binding signal to effector molecules via heterotrimeric G proteins. Understanding the binding interface or contact sites for the receptor and G-proteins has been a major goal in the field of signal transduction. Most of the research in deciphering coupling regions has focused on the areas of receptors and G-proteins required for activation. Studies using anti-receptor antibodies, synthetic peptides and mutational analysis have all revealed that several cytosolic regions of G-protein coupled receptors, particularly residues in close proximity to the transmembrane domains, participate in the functional coupling and interaction of receptors and G-proteins.

In 1983, a classic study was published where proteolytic digestion of rhodopsin's third intracellular loop inhibited the coupling to Gαt and activation of cGMP phosphodiesterase (89). The same concept was applied to the Gs-coupled receptor, the β2-adrenergic receptor, where a large deletion in the third intracellular loop (239-272) resulted in a complete loss of adenylyl cyclase stimulation (90). This was followed by a more detailed deletion analysis which demonstrated that residues at the N and C-terminal ends of the third intracellular loop were critical for receptor-Gs coupling (91, 92). Furthermore, mutations in the second intracellular loop and the proximal region of the C-terminal tail were also shown to diminish the efficiency of coupling for the β2-adrenergic receptor (93, 94). These results implicating the second loop (i2), third loop (i3) and C-terminal regions in receptor-G-protein coupling have been corroborated with other receptors such as the muscarinic receptor family, peptide, rhodopsin and hormone receptors (68, 69, 76,

95). The following section will summarized findings for each intracellular domain of various G-protein coupled receptors.

First Loop. Calcitonin receptors are alternatively spliced and are coupled to different effector systems. The CTR-1 containing an insertion of 16 amino acids in the i1 loop only couples to the adenylyl cyclase pathway whereas other calcitonin receptors (CTR-2 and pCTR) couple to the phospholipase C and adenylyl cyclase effector systems. To investigate the possible role this insertion might play in coupling, a series of chimeric receptors were constructed in which the intracellular loops were exchanged between p-CTR, hCTR-1 and CTR-2 receptors. The chimeric mutants demonstrated that the 16 amino acid sequence in the i1 loop of h-CTR-1 abolished the stimulation of the phospholipase C pathway while retaining the ability to stimulate adenylyl cyclase suggesting a possible inhibitory domain for the phospholipase C pathway (96). Interestingly, there exist two receptor isoforms for the corticotropin-releasing hormone receptor where a 29 amino acid insertion is present in the i1 loop. But there have not yet been any reports on differentially functional signalling for these receptors (97).

Second Loop. It has been postulated that the i2 loop plays a critical role in the activation of G-protein coupled receptors as assessed by peptide, chimeric and mutational studies (68, 69,76, 92).

Peptides derived from the i2 loop of several G-linked receptors have been shown to mimic receptor activation. A synthetic peptide corresponding to the entire i2 loop of the turkey erythrocyte β1-adrenergic receptor abolished the adenylyl cyclase activation (98). This approach was also demonstrated with Gi-linked receptors particularly with the 5-HT1A receptor, the muscarinic m2 receptor and the neutrophil N-formyl peptide receptor. Peptides composed of amino acid residues derived from the i2 loop of the 5-HT1A receptor and the muscarinic m2 receptors were found to strongly inhibit the forskolin-stimulated adenylyl cyclase activity (99, 100). Whereas, peptides derived from the second intracellular loop of the neutrophil N-formyl peptide receptor inhibited the binding of Gαi antibody in a competitive enzyme-linked immunoassay and antagonized the pertussis-toxin catalyzed ADP-ribosylation reaction of Gi alpha (101).

Chimeric studies have also contributed to our knowledge regarding the importance of the i2 loop in Gαi/s/q-protein receptor activation. A study on chimeric muscarinic/β2-adrenergic

receptors suggested that the i2 domain is an important requirement for receptor and Gs-protein coupling (102). This observation was later confirmed with chimeric endothelin A/B receptors showing a similar need for receptor-Gs-protein activation (103). In addition, the adenylyl cyclase-coupled mGluR3 and the PLC-coupled mGluR1c revealed that the second intracellular loop of mGluR1c was a critical and necessary feature for the specific activation of Gαq-PLC coupling (104, 105). The dopamine D1/D2 chimeric receptor also suggested that proper inhibition of adenylyl cyclase via Gαi required the second cytoplasmic loop of the D2 receptor (106).

Sequence alignment of many G-protein coupled receptors demonstrates the presence of highly conserved residues in i2 loop region namely the "DRY" motif located at the junction of the second transmembrane domain and the N-terminal region of the i2 loop and predicted to significantly contribute to the process of signal transduction (68). Site-directed mutagenesis studies have shown that substituting the conserved acidic residue at the beginning of this motif protects high affinity binding but significantly reduces or completely abolishes the coupling efficacy for some receptors. This was shown for the β2-adrenergic, the muscarinic m1, α-2A adrenergic receptors and the TSH receptor (81, 107- 109). The analogous mutation in the LH/CG receptor did not have a major effect on the signal transduction ability (a slight increase in the EC50 for cAMP accumulation) but remarkably one of the mutant receptors (E441D) could only be detected with detergent solubilization suggesting internalization (110).

The arginine in the "DRY" motif is also conserved and has been extensively documented for its importance in signal transduction. In bovine rhodopsin this mutated arginine did not affect retinal binding but precluded the interaction of rhodopsin with transducin (111). Interestingly, two naturally occurring mutations of the same residue, R135L and R135W, have been identified in individuals with autosomal dominant retinitis pigmentosa and have been shown to inhibit the retinal binding (112). The equivalent mutation in gastrin-releasing peptide receptor and the V2 vasopressin receptor were shown to inhibit receptor-G-protein interaction and impair the stimulation of adenylyl cyclase and PLC β, respectively (113, 114). The equivalent residue in the LC/CH receptor not only displayed a decrease in hCG-induced cAMP accumulation but also interfered with receptor-mediated endocytosis (115). Mutations of the conserved triplet in the Angiotensin II receptor did not lead to major changes in agonist affinity but abolished the binding

of GTP and subsequent activation of PLC (116). This arginine has also been reported to be a critical site for functional coupling in muscarinic m1 and m2 receptors (117, 118).

Another residue which seems to be conserved within this superfamily is the Leu residue located in the middle of the i2 loop. In the muscarinic m1 and m3 receptors this specific amino acid has been implicated in the inhibition of agonist-stimulation of the PLC β and receptor sequestration (119, 120). Replacement of the analogous Leu residue with an alanine in the GnRH receptor not only impaired receptor coupling and subsequent activation of PLC but also inhibited receptor internalization (121). This observation was also confirmed with the β2-adrenergic receptor where the Phe residue was replaced with a Leu. Mutating the Phe residue abrogated the stimulatory effect on adenylyl cyclase activity (119). Other mutational studies using the muscarinic m3 and the TSH receptors have demonstrated that the second intracellular loop is critical for coupling to both the phosphoinositol pathway and adenylyl cyclase (122, 123).

To conclude, highly conserved amino acids present in the i2 loop of many distantly related G-protein coupled receptors have been shown to play an active role in receptor-G-protein interactions. The entire i2 loop seems to be critical for receptor folding and proper receptor-G-protein activation. This reinforces the concept that the i2 loop plays a central role in receptor-G-protein interactions for activation of the adenylyl cyclase and phospholipase C effector systems.

Third Loop. The third intracellular loop of G-protein coupled receptors is the region which has the highest sequence diversity and has unequivocally been shown to be important for receptor G-protein coupling or activation (68, 69, 76, 92). The lack of obvious consensus sequence for receptor coupling was proposed as a coupling domain.

Experiments using synthetic peptides derived from the N- and C-terminal regions of the third intracellular loop of differentially regulated receptors have determined the importance of these regions including the rhodopsin receptor system (111). Peptides corresponding to regions of the i3 loop of the β2-adrenergic receptor were shown to specifically activate Gs (124). Furthermore, two independent groups have demonstrated that synthetic peptides derived from the N-terminal loop and C-terminal region of i3 loop of the dopamine D2 receptor attenuated the dopaminergic inhibition of adenylyl cyclase. (125, 126). Peptide studies have clearly established the role of these regions in the i3 loop of Gi-coupled receptors such as the muscarinic m4 cholinergic, the α-

2A-adrenergic receptors and Gq-coupled Angiotensin II type 1 (AT1) receptors (127-129).

Mutational analyses have also confirmed that the N and C-terminal regions of the third cytoplasmic loop unmask G-protein coupling domains and affect receptor sensitivity to properly couple to effector systems. A deletion in the i3 loop of the β2-adrenergic receptor impaired agonist activation of the adenylyl cyclase pathway in mammalian and *Xenopus* cells (90, 130). The equivalent mutation in the rhodopsin receptor allowed αt to bind but failed to stimulate the GTPase activity presumably due to the failure of dissociation between receptor/Gprotein complex (131). Mutational, deletional and chimeric analyses have all confirmed that the third loop and vicinal transmembrane portions are critical sites for selective G-protein coupling for the muscarinic m1, m2, m3 acetylcholine receptors, the α-2A, α-1B and α-2C adrenergic receptors, the receptors for pituitary adenylyl cyclase activating peptide (PACAP) and TSH, the angiotensin II type 1 and 5-HT2A receptors (68, 76, 79, 116, 132-135). The N-terminal end of the third cytoplasmic domain of the glucagon-like peptide receptor was recently shown to be critical for coupling to the adenylyl cyclase system (136).

Constitutively active receptors have also contributed to our understanding of the underlying receptor-G-protein activation mechanisms. Mutations in the C-terminal region of the i3 loop of the α-1B adrenergic receptor constitutively activated the G-protein-mediated stimulation of phospholipase C (137). Related residues in the Gs-coupled β2-adrenergic receptor and the Gi-coupled α2-adrenergic receptor were also shown to have the same effect when mutated (138). The analogous alanine mutation in the i3 loop of the TSH receptor not only resulted in a loss of Gq-phospholipase C coupling but constitutively activated the Gs-adenylyl cyclase pathway (139). The E360A residue located in C-terminal region of the i3 loop of the muscarinic m1 acetylcholine receptor was recently shown to constitutively activate the PLC pathway in the absence of agonist (140). Interestingly, a mutation in the third transmembrane domain of the α-1B receptor (Cys-Phe) was shown to be constitutively active in the absence of agonist affecting a single effector system, the PLC pathway and not PLA (141). These are examples of constitutively active receptors that were created *in vitro* . There also exists examples of receptors that display properties similar to the constitutively activated receptors as described above and are examples of *in vivo* constitutively active receptors (142, 143). Parma and colleagues demonstrated that the constitutively active TSH

receptor (Gs-adenylyl cyclase) contained a similar mutation in its C-terminal tail of the i3 loop as the *in vitro* mutated receptors (142). Whereas, a mutation in the LH receptor was identified in the the sixth transmembrane spanning domain in close proximity to the C-terminal region of the i3 loop rendering the receptor constitutively active (143). These mutations resulted in the clinical conditions of hyperthyroid adenomas and familial male precocious puberty, respectively. Point mutations in the second transmembrane spanning domain and first intracellular loop of the MSH receptor caused constitutive activation of the receptor and resulted in a darkened colour coat (138). These observations underscore the importance of i3 loop region in mediating the transition from an inactive receptor state to an active one.

Hence, the i3 domain of G-protein coupled receptors appears to be a critical region for G-protein activation and may in fact be harbouring a structural constraint on the unbound receptor so that when a ligand binds, it induces a conformational change and releases the structural constraint of the unbound receptor to allow effective receptor and G-protein coupling. Physiologically, the consequences of constitutive activation of receptors can be severe, as demonstrated for the TSH and LH receptors. However, some receptors (eg. 5-HT2C, dopamine-D1 receptors) have elevated constitutive activity in the wild-type receptor as assessed by overexpression. This gave rise to a novel class of agonists termed inverse agonists. Their importance is based on their potential ability to inhibit these constitutively active receptor states (144). Thus, these drugs might be helpful in alleviating disorders caused by constitutive receptor activation.

These scientific observations indicate that the N-and C-terminal regions of the i3 loop of G-protein coupled receptors are required for G-protein activation. A general consensus sequence for coupling does not exist but what has been hypothesized is that the intracellular cytoplasmic regions form an amphiphatic α-helical structure capable of promoting G-protein activation and binding. This has been shown to occur with mastoparan, an amphiphatic helix-forming peptide derived from the wasp venom and capable of activating G-proteins in a receptor-like manner (145). (However, there is evidence with the LH receptor and the insulin-like growth factor receptor II that regions with structural similarity to the mastoparan peptide failed to activate their respective G-proteins (68)). This suggests that the amphipathic α-helix complex may not be the main structural characteristic in determining receptor-G-protein interactions or maybe this peptide or the α-helical

complexes of receptors could be responsible for rearranging domains and exposing certain critical regions of the receptor vital for proper G-protein coupling.

Current scientific evidence using peptides, chimeric receptors and mutational analyses suggests that G-protein coupling depends on a combination of multiple cytoplasmic domains. Peptides have demonstrated that the second intracellular loop and the carboxyl-terminal tail of the N-formyl peptide receptor are more effective in receptor-G-protein coupling whereas the third intracellular loop seems to be less effective in this process (101). Construction of chimeric receptors between the Gi-coupled mGluR3 metabotrobic glutamate receptor and the Gq-coupled mGluR1C have unequivocally demonstrated that both the C-terminal end of the i2 loop and the N-terminal region of the third cytoplasmic loop are essential for specific G-protein stimulation. Similar conclusions can be drawn for m1 and m3 muscarinic receptors and the β2-adrenergic receptor where contribution from the i2 and i3 loops are required for effective receptor-G-protein interactions (68-93).

The C-terminal tail of certain G-protein coupled receptors has also been implicated in G-protein coupling. A splicing event in the C-terminal region is a characteristic shared among several G-protein coupled receptors such as the somatostatin receptor SST2, the μ opioid receptor, the mGluR1 receptor, the neurokinin NK1 receptor, the prostaglandin receptor EP3 and the pituitary adenylate activating polypeptide receptor (PACAP): this event gives rise to a diversity of receptors which can couple to different G-proteins and effector systems (68, 146-147).

Taken together, these results support a model in which the intracellular loops and portions of the intracellular ends of the membrane spanning domains of receptors form potential G-protein contact sites critical for activation and the binding of agonists to induce conformational changes within the receptors so that these domains become accessible to the G-proteins, mediators of signal transduction.

1.7 Desensitization

Desensitization is a process which occurs in a multitude of organisms in response to a variety of stimuli and can be observed from yeast to man which points to its importance in biological signalling. This section will focus mainly on the functional desensitization of G-protein-coupled receptors defined as the impairment of functional interactions between receptors and G proteins leading to an attenuation in the cellular response to a hormone or neurotransmitter. Desensitization serves a two fold purpose: A) it attenuates the cellular response to a stimulus and B) it triggers a reduction in receptor responsiveness. Hence, the general task of desensitization is to diminish the stimulatory effects and maintain biological equilibria in a living organism. Many receptor systems have been thoroughly studied in order to elucidate the mechanisms of desensitization including the β2-adrenergic receptor and the rhodopsin receptor system. Cellular events contributing to this process include phosphorylation of the receptor and physical removal of the receptor from the plasma membrane (148).

The kinetics of desensitization reveals that it is biphasic in nature and is either short- or long-term. Short exposure of receptors to agonists (milliseconds-60 minutes) leads to the attenuation of the effector system but does not require the synthesis of novel RNA or protein. Whereas, to recover from long-term desensitization (prolonged exposure to agonists > 60 minutes) may require the synthesis of novel RNA and protein and may take a few days (149).

Desensitization has historically been viewed as two distinct processes: homologous and heterologous. **Heterologous desensitization** is defined as the attenuation in receptor responsiveness of stimulated and non-stimulated receptors and is generally considered to be receptor non-specific. This process is usually mediated by second-messenger kinases such as Protein kinase A (PKA), Protein kinase C (PKC) and cGMP dependent protein kinase. **Homologous desensitization** is classically defined as being a decrease of the stimulated receptor only, in a multi-receptor system. The kinases responsible for this mechanism are known as G-protein receptor kinases (GRKs) which phosphorylate the ligand-activated for of the receptor. This form of receptor desensitization is considered a specific event (150). The extent to which each of these mechanisms is responsible for desensitization is cell type and receptor specific.

1.7.1 Role of Receptor Phosphorylation

Protein phosphorylation is a major mechanism involved in the control of most cellular processes and is the most frequent form of post-translational modification. Interestingly, this type of modification is involved in the regulation of neurotransmitter receptor function and more importantly, has been shown to be responsible for the regulation of synaptic plasticity and transmission. In essence, protein phosphorylation involves the reversible covalent modification of hydroxyl groups of serine, threonine and tyrosine residues of receptors by a phosphotransferase reaction. The addition of a highly charged phosphate moiety to the hydroxyl group of receptors induces the receptor to undergo a conformational change such that the functional properties of the receptor are altered and attenuation of signalling occurs (151, 152).

The process of receptor desensitization has been extensively characterized with the β2-adrenergic receptor. Many of the mechanistic events operative for the β2-adrenergic receptor have also been observed with other G-protein coupled receptors (149,150).

1.7.2 Heterologous Desensitization

A critical step in rapid desensitization of receptor function is triggered by a phosphorylation reaction. Many G-protein-coupled receptors are known to be phosphorylated by second-messenger kinases such as PKA and PKC. This genre of phosphorylation provides a direct negative feedback, whereby the effector enzyme turns off its own activation and provides a generalized and non-specific form of desensitization.

1.7.3 Second Messenger Kinase.

A) Protein Kinase C

Active signal transduction is mediated by the generation of bioactive compounds derived from the stimulation of second messenger effectors. These bioactive substances are known to cause changes in regulatory proteins present in the cellular milieu. One of these proteins is PKC, believed to be a key player in signal transduction. PKC is involved in a multitude of diverse processes including growth, differentiation, neural development, synaptic transmission, tumor promotion, aging, endocrine and exocrine secretion and receptor desensitization. Since its initial discovery in the late seventies as a proteolytically activated protein enormous amount of information has been accumulating (153). The purpose of this section is to review PKC's activation, regulation and interaction with major substrates.

Members. To date, 11 protein kinase C isozymes have been identified in mammalian tissues and classified according to their organizational structure and regulation. These isoforms are divided in three distinct families: A) the classical/conventional or Ca^{2+} dependent PKC (cPKC) comprised of α, βI, βII and γ, : B) the novel or Ca^{2+} independent PKC (nPKC) which include the following members δ, ε, η, θ : C) the atypical PKC (aPKC) including ξ and $\lambda(\upsilon)$ are named because they are not activated by phorbol ester nor Ca^{2+} D) PKC μ which takes an intermediate position between nPKC and aPKC. The biochemical characteristics for PKC isoforms are illustrated in **Table 2** **(for abbreviations refer to page 12)**. Most members of the PKC multigene family require phosphatidylserine (PS) for activation except for PKC μ (154).

Table 2: The Biochemical Differences Between PKC Isoforms.

Family	Activators
1) cPKC	
α	PS, Ca 2+, DAG, FA, PMA
β1	PS, Ca 2+, DAG, FA, PMA
β11	PS, Ca 2+, DAG, FA, PMA
γ	PS, Ca 2+, DAG, FA, PMA
2) nPKC	
δ	PS, PI, DAG, FA, PMA
ε	PS, DAG, FA, PMA
η (L)	PI, PMA
θ	PI, PMA
3) aPKC	
ζ	PS, FA, PIP3, ceramide, PA
λ	unknown
4) PKC	
μ	unknown

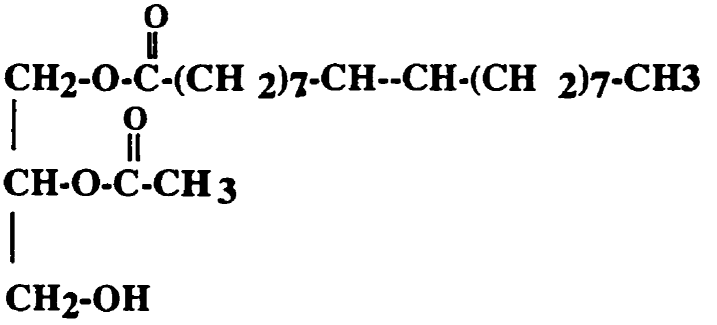
Members of this family are composed a single polypeptide and contain a regulatory N-terminal region and a catalytic C-terminal region. Each isoform is a functional entity with four conserved domains C1-C4 (most PKC members have C1 or C2). The function of each domain has been extensively studied and has revealed that the C1 domain contains a Cys-rich motif which is the DAG/phorbol ester binding site. This is preceded by an autoinhibitory pseudosubtrate domain which folds onto the C4 domain and maintains the enzyme in an inactive state. The C2 region functions either as a region where acidic lipids are recognized or as the Ca+2 binding site. The C3 contains the ATP-binding consensus sequence and the C4 region is responsible for protein substrate binding (153-156).

Activation. Classically, activation of PLC by a neurotransmitter or hormone leads to the hydrolysis of inositol phospholipids which ultimately generates second messengers, 1,4,5,-inositol triphosphate (IP3) and DAG responsible for mobilization of intracellular calcium stores and activation of PKC, respectively. Activation of PKC requires the association of the enzyme with phospholipids within the plasma membrane particularly phosphatidyl serine (PS). PKC is able to bind to PS in the absence of divalent cations via electrostatic interactions provided by the regulatory and catalytic domains. This binding is highly cooperative and is remarkably enhanced in a concentration-dependent fashion when calcium is present which binds to the C2 region. PKC associates with this phospholipid-Ca+2 complex but only becomes active when DAG also becomes part of this complex. PKC is autophosphorylated at three distinct sites at the C-terminal end. At this point, PKC is still inactive due to the pseudosubstrate which occupies the active site of the enzyme. Generation of DAG causes a selective increase in c/nPKC's affinity for PS accompanied by an allosteric change and the release of the pseudosubstrate from PKC's active site thus rendering PKC catalytically functional. Phorbol esters mimic the action of DAG and in a similar manner, they bind to the C1 region and activate PKC. The lipid regulation for the novel PKCs is analogous to cPKCs except that it occurs in the absence of calcium. Once activated PKC is translocated from the cytosol to the plasma membrane where phosphorylation of the receptor takes place (153-156).

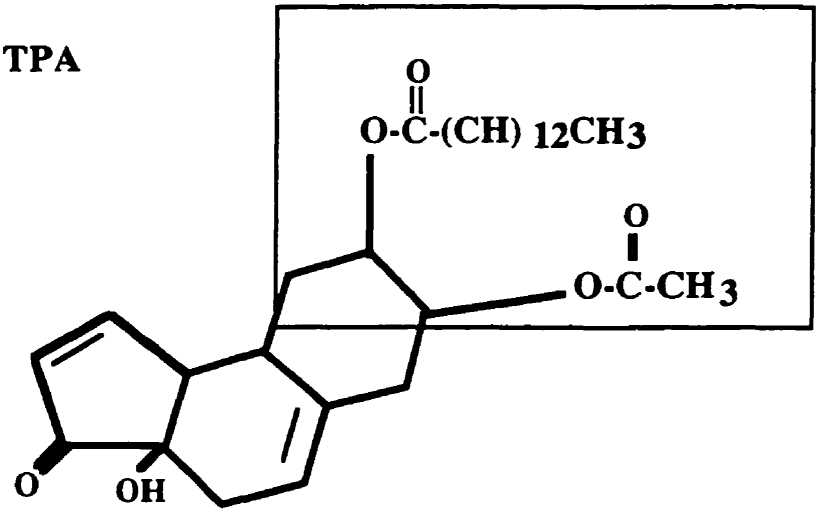
Phorbol esters such as TPA are potent tumour activators. PKC is considered the major cellular target of phorbol esters. In the early eighties, PKC was shown to be a target for phorbol

Figure 4: Structures of DAG and TPA.

A) DAG



B) TPA



esters (157). These agents are structurally related to DAG as depicted in **Figure 4** and can activate PKC *in vitro* and *in vivo*. Similarly to DAG, phorbol esters act as hydrophobic anchors to recruit and dramatically increase the affinity of PKC for Ca+2 resulting in the activation and translocation of PKC to the plasma membrane. Phorbol esters bind to the C1 region of PKC. This not only alters the surface hydrophobicity but it promotes interaction with the membrane in the absence of any conformational change. Phorbol esters are slowly metabolized and persist in cells for a longer time as compared to DAG. Thus, phorbol esters may cause a prolong activation of PKC and may alter or perturb the integrity of the plasma membrane and contribute to unknown biological behaviours (153,154,157).

Phosphorylation sites . Like many serine and threonine kinases, PKC catalytically transfers a phosphate group from ATP to the free hydroxyl groups of serine or threonine residues of substrate regions. These substrates contain motifs which determine the specificity of the kinase. These putative sequences are usually rich in basic amino acid residues and can either be S*/T* XK/R, KRXXS*/T*, K/RXXS*/T*K/R, K/RXS*/T* or K/RXS*/T*XKR where X codes for a single amino acid. PKC is known to phosphorylate a large number of Arg-rich proteins (158, 159) and these substrates are capable of displacing the pseudosubstrate domain. A large number of potential substrates for PKC have been reported such as several G-protein coupled receptors including the adrenergic, acetylcholinergic, serotonergic and the catecholaminergic receptors, neuromodulin, the MARCKS protein , growth-factor receptors and several proto-oncogene products (153-157, 160).

Involvement in receptor desensitization. Several PLC-coupled receptors have been shown to undergo PKC-mediated desensitization. Receptor-mediated activation of PLC generates two second messengers IP3 and DAG which are known to mobilizes intracellular calcium stores and activate PKC, respectively. When PKC is preactivated by phorbol esters or DAG it induces a loss in receptor-mediated PI turnover and calcium mobilization. Hence, PKC acts in a negative feedback loop inhibiting the PLC pathway and itself. Many Gq, Gs and Gi/o-coupled receptors and ligand-gated ion channels have been shown to be regulated by PKC including the thrombin receptors, the serotonergic receptors 5-HT1A, 5-HT1B, 5-HT2 receptors, the angiotensin-II receptors, the dopamine D2 receptors, P2Y purinoceptor, the muscarinic receptors, the α–1B and β2-adrenergic receptors, the GABA-A receptor, the cholecystokinin receptor, the neurokinin

receptor types 1 and II, gastrin-releasing peptide, the formyl peptide receptors, μ and δ-opoid receptors and the parathyroid hormone receptors (12, 19, 38, 39, 150, 161-176). All these receptors have been shown to be heterologously desensitized and/or phosphorylated by preactivation of PKC with phorbol esters (153-176). Receptor phosphorylation plays a pivotal role in this type of desensitization by transferring negatively charged phosphate moieties to the receptor thereby sterically hindering effective receptor-G-protein interactions.

B) Protein kinase A

Eukaryotic PKAs are tetramers composed of two regulatory (or inhibitory domain) and two catalytic subunits. The enzyme is inactive when the catalytic and regulatory subunits are associated with each other. Once cAMP is generated the second messenger binds to the regulatory subunits since they have two cAMP binding sites per subunit and causes a conformational change resulting in the dissociation of the catalytic and regulatory subunits. At this point the active catalytic subunits exert their effects by phosphorylating cell proteins which will ultimately mediate the effects of cAMP (177).

The advent of molecular biology has also highlighted the diversity of cAMP-dependent protein kinase and its subunit composition. There are presently three species of catalytic subunits, α, β and γ. The α and β subunits are highly homologous, have a wide tissue distribution and do not exhibit any differences in either substrate specificity or affinity. In contrast, the γ subunit does not only display some distinctive substrate specificity but has only been detected in the primate's testis (178). PKA has been shown to be anchored by proteins that target the kinase to specific subcellular locations within the cell (179). A key factor in substrate recognition for PKA is the presence of basic amino acid residues particularly arginine in close proximity or N-terminal to a phosphoacceptor residue such as serine or threonine moieties. Studies have demonstrated a consensus motif for PKA phosphorylation composed of the following sequences, R-R/K-X-S*/T* > R-X2-S*/T* = R-X-S*/T* ; these putative phosphorylation domains describe more than 95% of the sequences studied (159, 177, 178).

PKA is known to be positively regulated by cAMP. Thus, PKA is stimulated by receptors which activate the adenylyl cylase effector pathway such as the β2-adrenergic receptor. There exists two potential PKA phosphorylation consensus sites in the β2-adrenergic receptor, one

located in the third loop and the other in the C-terminal region. Site-directed mutagenesis of these sites and/or inhibition of PKA by selective inhibitors prevented the desensitization and phosphorylation of the β2-adrenergic receptors at low agonist concentration (180). When purified and reconstituted in phospholipid vesicles the β2-adrenergic receptors were shown to be phosphorylated by PKA and poorly coupled to Gαs (181). Many receptors are regulated and down-regulated by PKA including the PGE1 receptor, the peripheral dopamine D1 receptor, the β1-adrenergic receptor, thromboxane A2 receptor, μ opioid receptor and GluR6 glutamate receptor (149, 174, 180-186). In 1991, our laboratory demonstrated that PKA could potentiate the action of PKC-induced heterologous desensitization of the 5-HT1A receptor-mediated increase in intracellular calcium but had no effcet on its own (19). Harrington and colleagues have shown that the pretreatment of 5-HT1A receptor with forskolin eliminated the inhibition of cAMP accumulation mediated by 5-HT1A receptors in Hela cells. Acute PKA activation was shown not only to be capable of phosphorylating the 5-HT1A receptors but also to functionally regulate the desensitization process with respect to the inhibition of adenylyl cyclase (187). Interestingly, this was followed by Raymond and co-workers' observation that a cooperativity existed between PKA and PKC in desensitizing and phosphorylating the 5-HT1A receptors in CHO-K1 cells (188). This suggests that the cell may "turn-off" the 5-HT1A receptors when both PKA and PKC are active. To conclude, cooperative cross-talk between PKA and PKC may be an important element in desensitizing the 5-HT1A receptors in certain cell lines.

1.7.4 Homologous Desensitization.

Agonist-induced desensitization is a key element in receptor regulation. Homologous desensitization involves several processes which are known to occur sequentially following the binding of a ligand: a phosphorylation event mediated by a G-protein receptor kinase (GRK) followed by uncoupling (sec-min) and the binding of an arrestin protein to the phosphorylated receptor which sterically hinders the interaction between the receptor and the G-protein. Phosphorylation essentially reduces the receptor's ability to couple to its effector system and this very process has been shown to occur and regulate many G-protein-coupled receptor systems

including the β2 and α-2A- adrenergic receptors, the m2 muscarinic acetylcholine receptors, the rat olfactory and the rhodopsin photoreceptor systems (189-191).

GRKs are a family of serine/threonine kinases that phosphorylate G-protein coupled receptors. They belong to a family of kinases comprised of six members (GRK1-GRK6) whose activities are differentially regulated by G-protein βγ subunits, phospholipids and post-translational modifications. The hallmark of these GRKs is that they phosphorylate the receptor only when active or ligand-bound (189-191).

Members. There are currently six distinct members in this family. They have been categorized into three subfamilies according to sequence and functional similarities: a) rhodopsin kinase (GRK1), b) the GRK2 or β-adrenergic receptor kinase (βARK) subfamily comprised of GRK2 and GRK3 and c) the GRK4 subfamily which includes GRK4, GRK5 and GRK6. These kinases share 50-93% sequence identity overall and a similar structural organization. The N-terminal is believed to be important for receptor recognition whereas the C-terminal region is responsible for enzyme localization. The central region forms the core of the kinase catalytic domain (189-191).

A) Rhodopsin kinase (GRK1):

This kinase is found almost exclusively in the rods and cones of the retinal cells with limited expression in the pineal gland. Rhodopsin kinase phosphorylates the light-absorbed rhodopsin but not the dark-adapted rhodopsin. Consensus sequences for farnesylation (a *CAAX* box via a thioester linkage) and carboxymethylation are present at the C-terminal region whereas a consensus sequence for myristoylation is present at the N-terminal end of this kinase. The post-translational farnesylation modification has been shown to be essential for translocating the rhodopsin kinase from the cytosol to the retinal rod outer segment membranes and is also necessary for full enzymatic activity. The GRK1 or rhodopsin kinase was shown to phosphorylate and desensitize the β2-adrenergic receptor and the light-activated rhodopsin (189-191).

B) The βARKs, GRK2 (βARK1) and GRK3 (βARK2).

These kinases were initially named because of their ability to phosphorylate the β2-adrenergic receptors. The bovine GRK2 (βARK1) was the first member of the GRK family to be cloned (192). They are distributed throughout the body but GRK2 is more abundant than GRK3. GRK3 was shown to be expressed in high amounts in the olfactory bulb (193). These kinases do

not possess a *CAAX* box but require an isoprenylation modification at the C-terminal for the translocation process from the cytosolic milieu to the plasma membrane. Rather than being isoprenylated, the translocation process is accomplished due to the association of the GRK2 and GRK3 with free geranylgeranylated βγ subunits in the presence of PIP2 via the carboxy-terminal region of these kinases which possess a pleckstrin (PH) domain (189-191, 194, 195). The mastoparan peptide has recently been demonstrated to also activate GRK2 and GRK3 (196).

GRK2 is capable of not only phosphorylating the β1-, β2- and α2- adrenergic receptors but also distantly related receptors such as the 5-HT1B, rhodopsin, m2 and m3-muscarinic, δ opioid, substance P and A1-adenosine receptors (183, 190-192, 197-199). GRK2 has been demonstrated to impair the thrombin-mediated calcium response in *Xenopus* oocytes and desensitizes the α2-adrenergic receptors and the angiotensin II type 1A receptor (167, 200, 201). The α-1B receptor-mediated PI turnover and the dopamine D1 receptor-mediated cAMP accumulation were recently shown to be attenuated by GRK2 and GRK3 overexpression (202, 203). Substrates for GRK3 phosphorylation include, the β2- and α2- adrenergic receptors, m2 and m3 muscarinic receptors, rhodopsin, substance P receptor and the dopamine D1 receptor (189-191, 202, 203). In addition, GRK3 was reported to be capable of not only desensitizing the inhibition of voltage-dependent Ca+2 mediated by the α2-adrenergic receptor in isolated intact chick sensory neurons but also to selectively and functionally associate with olfactory receptor desensitization in excised cilia of olfactory neurons (204, 205).

Interestingly, the first *in vivo* demonstration that a GRK functionally desensitizes receptors was recently published. With heart failure, the β–adrenergic receptor G protein cyclase system is known to be downregulated in the human myocardium and the levels of GRK2 have been shown to be elevated (206). This suggested a reason for heart failure, an increase in the desensitizing protein GRK2 and/or a decrease in β–adrenergic receptors. Therefore, transgenic mice overexpressing either the β2–adrenergic receptor or a GRK2 kinase inhibitor (C-terminal region to soak up the βγ subunits) were generated in order to bypass these myocardial defects. Transgenic mice overexpressing the β2–adrenergic receptors demonstrated enhanced myocardial activity whereas transgenic mice overexpressing the GRK2 or the GRK2 inhibitor demonstrated reduced cardiac contractility in response to isoproterenol and increased contractility, respectively.

Moreover, mice overexpressing the GRK2 inhibitor displayed an enhanced cardiac activity under non-stimulated conditions (207, 208). These results imply that 1) the GRK2 kinase is extremely critical *in vivo* for proper cardiac function and 2) under basal conditions the rate limiting step for cardiac function may be the availability of βγ subunits since basal cardiac function was not altered in mice overexpressing the GRK2 only. Transgenic manipulation of the β–adrenergic receptor G protein adenylyl cyclase system has not been limited only to the receptor and GRKs. Recently, transgenic mice overexpressing Gαs were generated and shown to have no change in the maximal activity of adenylyl cyclase thereby reinforcing the notion that the desensitizing kinase plays a central role in the attenuation of myocardial activity (209). A dominant negative mutant for GRK2 was generated, where an invariant lysine in the catalytic domain responsible for the phosphoryl-transferase reaction, was shown to not only abolish the kinase activity of GRK2 but also attenuate short-term desensitization of β2-adrenergic receptors endogenously expressed in a bronchial epithelial cell line (210). These observations provide evidence suggesting that GRK2 plays a critical role *in vivo*.

C) The GRK4 subfamily.

The GRK4 was identified by positional cloning in the search for Huntington's gene. This member is expressed in significant levels in the testis and is the only GRK kinase to be alternatively spliced giving rise to four distinct variant forms (189). Recently, the GRK4 was shown to phosphorylate and desensitize the ligand-activated β2-adrenergic receptor and also increase the desensitization process of the LH/CG receptor expressed in HEK293 cells (211). The GRK5 is expressed predominantly in the heart, placenta and lung with a low level of expression in the brain (189, 191). It is a functional kinase since it is capable of phosphorylating and homologously desensitizing several receptors including rhodopsin, α2 and β2-adrenergic receptors, the m1 and m2 muscarinic receptors, the TSH receptor and the dopamine-D1 receptor (189, 191, 202, 203, 214, 213). The GRK6 has also been cloned and shown to have a complex pattern of expression because there are two forms which are expressed in a tissue-specific manner and in different ratios (214). GRK6 not only phosphorylates the rhodopsin receptor and muscarinic receptors but also enhances the desensitization process of the TSH receptor, the dopamine-D1 receptor and the α–1B and β2-adrenergic receptors (189, 191, 202, 203, 212). All

members of this subfamily lack a *CAAX* box and a βγ binding site but appear to exhibit a high degree of association with cellular membranes or phospholipids via electrostatic interactions (189-191).

Phosphorylation sites. The GRKs are serine/threonine kinases that phosphorylate G-protein-coupled receptors. The only G-protein-coupled receptor where GRK-mediated phosphorylation has been mapped concisely is rhodopsin. Many mutational studies have demonstrated that GRK1 phosphorylates rhodopsin on specific serine and threonine residues at the distal portion of the C-terminal region (215). Interestingly, several other GRKs including GRK2, GRK3 and GRK5 have been reported *in vitro* to phosphorylate essentially the same residues on rhodopsin but in a preferential manner (215). *In vitro* phosphorylation studies using peptides have demonstrated that GRK1, GRK2, GRK3 and GRK5 prefer acidic residues in close proximity to the residue being phosphorylated (189). Deletional and mutational studies indicated that the sites for GRK2-mediated β2-adrenergic receptor phosphorylation are located at the C-terminal region of the receptor (180, 181, 216). Using the mutational approach, Eason and colleagues have reported a GRK2-mediated phosphorylation motif in the third intracellular loop of the α2A-adrenergic receptor (α2-10) comprised of serine and threonine residues flanked by several acidic moieties (217). This was followed by the elegant work of Fredericks and coworkers demonstrating the specific phosphoacceptor sites in the C-terminal region of the β2-adrenergic receptor mediated by GRK2 and GRK5 (218). Remarkably, many G-protein-coupled receptors which contain similar sequences have been shown to be phosphorylated *in vitro* by GRK2 including the β2 and β1-adrenergic receptors, the α1B-adrenergic receptor, the muscarinic m2 and m3 receptors, the adenosine A1 receptor, the substance P receptor, the opossum 5-HT1B receptor, the thrombin receptor, the N-formyl peptide receptor and recently reported the dopamine D1 receptor (167, 189, 190, 197, 200-203, 213, 215, 219, 220). Many of these putative phosphorylation sites are located either in the third intracellular loop or the C-terminal region of the receptors. Hence, many members of the GRK family appear to be acidotropic kinases where a clear preference for acidic residues exists in close proximity to the serine and threonine residues.

Recently, a study was published reporting the finding of a novel GRK distinct from the other members present in this family. This kinase has not yet been cloned but has been purified

from the porcine cerebellum and found to phosphorylate the PLC-coupled m3 muscarinic receptor in an agonist-dependent manner. Studies based on chromatographic mobility, molecular weight, and kinase inhibitors demonstrate that this kinase, designated MRK, is distinct from previously characterized second messenger regulated protein kinases (221). Cloning will determine if it is indeed a novel member of the GRK family.

The Arrestin Family. The arrestins constitute a multigene family of regulatory proteins which bind to phosphorylated G-protein coupled receptors leading to receptor attenuation, including the rod and cone photoreceptors, adrenergic and muscarinic receptors. A mutational analysis has demonstrated that arrestin plays a critical role in the regulation and function of rhodopsin in *Drosophila* (222). The arrestin superfamily consists of four distinct subfamilies comprising: 1) rhodopsin arrestin or visual arrestin predominantly expressed and localized in retinal photoreceptor cells and has the following receptor binding preference rhodopsin >> β2-adrenergic receptor ≈ m2 muscarinic receptor; 2) β-arrestin 1 which is known to play a role in the desensitization of β2-adrenergic receptors. The receptor binding selectivity for this arrestin member is as follows m2 muscarinic receptor >> β2-adrenergic receptor ≈ rhodopsin; 3) β-arrestin 2 or arrestin 2 which has been reported to interact and mediate the attenuation of odorant receptors (193, 204); and the recently cloned cone retinal-specific arrestin termed X-arrestin or 4) C-arrestin (cone arrestins). Molecular studies done on arrestins have revealed that at least three of the mammalian arrestins have splice variants due to alternative splicing events (189-191).

1.7.5 Paradigm for GRK2 and GRK3 Agonist-Induced Homologous Desensitization.

Homologous desensitization involves several processes which occur sequentially once the ligand binds to the receptor: A) coupling to the G-protein-effector B) a phosphorylation reaction resulting in uncoupling C) internalization of the phosphorylated receptor in vesicles and D) dephosphorylation of the receptor in an acidic compartment and recycling of receptors back to the cell surface (150). Once the G-protein coupled receptor is stimulated or bound by a neurotransmitter, it undergoes a conformational change to activate its effector system. For

biological equilibrium the receptor's response must become attenuated.

How does this happen? The conformational change within the receptor also mediates a structural change in the G-protein by dissociating. The dissociated βγ subunits act as a docking site for the GRK2 and GRK3 via an isoprenylation modification at the C-terminal region of the kinases where the pleckstrin domain is located (189-191). This modification allows the kinases to translocate from the cytosol, come in close proximity to the agonist-bound receptor to form a stable tertiary complex between the receptor and kinase-βγ subunit and phosphorylate multiple serine and threonine residues flanked by acidic entities (217, 218). This increases the binding affinity of the phosphorylated receptor for arrestin whose primary function is to bind the receptor and sterically hinder the interaction between the receptor and G-protein (189-191). This ultimately and effectively prevents the signal transduction mechanism of the receptor and turns off the effector pathway. Interestingly, this proposed mechanism is actually a very dynamic one. It provides evidence for the close association between activation and desensitization where two purposes are served when the receptor is activated. The receptor becomes a substrate for the GRK(s) and the free βγ subunits generated from the activation of the receptor provide the impetus for the translocation mechanism. Effectively, only when the receptor becomes activated are the means for desensitization set in motion.

The next question is, how is phosphorylation and receptor desensitization reversed? Phosphorylated receptors become sequestered in acidic endosomal vesicles which probably alter the conformational change of the receptor (150). Recently, a unique protein termed a G-protein coupled receptor phosphatase was found to not only remove the phosphorylation groups on the receptor but also reverse the desensitization process (223). *In vitro* studies have demonstrated that receptors phosphorylated only by GRKs and not by second messenger kinases are substrates for this specific phosphatase. This phosphatase probably functions in an acidic environment so that when the receptors are internalized in the vesicles, the acidic milieu induces a conformational change within the receptor and the phosphorylated sites on the receptor become available to the phosphatase which removes these phosphate moieties and the receptors are recycled to the plasma membrane. Recently, Ferguson *et al.*, reported that the arrestin protein played a strategic role in G-protein-coupled receptor internalization (224). It was suggested that arrestin had a dual role in the

regulation of receptors; it participated in receptor desensitization by associating with the phosphorylated receptor and also mediated receptor internalization leading to receptor resensitization. This model of homologous desensitization has been demonstrated and proposed for the β2- α1B-adrenergic receptor, the rhodopsin system, muscarinic receptors, the 5-HT1A receptor and dopamine receptors (189-191, 202, 203, 225).

1.7.6 Cross-Talk Between Second Messenger Kinases and GRKs.

The pivotal role of receptor phosphorylation in desensitization has been extensively documented using reconstitution experiments, receptor mutagenesis and overexpression of protein kinases. An intriguing question is, do second messenger kinases regulate the activity of GRKs? This would provide a feedback loop by which these second messenger kinases could modulate the homologous desensitization process of receptors in a heterologous manner. Using an antisense RNA approach, Shih and Malbon elegantly demonstrated the potential cooperative role between second messenger kinases (PKA and PKC) and GRK2 in cell-specific desensitization of β2-adrenergic receptors (226). This is in agreement with the recent finding that preactivation of PKC by phorbol esters phosphorylates GRK2 *in vitro*. Winstel and collaborators reported that preactivation of PKC phosphorylated GRK2 and enhanced its translocation process to the plasma membrane (227). Along the same line, Chuang *et al.*, demonstrated that the activity of GRK2 and GRK2-dependent homologous desensitization were increased in cells where PKC was preactivated (228). The specific mechanism mediating the interaction between PKC-GRK2 is not yet known but there is speculation that the pleckstrin (PH) domain at the C-terminal end of the GRK2 might be involved. The PH domains have been shown to bind PIP2 at the N-terminal region (229). Interestingly, recent studies have shown that PS and phosphatidic acid, activators of PKC, can activate GRK2 (230). All these studies demonstrate that there is potential cross-talk between GRKs and PKC. These results suggest a novel level of complexity governing the desensitization mechanisms of G-protein coupled receptors.

1.8 Physiological Relevance of Desensitization for the 5-HT1A and 5-HT1B Receptors:

The two major systems which have been reported to affect the pathophysiology of depression are the serotonergic and catecholaminergic systems. Several clinical studies have unambiguously demonstrated that an enhancement of serotonergic neurotransmission generally alleviates the symptoms of patients afflicted with depression. This observation comes from clinical investigations where Selective Serotonin Reuptake Inhibitors (SSRIs) have been found to be extremely effective for the treatment of major depression disorder (MDD). The common property amongst these drugs is their ability to inhibit the reuptake of serotonin and hence increase the level of 5-HT. Additional clinical evidence implicating the serotonin system in MDD comes from the following paradigms: A) using an inhibitor to tryptophan hydroxylase (para-chloro-phenylalanine: affects the rate-limiting step for the synthesis of 5-HT) and B) reducing the intake of the essential amino acid tryptophan, both have been shown to produce symptoms of depression in animals and patients. Hence, there is strong evidence to suggest that the serotonergic system is implicated in MDD. The clinical evidence for the involvement of the catecholaminergic system in MDD is not as strong although some noradrenaline reuptake inhibitors have produced some antidepressant therapeutic effects such as desipramine and maprotiline (6, 10, 12, 231).

Serotonin is known to play a role in behavioural functions. Since the discovery of multiple 5-HT receptor subtypes, the 5-HT1A receptor has received the most attention. This stemmed from the clinical observation that buspirone a very effective anxiolytic agent was a selective 5-HT1A receptor agonist. This was followed by the finding that several selective 5-HT1A agonists such as ipsapirone, gepirone and tandospirone all had anxiolytic and anti-depressant properties. From these classic clinical studies it was suggested that the 5-HT1A receptor may play a role in MDD and general anxiety disorder (GAD). Hence, understanding the functional regulation of the serotonergic system, especially 5-HT1A receptor is critical in deciphering the mechanisms involved in depression and anxiety (6, 10, 12, 231).

Generally the inhibitory somatodendritic or presynaptic autoreceptor found mainly on the dorsal and median raphe nuclei are of the 5-HT1A receptor subtype. When activated these

presynaptic or somatodendritic 5-HT1A receptors open potassium channels which hyperpolarize the cell membrane and cause a decrease in the firing rate of serotonergic neurons (*in vivo* and *in vitro*). The decrease in the firing rate also leads to a suppression in the rate of 5-HT synthesis, 5-HT turnover and 5-HT release in many regions of the brain. Terminal autoreceptors are also present close to the synaptic cleft belonging to the 5-HT1B subtype (in rodents and 5-HT1Dα and β in humans). Several serotonergic post-synaptic receptors located in limbic areas have also been implicated in MDD including 5-HT1A, 5-HT2A, 5-HT2C and 5-HT3 receptor subtypes. Activation of the postsynaptic 5-HT1A receptor also induces membrane hyperpolarization and decreases the firing rate of these neurons (6, 9, 10, 12, 13, 16, 21, 28, 31, 231).

Interestingly, SSRIs have been shown to inhibit serotonergic transmission within minutes but have a delayed onset of action usually subsequent to 2-3 weeks of treatment. This delay coincides with the desensitization of the 5-HT1A somatodendritic receptors as assessed by electrophysiological and binding studies following repeated administrations of SSRIs. The terminal autoreceptors (5-HT1Dα and β) which inhibit 5-HT release, when activated in the synaptic cleft are also desensitized following this treatment, allowing more 5-HT to be release at the terminals (231-235). Moreover, the number of carrier sites for 5-HT were recently shown to be decreased thus, accounting for enhanced 5-HT transmission. However, the postsynaptic 5-HT1A receptors appear not to be down-regulated (there may be a minor effect) with this treatment, but have an increase in neurotransmission due to the presence of more agonist at the postsynaptic receptors. The net effect is additive, more agonist in the postsynaptic area and also normal firing when SSRIs are administered. All these changes may explain why SSRIs have a delayed clinical onset for depression. The desensitization of other 5-HT receptor subtypes at the postsynaptic level have also been implicated in depression including the 5-HT2 receptor but still remains controversial (231- 234).

Similar observations have been made with anxiolytic agents. Partial agonists for the 5-HT1A such as 8-OH-DPAT and the azapirones behave differently, although this issue is still not fully resolved. They behave as full agonists at the somatodendritic level and consequently inhibit the release of 5-HT at the terminals and as antagonists postsynaptically. It is often argued that the presynaptic 5-HT1A receptors require lower doses of 5-HT1A agonists as compared to the

postsynaptic 5-HT1A receptors to mediate their effect and this behavioural discrepancy is attributed to the concept of receptor reserve (231-235).

The synthesis of selective silent antagonists for the 5-HT1A receptor will most likely clear this issue. Until then, it is extremely difficult to predict the net effect on serotonergic transmission in the brain when "selective" 5-HT1A agonists are used. To conclude, SSRIs and 5-HT1A receptor agonists have several beneficial therapeutic effects including anxiolytic and antidepressive.

1.9 G Proteins: Structure and Function

Heterotrimeric regulatory guanine nucleotide-binding proteins (G proteins) belong to the superfamily of GTPases and are conserved among the animal kingdom and hence underlie their critical role in cellular regulation. The GTP-binding proteins were discovered serendipitously while investigating the effects of glucagon on the accumulation of hepatic cAMP. Initially, both the stimulatory (Gs) and inhibitory (Gi) G-proteins were discovered on the basis of there effects on the adenylyl cyclase pathway following the treatment with either *Vibrio cholerae* (cholera toxin:stimulated) or *Bordetella pertussis* (pertussis toxin:inhibited). Over the last decade, enormous progress has been made in redefining the elements that are involved in transmembrane signalling and a large number of G-proteins have been cloned, characterized and subdivided into families. Mammals have over 20 different G-protein α subunits and the proteins are divided according to sequence similarity and the nature of the α subunit. Most G-proteins are ubiquitously expressed with at least four subtypes present in individual cells. But exceptions do exist where some G proteins are found exclusively in sensory organs such as αt, αgust, αolf and αz while α16 and αo are predominantly expressed in hematopoietic cells and neural tissues respectively (236-238).

1.9.1 The G-protein Cycle

The heterotrimeric membrane-associated G-proteins are composed of three distinct subunits: an α subunit (36-42 kD) which has an intrinsic GTPase activity and therefore binds and hydrolyzes GTP, a β subunit (≈37 kD) and a γ subunit (≈8kD). The β and γ subunits exist as a tightly associated complex that represent a functional entity. When GDP is bound to the α subunit, it associates with the βγ subunit and forms an inactive heterotrimeric G-protein coupled to the receptor. Both the α and βγ subunits bind to the receptor. The GDP-bound α subunits can interact with receptors but the association is greatly enhanced by βγ subunits. When a stimulus activates a receptor it induces a conformational change such that the affinity of GDP decreases for the α subunit and is released from the active site of the subunit. Since the concentration of GTP in the intracellular milieu is higher as compared to GDP, the GDP is replaced by GTP, which is closely associated with Mg^{2+}. Once GTP is bound, the α subunit assumes an activated conformational state, dissociates from the receptor and βγ subunit, and stimulates or inhibits an effector protein. The free α and βγ subunits are able to activate target effectors. This excited state lasts until GTP is hydrolyzed by the intrinsic GTPase activity of the α subunit. Following GTP hydrolysis, the inactive GDP-bound α subunit dissociates from the effector and reassociates with the βγ subunit to form the heterotrimeric G-protein. Essentially, all α subunits are GTPases and the intrinsic rate of GTP hydrolysis varies greatly among the α subunits. Hence, the rate of GTP hydrolysis dictates the timing mechanism which controls the duration of activation for both the α and the βγ subunits. Recently, it was postulated that the β subunit could act as a high-energy phosphate acceptor and it was suggested that following the GTPase reaction the free phosphate is transferred to a histidine residue on the β subunit allowing the phosphorylated β subunit to act similarly to the GTP-bound activated α subunit (236-240).

1.9.2 Gα subunits.

Structure

Crystal structures of GTP and GDP-bound transducin and αi were recently generated revealing the residues in contact with the guanine molecule and how it changed from an inactive state to an active form (236). The α subunit is composed of two domains: a GTPase domain which is topologically identical in elongation factor Tu (EF-Tu) and $p21^{ras}$ and binds the guanine moiety and an α-helical domain common to all α subunits containing different binding sites for the receptor, effectors and βγ subunits. The GTPase activity was mapped to Arg178 and Glu200 in αt. The first 25 amino acid residues of the α subunit have been shown to be critical for βγ binding. Whereas the C-terminus plays an important role for some G-proteins in delineating the specificity of G-protein interactions (237-240).

Members and Functions.

Gs subfamily.

The cholera-toxin sensitive Gs proteins form a Gs subfamily and they all directly activate the adenylyl cyclase effector system and also regulate two ion channels (236-239). The CTX toxin ADP-ribosylates Arg201 which ultimately reduces the GTPase activity and renders the G-proteins constitutively active. Four splice variants exist and are ubiquitously expressed. The only exception is the αolf which shares 88% sequence similarity with αs and is exclusively expressed in sensory neurons such as the olfactory epithelium. Recently, an "extra-large" αs protein [Gαs(XL)] was cloned and shown to be associated with the trans-golgi network mediating CTX-sensitive effects on secretory vesicle formation (241).

This G protein subtype has been shown to be responsible for sporadic endocrine tumors. The most commonly described mutations are somatic ones which either affect the CTX site where the Arg201 is changed to Cys/Leu/Hys and alters the GTPase site and the Glu227 is mutated to an

Arg/Leu/Hys. These modifications lead to constitutively active αs and result in an increased level of basal cAMP (242, 243).

Gi subfamily.

The Gi subfamily or "inhibitory proteins" includes members that are sensitive to pertussis toxin. This toxin catalyzes the ADP-ribosylation reaction on Cys351 and conserves the G-protein in an inactive GDP-bound state hence preventing receptor interactions. They include at least seven members which are found in all cells and inhibit adenylyl cyclase and modulate the activity of several ion channels (236, 237, 242). In biological assays, the inhibitory effect of αi on adenylyl cyclase has been ascribed to Gαi2 & Gαi3 (236, 237, 244-246). All subtypes of αi1, αi 2&3 stimulate K^+ channels equally well. Certain neuronal K^+ channels are activated by purified Gαo1&2. The αi3 is particularly abundant in non-neuronal tissues and has been detected on golgi complexes suggesting a role in vesicular transport. The αo subtype is predominantly expressed in neuronal tissues such as in neural growth cones and may be an important modulator of early neuronal growth. Recently, a splice variant of Gαi2 was discovered [Gαi2L] which lacks the PTX-sensitive motif and appears to play a role in intracellular trafficking processes. The Gαi2 has also been implicated in adrenal and ovarian carcinomas (236, 243). Mutations of this G protein subtype causing consitutive inhibition have been ascribed to the Arg179 residue and Glu205 residue. Transducins or αt (the rod and cone photoreceptor G-proteins) also belong to this subfamily and are solely found in the retina and are coupled to a cGMP-phosphodiesterase. Gustducin, αgust is also a member of the transducins and shares a high structural identity with αt but is restricted to the taste papillae of the tongue. It has been postulated that it may be linked to a taste cell phosphodiesterase. Another member of this subfamily is the αz, it lacks the C-terminal cysteine residue for PTX modification, exhibits an extremely low basal GTPase activity and its effectors are presently unknown (236-243).

Gq subfamily.

There are currently 5 members in this family αq, α11, α14, α15 and α16 all of which are PTX-insensitive. Members of the αq/11 family prototypically activate the hydrolysis of phosphotidylinositol via phospholipase C β and indirectly activate pituitary voltage-dependent dihydropyridine-sensitive Ca+2 channels through a protein kinase C-sensitive pathway (via βγ

subunits see below). Most of these members are expressed ubiquitously except for α15/α16 which are only found in hematopoietic cells whereas α11 is detectable only in non-hematopoietic cells. Furthermore, it appears that α16 is the human homologue of murine α15 (236, 238).

G12/13 subfamily.

These PTX-insensitive G proteins share 67% identity with each other and 45% sequence similarity with other members of the G-protein family. Although ubiquitously expressed, the Gα12/13 subtypes are extremely abundant in the kidney. Both have been shown to be activated, albeit in a slow manner by thromboxane A2 and by thrombin receptors in human platelets. In the literature there are reports that α13 in a glioma cell line can mediate the closure of Ca+2 channels induced by bradykinin and also stimulate a Na^+/H^+ exchanger. Interestingly, the α12/13 proteins have been implicated in cellular proliferation since mutants lacking the GTPase activity were shown to induce transformation in NIH-3T3 cells. The wild-type gene products for these G proteins were also shown to harbour significant mitogenic activity (236-243).

1.9.3 βγ Subunits.

In recent years, much attention has been dedicated to βγ subunits. They were previously thought to act merely as a membrane anchoring device for the α subunit. To date, there exist five β subunits and seven γ subunits. The 5 β subunits are 53% to 90% identical to each other while the γ subunits are even more divergent amongst each other. Not all pairs can form and the subunit composition may contribute to the specificity of the cellular response that a receptor elicits (245).

Structure. The β subunit contains two structural features: an N-terminal region believed to form an amphipathetic α helix thought to form coiled-coils and seven repeating units of approximately 43 amino acids with the following motif;

$$\{\ X6\text{-}94\ \text{---}[GH\text{----}X23\text{-}41\text{---}WD]\}^{N4\text{-}8}$$

Variable length loop Constant length loop

This motif is present in various proteins such as proteins involved in cell division, transcription, processing of mRNA, cytoskeletal assembly and vesicle fusion.

What are the WD repeats important for? Many proteins with WD repeats help to assemble macromolecular complexes. The β subunit is shaped like a 7-blade propeller and the γ subunit is draped like a thread over the β subunit. The βγ subunits may facilitate the association of the α subunit with membrane receptors to form a ternary protein complex which will enable the receptor to bind ligands. In addition, it may also enable the formation of a membrane receptor-receptor kinase complex which will phosphorylate the bound receptor (189, 236-240, 247).

These subunits exhibit higher lipophilicity as compared to the α subunits, the reason being the γ subunit contains a *CAAX* motif which is an isoprenylation site and enables the βγ dimer to dock to the membrane and in some cases associate with the α subunits (247).

Effectors. The number of effector systems regulated by βγ subunits are increasing rapidly. The initial discovery was made in 1987 with βγ subunits being able to activate the muscarinic K+ channel in the heart (248). Subsequently, the βγ subunit was shown to regulate many effectors such as adenylyl cyclases, this functional interaction is dependent on the type of adenylyl cyclase (AC) expressed. For example, type I AC s are activated by αs and calmodulin but are inhibited by free βγ subunits. Moreover, type II and type IV ACs are stimulated conditionally (in the presence of αs) by the βγ complex (249). Other effectors that the βγ subunits can interact with are the phospholipase C β, phospholipase A2 and phosphoinositide 3-kinase (236). These dimers also participate in desensitizing the receptors by a negative mechanism via receptor kinases. They act as anchoring or docking devices for GRK2/3 and enable the kinases to phosphorylate the receptor and desensitize the system (189-191). There is now evidence that implicates the βγ subunits in the activation of GTPase *ras* (250, 251) by binding via ras-regulatory proteins and activating the growth factor signalling pathway. Recently, the βγ subunits were shown to occlude the voltage-dependent N-type and P/Q type Ca^{2+} channels localized in presynaptic terminals (252, 253).

Most of these proteins bind to βγ subunits via a pleckstrin homology domain (PH). This region is present in many of the aforementioned proteins and has been implicated for effectively binding the βγ subunits. Pleckstrin is the major substrate of PKC in platelets, the PH domain was identified by sequence similarity. It has been found in a variety of proteins involved in cellular signalling and cytoskeletal organization. It was suggested that the PH domains are capable of mediating protein-protein interactions critical in signal transduction. These domains can be found in GRK2 and

GRK3, PLC β, γ, δ, cdc25, SOS, dynamin and GAP-ras (236-240, 254).

In less than a decade science has uncovered that the heterotrimeric G protein, the α and βγ subunits can interact with effectors and receptors and both entities play an active and dynamic roles in the process of effective signal transduction between G-protein and receptors.

2. Thesis

The thesis is in manuscript format and is divided into three chapters. Chapter 1 examines PKC-mediated heterologous desensitization of the 5-HT1A receptor in Ltk- cells. Chapter 2 examines the coupling selectivity of the 5-HT1A receptor in Ltk-and GH4C1 cells with respect to the IP3-mediated calcium mobilization in fibroblasts, inhibition of calcium channel opening in pituitary cells and inhibition of cAMP levels in both cell types. Finally, Chapter 3 investigates the components of agonist-induced desensitization of endogenously expressed 5-HT1B and α2C-receptor in the opossum kidney cell line (OK).

3. Chapter 1. Multiple phosphorylation sites are required for pathway-selective uncoupling of the 5-HT1A receptor by protein kinase C. P.M.C. Lembo and P.R. Albert. *Mol. Pharmacol.* 48:1024-1029 (1995).

In 1991, Raymond and colleagues demonstrated concomitant phosphorylation and desensitization of 5-HT1A receptor and suggested receptor phosphorylation as the prime mechanism (163). The same year our laboratory also examined and confirmed the selective modulation of this receptor by PKC in Ltk- cells (19). There are four putative PKC sites located in the cytoplasmic regions of the 5-HT1A receptor, one in the second loop and three in the third intracellular loop. The hypothesis was that PKC mediated acute desensitization by phosphorylating the receptor. We therefore addressed this hypothesis using site-directed mutagenesis as the approach to investigate this question. We found that multiple PKC sites in the third intracellular loop of the 5-HT1A receptor are involved in the selective uncoupling of the 5-HT1A receptor from calcium mobilization and have established that phosphorylation of the 5-HT1A receptor mediates this process (176).

Multiple Phosphorylation Sites are Required for Pathway-selective Uncoupling of the 5-HT1A Receptor by Protein Kinase C

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Abbreviations:
[3H]-DPAT, 8-hydroxy-(2-(N,N-di-[2,3,3H]propylamino)1,2,3,4-tetrahydronaphthalene; PI, phosphatidyl inositol; PK C, protein kinase C; PLC, Phospholipase C; TPA, 12-O-tetradecanoylphorbol 13-acetate; 5-HT, serotonin; IBMX, 3-isobutyl-1-methylxanthine; PTX, pertussis-toxin; PGE1, prostaglandin E1.

ABSTRACT

Classically, acute uncoupling of G protein-linked receptors has been presented as a non-selective process in which modification of the receptor by phosphorylation leads to a reduction or loss of coupling to all effectors. Investigation of multiple signalling pathways has modified this view: for example, when expressed in Ltk- fibroblasts, the 5-HT1A receptor couples to both stimulation of intracellular calcium mobilization (via inositol phosphate generation) and inhibition of cAMP accumulation. Acute pretreatment for two minutes with 100 nM TPA, an activator of protein kinase C (PK C), abolished the intracellular calcium response induced by 100 nM 5-HT, but did not reduce 5-HT1A receptor-mediated inhibition of cAMP. In the present report, mutant 5-HT1A receptors lacking one, two, or three putative PK C phosphorylation sites located in the receptor's third cytoplasmic loop were constructed and transfected separately into Ltk- cells. The receptor mutants displayed similar agonist affinities as the non-mutated receptor. The relative magnitudes of 5-HT-induced changes in intracellular calcium or forskolin-stimulated cAMP levels were also similar to the wild-type receptor for all but one of the mutants. In addition, TPA treatment did not change the magnitude or potency of 5-HT-induced inhibition of PGE1-stimulated cAMP accumulation in either the wild-type or triple mutant 5-HT1A receptor clones. Upon pretreatment with TPA, there was a progressive recovery to 74% of the control 5-HT-induced increase in calcium mobilization as PK C sites were eliminated from the receptor, indicating that multiple phosphorylation sites are required for PK C-mediated receptor uncoupling. Additionally, these results indicate that receptor phosphorylation selectively uncouples the 5-HT1A receptor from enhancement of calcium mobilization without reducing receptor-mediated inhibition of cAMP. Thus, phosphorylation by PK C can sculpt receptor signalling by pathway-selective uncoupling.

INTRODUCTION

Desensitization is defined as a decrease in receptor responsiveness upon prolonged exposure to agonist (1). Uncoupling, the immediate phase of the desensitization of G protein-coupled receptors, is characterized by the activation of protein kinases and phosphorylation of the receptor protein leading to impaired activation of G proteins (2-4). The prototypic b-adrenergic receptor, which stimulates adenylyl cyclase, is uncoupled by phosphorylation of distinct sites by protein kinase A and receptor kinases (2-6). Elimination of these sites by receptor mutagenesis reduces the extent of agonist-induced desensitization (7,8).

The 5-HT1A receptor is a member of the family of "inhibitory" receptors which couples to PTX-sensitive G proteins to inhibit adenylyl cyclase, open potassium channels and close calcium channels leading to a decrease calcium levels in pituitary and neuronal cells (9-13). The 5-HT1A receptor, as well as other 5-HT1 and dopamine-D2 receptors, induces a cell-specific enhancement of PI turnover and increase in intracellular calcium levels when expressed in fibroblast cells such as Ltk-, Balb/c-3T3 cells or Hela cells; these responses are blocked by PTX (13-17). In these cells acute (2 min) pretreatment with TPA, which activates several isoforms of PK C, selectively abrogates 5-HT-induced increases in PI turnover and intracellular calcium, but not inhibition of cAMP level. The rapid time course and concommitant phosphorylation of the 5-HT1A receptor (18) suggests that the uncoupling could be mediated by receptor phosphorylation. However, several targets phosphorylated by PK C, including the receptor (18), specific G proteins (19) or the effector, PLCb (20), might be involved in pathway-selective modulation of receptor signalling by PK C. We addressed the mechanism of PK C action by mutating the receptor, and show that PK C activation induces selective uncoupling from calcium mobilization via phosphorylation of the 5-HT1A receptor at multiple sites.

EXPERIMENTAL PROCEDURES

Materials–Restriction endonucleases and other molecular biology reagents were purchased from Boehringer Mannheim and GIBCO-BRL. Sequenase was from U.S. Biochemicals. TPA, forskolin, IBMX, 5-HT and PGE1 were from Sigma; [3H]-DPAT (135 Ci/mmol) and [a-32P] ATP (2,200 Ci/mmol) were obtained from Amersham Corp. Geneticin was from GIBCO and Fura 2-AM from Molecular Probes, Eugene, OR.

METHODS

Cell Culture–All cells were grown as monolayer in a-minimum Eagle's medium supplemented with 5% fetal bovine serum, at 37°C in a humidified atmosphere, with 5% carbon dioxide. Media were changed 12-24 h prior to experimentation.

Construction and Expression of 5-HT1A Receptor Mutants–The BamHI/XbaI fragment of the rat 5-HT1A receptor gene in the pZEM-3 vector (12) (containing the mouse metallothionein promoter) was subcloned into p-Select to use as a template for site-directed mutagenesis (Altered-sites mutagenesis, Promega). Three putative PKC sites in the third loop were mutagenized individually to T229A, S253G, T343A using oligonucleotides incorporating point mutations (nucleotides 662-682, ATCCGCAAGGCTGTCAGGAAG; nucleotides 749-766, CCAAGAAGGGCCTGAACG; and nucleotides 1018-1038, GAAAGGAAGGCGGTGAAGA, respectively (12)). Double and triple mutants consisting of T229A/S253G and T229A/S253G/T343A were also constructed. All mutations were confirmed by DNA sequencing. Mutated and wild-type (without metallothionein promoter) 5-HT1A receptor clones were subcloned into the eukaryotic expression vector pcDNA I (Invitrogen) and were co-transfected with pSV-Neo in Ltk- cells using calcium phosphate co-precipitation (12). Neomycin-resistant cells expressing 5-HT1A receptors were selected and grown in a-MEM supplemented with 10% fetal calf serum and 700 µg/ml Geneticin. Isolated clones were screened by Northern blot analysis.

Ligand Binding–Cell membranes were prepared from 10- or 15-cm dishes by replacing the growth medium with ice-cold hypotonic buffer (15 mM Tris, pH 7.4, 2.5 mM MgCl2, 0.2 mM EDTA). After swelling for 10-15 min at 4°C , the cells were scraped from the plates, sonicated on ice and

centrifuged (20,000 rpm for 10 min) and resuspended in ice-cold TME (75 mM Tris, pH 7.4, 12.5 mM MgCl2, 1 mM EDTA). Aliquots of thawed and sonicated membrane preparation were added to tubes containing 200 µl TME and [3H]-DPAT (Amersham) and indicated drugs. 5-HT (10 µM) was used to define nonspecific binding, which was less than 10% of total binding at concentrations of radioligand near the Kd value. Incubations with 6-8 different concentrations of [3H]-DPAT (in triplicate) were initiated by the addition of 100 µg of membrane protein, carried out at room temperature for 30 minutes and stopped by filtration through GF/C (Whatman) filters and immediate washing with 3 x 4 ml of ice-cold buffer (50 mM Tris, pH 7.4). Radioactivity retained on the filter was dissolved in 5 ml of HiSafe3 (Wallac) and quantitated by liquid scintillation counting. Protein was assayed with the BIO-RAD protein assay kit with bovine serum albumin as standard.

cAMP Assay–Measurement of cAMP was performed as described previously (12). Briefly, cells plated in six-well 35-mm dishes were washed twice with 1 ml of HBBS containing 100 µM IBMX and resuspended with 1 ml of buffer containing various test compounds for test incubation of 20 minutes at 37°C. The buffer was collected and stored at -20°C until assayed for cAMP by a specific radioimmunoassay (ICN) as described (13). Standard curves displayed average IC50 of 0.5 ± 0.2 pmol using cAMP as standard. Data for cAMP assays are presented as mean ± S.E. for triplicate wells.

Intracellular Calcium Measurement–As described previously (13) cells were harvested by incubation in HBBS (118 mM NaCl, 4 mM KCl, 10 mM D-glucose, 20 mM HEPES pH 7.2) + 5 mM EDTA, 0.05% trypsin and incubated with Fura-2, for 20 minutes at 37°C . The cells were centrifuged, washed twice with HBBS +1 mM CaCl2 and placed in a fluorescence cuvette. Change in fluorescence ratio was recorded on a Perkin-Elmer (Buckinghamshire, U.K.) LS-50 spectrofluorometer and analyzed by computer, based on a Kd of 227 nM for the Fura 2-Ca2+ complex. Calibration of Rmax was performed by addition of 0.1% Triton X-100 and 20 mM Tris base and of Rmin by addition of 10 mM EGTA. All experimental compounds were added directly to the cuvette from 200-fold concentrated test solutions as indicated in the figures.

RESULTS

The close association between PK C-induced phosphorylation and desensitization of the 5-HT1A receptor (18) suggested that TPA may act by inducing receptor phosphorylation rather than via phosphorylation of downstream components (eg., G proteins or PLC). We addressed this possibility by generating 5-HT1A receptors mutated at PK C consensus phosphorylation sites located in the third intracellular loop of the receptor (Fig. 1). The single point mutants included T229A, S253G, and T343A, which substitute alanine and glycine for the phosphate acceptors threonine and serine, respectively. A double mutant (T229A/S253G) and the triple mutant were also constructed. These mutants were stably transfected into receptor-negative Ltk- cells, and positive clones expressing receptor RNA were selected for further characterization. Membranes prepared from positive clones were subjected to saturation binding analysis using [3H]-DPAT, a selective 5-HT1A receptor agonist (12). The levels of 5-HT1A receptor expression of the various clones varied due to different efficiencies of transfection expression, with Bmax values ranging from 0.30 to 0.76 nmol/mg protein as compared to 1.64 nmol/mg protein for the wild-type receptor. The Kd values calculated for the mutant receptors were all in the nanomolar range (Table I), similar to the affinity of the non-mutated receptor (12). Thus, the mutations did not greatly alter agonist affinity consistent with previous observations that mutations of cytoplasmic domains have little effect on ligand binding in other monoamine receptors (21,22).

The ability of wild-type and mutant 5-HT1A receptors to inhibit adenylyl cyclase was examined by measuring cAMP accumulation in transfected clones in the absence and presence of forskolin, an activator of adenylyl cyclase. Neither mutant nor wild-type receptors inhibited basal cAMP level (without forskolin) when expressed in Ltk- cells (data not shown), as observed previously for the wild-type receptor (13). The wild-type 5-HT1A receptor markedly inhibited forskolin-stimulated cAMP level by >80% (Table 2). Each of the mutants was approximately as effective at inhibiting forskolin-induced cAMP (ranging from 70-75%) despite a 50-70% lower receptor number (Bmax) in cell lines expressing the mutant receptors (Table 1). Only the T229A mutant (30% inhibition) had a markedly reduced coupling, a result that was confirmed in three

independent clones. This 5-HT1A receptor mutant was atypical, since the double (T229A/S253G) and the triple mutant inhibited cAMP levels to a similar extent as the wild-type receptor. The reduced coupling of the T229A mutant may be due to a confirmational change introduced by the alanine substitution in an intracellular segment of the receptor thought to be important for coupling (3), which could be reversed when the double and triple mutations were reintroduced.

The effect of TPA on 5-HT-induced inhibition of cAMP accumulation in both wild-type and triple mutant 5-HT1A receptor clones was determined. Cells were incubated with PGE1, which activates a Gs-coupled enhancement of cAMP levels, and the potency of 5-HT to inhibit this response was examined in the absence and presence of TPA (Fig. 2). The magnitude and potencies of the wild-type and the triple mutant receptors did not greatly differ in the presence and absence of TPA. These results confirm and extend the conclusion that acute PK C activation does not uncouple the 5-HT1A receptor from inhibition of adenylyl cyclase in this cell line (13).

Coupling of wild-type and mutant 5-HT1A receptors to calcium mobilization and its modulation by PK C were examined by monitoring cytosolic free calcium levels in cells loaded with the calcium indicator, Fura 2. 5-HT (100 nM) induced an immediate 2.6-fold peak increase in intracellular calcium levels in Ltk- cells transfected with wild-type 5-HT1A receptor (Fig. 3A, Table 3). The response elicited by activation of the mutant receptors was in the same range and varied from 1.9-fold to 3.0-fold basal level, except for the atypical mutant T229A, which was only 1.4-fold basal (Table 3). Thus, the mutations did not eliminate coupling of the 5-HT1A receptor to enhance calcium mobilization. Acute pretreatment (for 2 min) with 100 nM TPA, to activate PK C, abolished the action of 5-HT to enhance calcium levels for the wild-type receptor and for each of the single mutant 5-HT1A receptors (Fig. 3; Table 3). However, upon acute pretreatment with TPA of cells expressing the double (T229A/S253G) and triple mutant, the 5-HT-induced calcium response was still observed (54 ± 6% and 74 ± 7% of treated, Table 3). The potency of TPA action on calcium mobilization for the wild-type receptor was 20 nM, whereas the EC50 value for triple mutant was greater than 1 mM (data not shown). These results indicate that the 5-HT1A receptor mutants with multiple mutations became progressively more resistant to PK C as the number of available PK C sites on the receptor decreased. Thus, the action of PK C to uncouple the 5-HT1A receptor from stimulation of calcium mobilization may involve multiple

phosphorylation sites located on the receptor itself. By contrast, TPA did not change receptor-mediated inhibition of cAMP in either wild-type or triple mutant receptor, indicating that PK C preferentially uncouples the calcium mobilization pathway.

DISCUSSION

Activation of the multiple subtypes of PK C by TPA or DAG (23), the endogenous product of PLC activation, results in the phosphorylation of substrate proteins at the minimal consensus sequences (S/T)-X-(K/R) or (K/R)-X-(S/T) (24, 25). Phosphorylation of these consensus sequences found in the intracellular domains of the b-adrenergic receptor has been suggested to account for the action of acute TPA treatment to reduce receptor potency (26, 27). Likewise, the 5-HT1A receptor is rapidly phosphorylated following addition of TPA, which correlates with a rapid and complete uncoupling from PLC activation (18). However, the site of action of PK C has not been identified. While PK C-dependent phosphorylation of downstream signalling proteins (Gi2 (19, 28, 29) and PLCb (20)) has been demonstrated, the functional importance of these events in receptor signalling in intact cells is not known. The increasing insensitivity to PK C activation of 5-HT1A receptors mutated at one, two and three consensus phosphorylation sites indicates that phosphorylation of the 5-HT1A receptor serves as the primary site for uncoupling mediated by PK C activation. The residual inhibitory effect of PK C activation on the triple mutant could be mediated by phosphorylation at other sites on the receptor, G proteins, or effector. These results represent the first evidence that elimination of multiple sites for receptor phosphorylation blocks the complete uncoupling of a receptor signalling pathway induced by acute activation of PK C.

Suppression of receptor-mediated calcium mobilization by acute activation of PK C occurs for a large variety of receptors which couple to PLC activation, and may serve as an important negative feedback pathway (23). Several receptors couple via PTX-sensitive G proteins (Go/Gi) to enhance PLC activity and calcium mobilization in cells of mesenchymal origin (16). For example, both transfected (16) or endogenously expressed 5-HT1B receptors in OK opossum kidney cells (30,31) couple similarly to induce calcium mobilization. In Ltk- cells transfected with 5-HT1B receptor, acute TPA treatment completely inhibits coupling to calcium mobilization, without blocking receptor-mediated inhibition of cAMP accumulation. Since the PK C sites we have mutated in the 5-HT1A receptor are conserved in the human and opossum 5-HT1B receptors,

these sites may also mediate PK C-induced uncoupling of the 5-HT1B receptor from PLC. Whether the mechanisms implicated for the 5-HT1A receptor can be extended to regulation of this or other receptor subtypes remains to be seen. Findings using PK C inhibitors have suggested that PK C may not mediate homologous receptor desensitization of the Gq-coupled a1B-adrenergic receptor (32). By contrast, mutational analyses of the gastrin-releasing peptide receptor indicate that PK C sites mediate homologous desensitization of this Gq-coupled receptor (33). Using an antisense approach, Shih and Malbon have shown that the importance of second messenger-activated kinases (PK C) and receptor kinases can vary depending on the cell type studied (34). Thus, further investigation of the role of PK C in 5-HT1A receptor desensitization may be more appropriately addressed in cells which endogenously express and regulate the receptor. Nevertheless, PK C-induced receptor phosphorylation may be a general signal to selectively inactivate receptor coupling to the PLC pathway.

Results obtained using 5-HT1A receptor mutants indicate that receptor phosphorylation by PK C selectively modulates one pathway (calcium mobilization) without altering the other pathway (inhibition of cAMP level, (13) and data not shown). This may represent an amplified version of a negative feedback mechanism whereby the product (DAG) inhibits its own generation via PLC but not receptor coupling to other effectors (ie., adenylyl cyclase). This novel "pathway-selective" view of desensitization (16) contrasts with b-adrenergic receptor uncoupling, where one signalling pathway (enhancement of adenylyl cyclase) has been investigated exhaustively, but other pathways have not been examined (1-8). We demonstrate that the pathway-selective modulation of the 5-HT1A receptor by PK C involves phosphorylation sites on the receptor, suggesting that receptor phosphorylation can sculpt receptor signalling, in this case converting a mixed phenotype into a strictly inhibitory one.

The selective action of TPA on the pathways could result from an apparently greater receptor reserve for the cAMP pathway than the calcium pathway of the 5-HT1A receptor (35). Receptor reserve is observed when a maximal response is elicited by an agonist at a concentration which does not occupy all of the available receptors, resulting in a higher agonist potency (EC50 = 4.4 ± 1.3 vs. 20 nM for calcium response) and lower dependence on receptor number (16; compare Tables 2 and 3), which was observed for the cAMP response. Hypothetically,

incomplete receptor phosphorylation by PK C could result in a population of non-phosphorylated 5-HT1A receptors capable of mediating the cAMP response, but insufficient to transduce a calcium response. However, the presence of TPA during the 20-minute duration of the cAMP assay is sufficient time to allow maximal 5-HT1A receptor phosphorylation by PK C (18). Thus, maximal activation of PK C for 20 minutes would be predicted to increase agonist EC50 or decrease maximal response, but had no effect on 5-HT-induced inhibition of cAMP (Fig. 3). This observation argues against change in receptor reserve as an explanation for the selectivity of PK C action. Alternatively, utilization of different domains of the receptor to conduct separate signalling pathways may explain the pathway-selectivity of PK C action (see below).

The structural basis for the specificity of PK C to selectively block receptor-mediated calcium mobilization but not cAMP accumulation has not been specifically addressed by these studies. The third cytoplasmic loop is clearly important for G protein coupling and discrete modifications (by phosphorylation or point mutation) in this domain modify receptor (3, 22) signalling. In the b-adrenergic receptor, PK A sites in the third cytoplasmic loop and C-terminal tail have been implicated in cAMP-mediated receptor uncoupling from adenylyl cyclase (7). Point mutations in the third loop adjacent to transmembrane domain VI cause constitutive activation in several receptors (36,37) including the TSH receptor. The A623I mutation of the TSH receptor selectively activated coupling to adenylyl cyclase, and no constitutive activation of PI turnover was observed (37). On the other hand, mutation of A623 to a charged residue (E or K) resulted in uncoupling from PLC but not adenylyl cyclase activation (38). Thus, side-chain modifications at a single amino acid can drastically alter the selection of receptor signalling pathways.

While the mutations we have introduced did not greatly affect the coupling of the 5-HT1A receptor to various effectors, the capacity for receptor phosphorylation at a few residues selectively inhibits one pathway but not the other. The finding that peptides derived from the second intracellular loop of the 5-HT1A receptor couple to inhibit adenylyl cyclase suggests that this region may be more important for coupling to this pathway; perhaps the third loop is more important for coupling to calcium mobilization. However, the latter results must be interpreted with care, since peptide-mediated actions were not sensitive to PTX (39).

In conclusion, multiple PK C consensus phosphorylation sites located in the third

cytoplasmic loop of the 5-HT1A receptor mediate pathway-selective uncoupling of the receptor from calcium mobilization by acute PK C activation.

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TABLES

Table 1: Binding characteristics of Ltk- cells expressing transfected wild-type or mutant 5-HT1A receptors. Kd and Bmax values were determined by nonlinear regression analysis of saturation binding data derived with the LIGAND program. Values shown are the mean ± S.E.M of data from at least three independent experiments. Membranes were prepared from cell clones stably transfected with wild type or mutant 5-HT1A receptors and subjected to saturation binding analysis with [3H]-DPAT (see Methods).

Table 2: Inhibition of cAMP accumulation for wild-type and mutant 5-HT1A receptors. The percent inhibition of (10 μM) forskolin-stimulated cAMP accumulation by 5-HT (10 μM) is tabulated as the mean ± S.D. of at least three independent experiments. Calculation for inhibition of forskolin-stimulated cAMP accumulation [(F-FS)/F] x 100, where the level of cAMP after forskolin (F) or forskolin and serotonin (FS) was measured.

Table 3: Effect of Protein Kinase C on 5-HT-induced changes in [Ca2+]i. The influence of acute pretreatment of TPA in Ltk- cells transfected with wild-type and mutant receptors. Serotonin-induced changes in [Ca2+]i are indicated as -fold basal level of [Ca2+]i and represent mean ± S.D. of at least three independent determinations in at least two different clones. Percent of control represents the % of the serotonin-induced response recovered post-TPA treatment. – indicates no response to 5-HT.

Table 1: Binding characteristics of the wild-type and mutant 5-HT1A receptors.

5-HT1A receptor mutants	B_{max} (pmol/mg)	K_d (nM)
W.T.	1.64 ± 0.38	2.74 ± 0.18
T229A	0.34 ± 0.12	5.12 ± 1.5
S253G	0.76 ± 0.10	1.31 ± 1.3
T343A	0.62 ± 0.10	7.8 ± 2.5
T229A/S253G	0.30 ± 0.06	7.9 ± 2.1
T229A/S253G/T343A	0.31 ± 0.04	1.4 ± 0.3

Table 2: cAMP accumulation for wild-type and mutant 5-HT1A receptor clones.

5-HT1A receptor mutants	%-Inhibition
W.T.	83 ± 10
T229A	31 ± 10
S253G	74 ± 10
T343A	70 ± 8
T229A/S253G	73 ± 5
T229A/S253G/T343A	75 ± 8

Table 3: Effect of PKC on 5-HT-induced changes on $[Ca^{2+}]_i$.

5-HT1A receptor mutants	5-HT induced increase in $[Ca^{2+}]_i$ (-fold basal)	Post-TPA treatment 5-HT induced increase in $[Ca^{2+}]_i$ (% of control).
W.T.	2.6 ± 0.30	——
T229A	1.4 ± 0.03	——
S253G	3.0 ± 0.20	——
T343A	1.9 ± 0.10	——
T229A/S253G	2.1 ± 0.10	54 ± 7%
T229A/S253G/T343A	1.8 ± 0.10	74 ± 6 %

FIGURE LEGENDS:

Figure 1: Model of targetted mutants of the 5-HT1A receptor. The proposed structure of the rat 5-HT1A receptor consisting of seven hydrophobic transmembrane domains forming three intracellular loops. The three putative PK C consensus phosphorylation sites which were mutated are depicted with circles.

Figure 2: The effect of TPA on 5-HT-induced inhibition of PGE1-stimulated cAMP accumulation in wild type and T229A/S253G/T343A 5-HT1A receptor clones. The indicated concentrations of 5-HT were added in the absence (CIRCLES, hatched line) and presence (SQUARES, solid line) of 100nM TPA. Data are means ± S.E. of triplicate samples and are plotted as % inhibition of cAMP accumulation induced by 1 mM PGE1 versus the concentration of 5-HT; curves were fit mathematically using the Kaleidograph program. The figure represents a single experiment which was repeated three times. A) Cells transfected with wild-type 5-HT1A receptor: EC50 = 6.0 nM ; EC50 (+TPA) = 5.1 nM. The EC50 values from three experiments for wild-type were 4.4 ± 1.3 nM and 4.1 ± 0.8 nM in the presence and absence of TPA, respectively. B) Cells transfected with T229A/S253G/T343A 5-HT1A receptor: EC50= 1.9 nM; EC50 (+TPA) = 2.9 nM. The EC50 values from three experiments for the triple mutant were EC50= 1.9 ± 0.1 nM and EC50 (+TPA) = 3.2 ± 0.5 nM.

Figure 3: Calcium mobilization by PK C site mutants of the 5-HT1A receptor transfected in Ltk- cells. Dark tracing represents control samples: 5-HT (100 nM) was added at 60 seconds as shown with the dark arrow. Light tracing represents TPA-treated samples: TPA (100 nM) was added at time 0, and 5-HT (100 nM) at 120 sec as depicted with the light arrow. A) Wild-type receptor, in TPA-treated samples (light tracing) the serotonin-mediated calcium response was completely inhibited. B) T343A (TPA completely blocked the 5-HT response). C) T229A/S253G, post-treatment with TPA, 50% of the 5-HT-induced calcium response was recovered. D) T229A/S253G/T343A, 74% of 5-HT-mediated calcium response was recovered post-TPA. All curves were generated by a computer and were from a single experiment which was repeated at

least 3 times and with at least 2 independent clones which gave similar results.

Figure 1 : Model of targetted mutants of the 5-HT1A receptor.

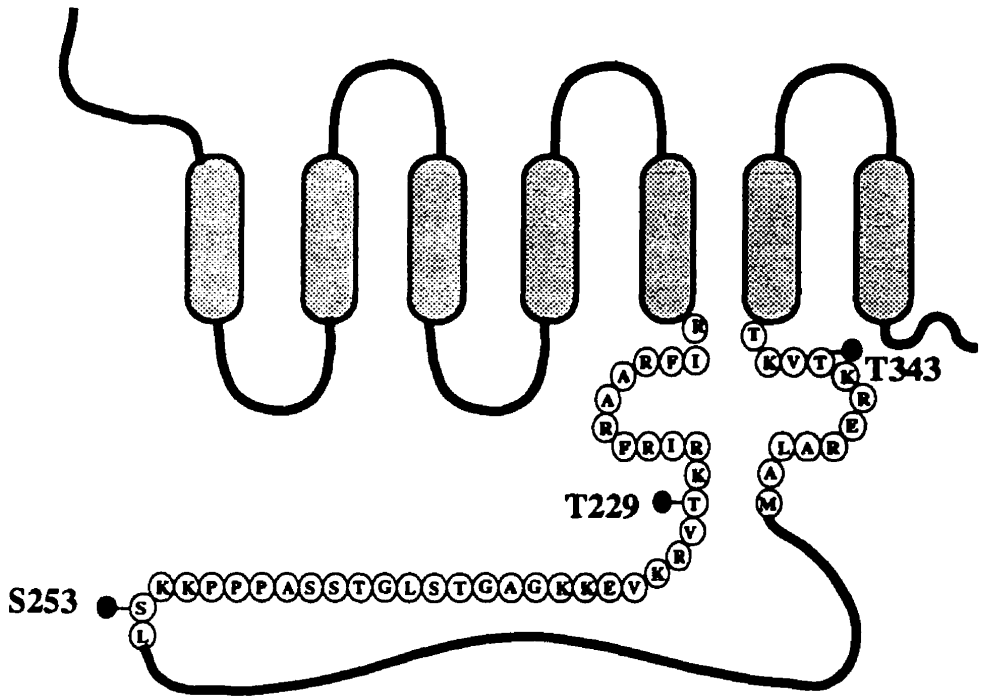
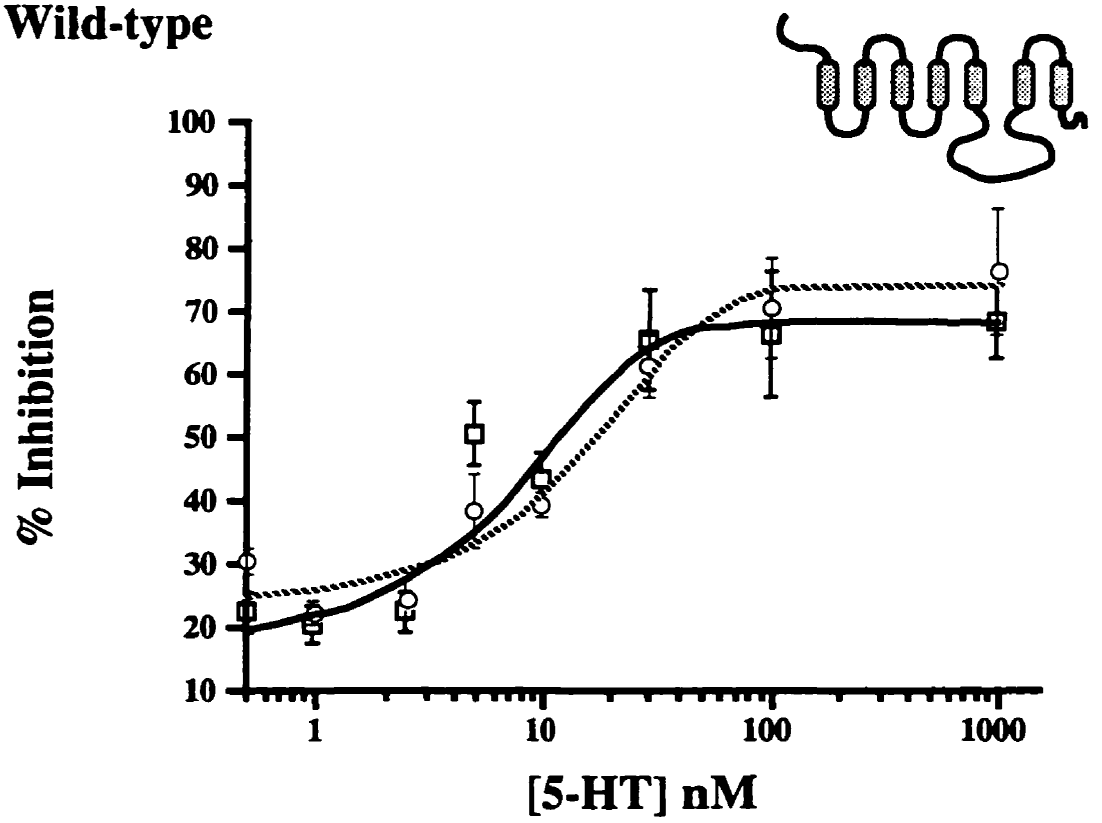


Figure 2: Effect of TPA on 5-HT-induced inhibition of cAMP accumulation in wild-type and T229A/S253G/T343A 5-HT1A receptor clones.

A) Wild-type



B) T229A/S253G/T343A

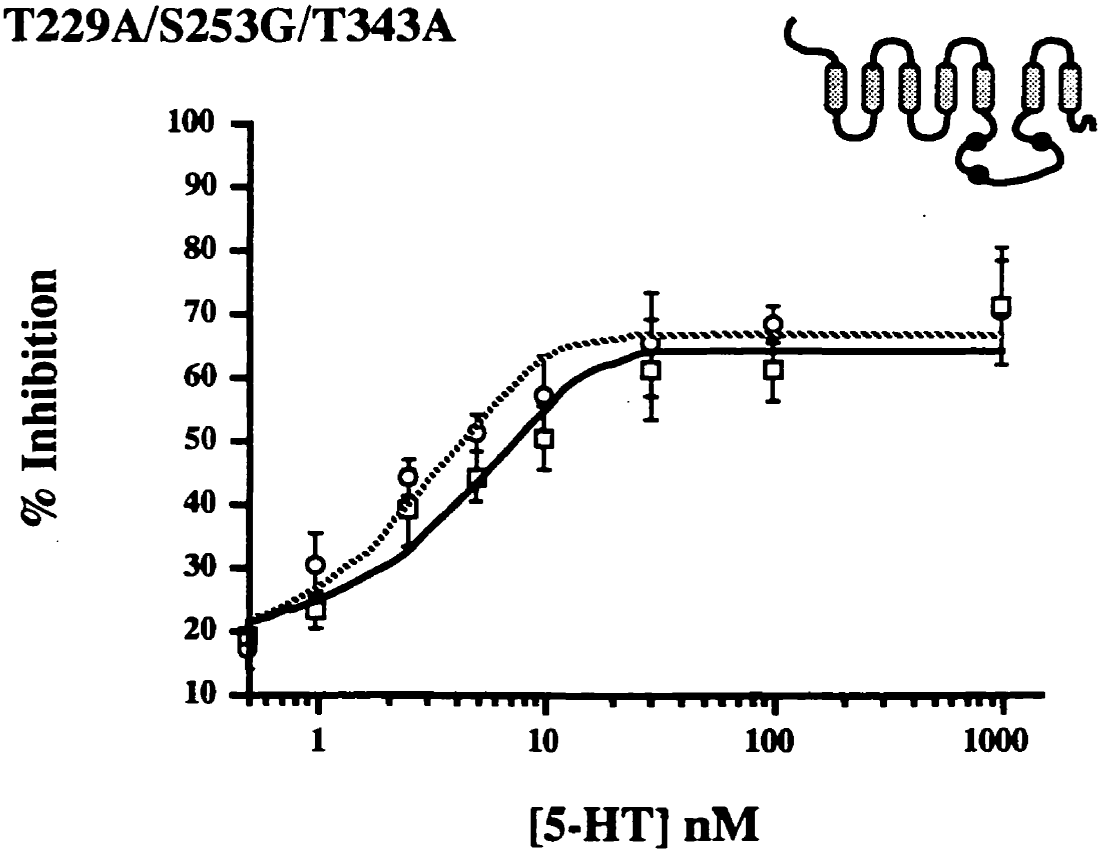
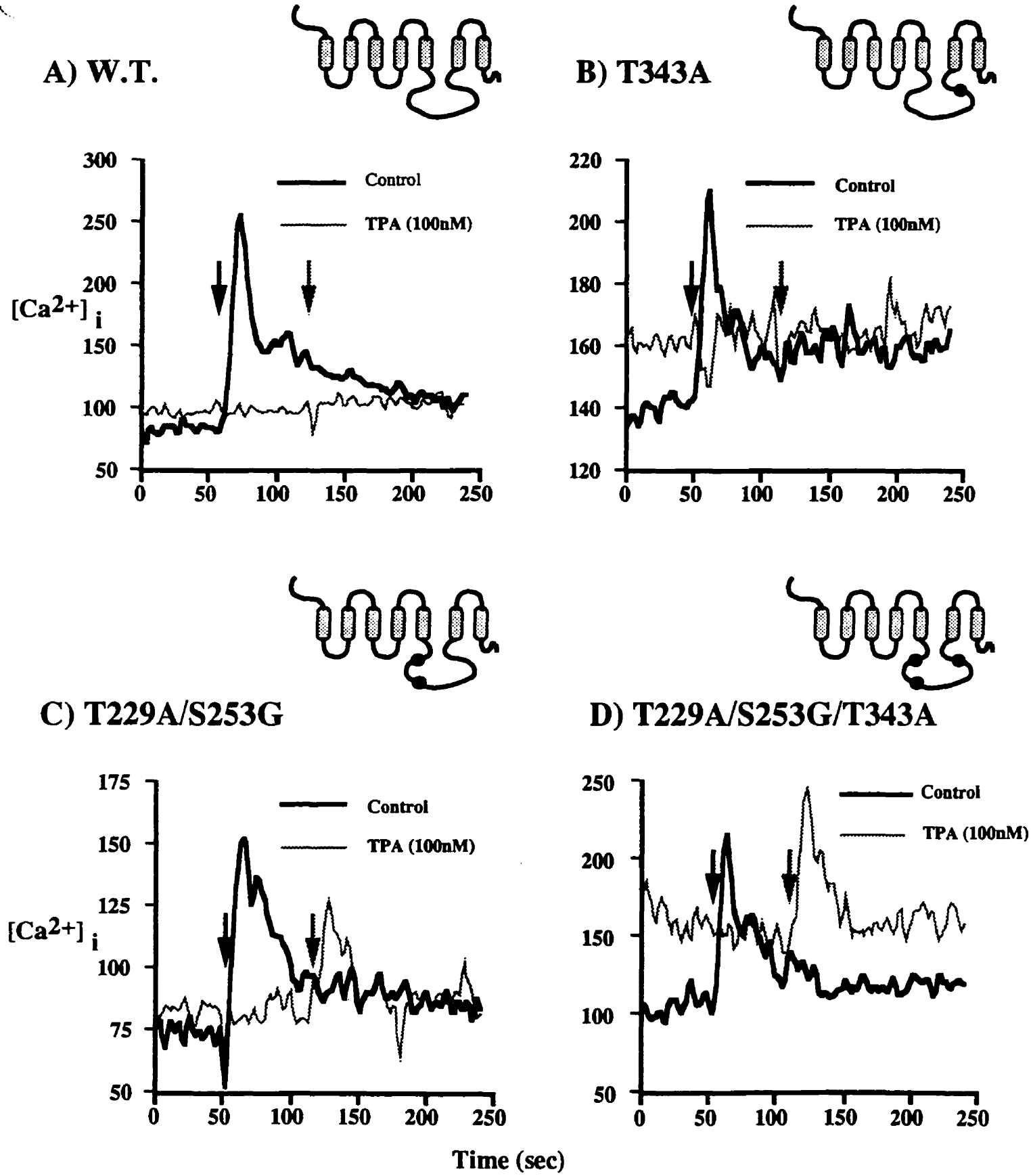


Figure 3: Calcium mobilization in PK C site mutants of the 5-HT$_{1A}$ receptor.



4. Chapter 2. A conserved threonine residue in the second intracellular loop of the 5-HT1A receptor directs signaling specificity. P.M.C. Lembo, M.H. Gharehmani and P.R. Albert (*under revision*).

Receptor mutational studies and experiments using receptor antibodies or short synthetic peptides that inhibit or mimic receptor interactions with various G-proteins have identified the i2 loop, the N-and C- terminal domains of the i3 loop, and the membrane-proximal portion of the C-terminal tail as receptor domains that participate in receptor-G-protein interactions (68, 69). The β2-adrenergic receptor and the rhodopsin system provide the most complete model of structure-function relationships for receptor-G-protein interaction. However, the role of individual amino acids in determining the selectivity of receptor-mediated signals has not been addressed. Sequence alignment of several G-protein coupled receptors revealed a highly conserved threonine residue in the i2 loop of the 5-HT1A receptor that forms a putative PKC phosphorylation consensus site located in a predicted amphipathic α-helical domain. To examine the role of this conserved threonine residue in 5-HT1A receptor coupling to Gi/Go proteins this residue was mutated to alanine (T149A mutant). We have demonstrate that the T149, a conserved residue in the i2 loop is part of a hydrophilic α-helical domain that directs specific interactions of the 5-HT1A receptor resulting in cell-specific coupling to calcium mobilization, inhibition of calcium entry and a partial role in the inhibition of cAMP accumulation.

A Conserved Threonine Residue in the Second Intracellular Loop of the 5-HT1A Receptor Directs Signaling Specificity.

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Abbreviations:

[^{3}H]-DPAT, 8-hydroxy-(2-(N,N-di-[2,3,^{3}H]propylamino)1,2,3,4-tetrahydronaphthalene; PI, phosphatidyl inositol; PK C, protein kinase C; PLC, Phospholipase C; 5-HT, serotonin; IBMX, 3-isobutyl-1-methylxanthine; PTX, pertussis-toxin; VIP, vasoactive intestinal peptide; $[Ca^{2+}]_i$, intracellular free calcium concentration; i2, i3, second (third) intracellular.

Abstract

Productive interaction between receptors and G proteins involves multiple intracellular receptor domains, but the role of individual receptor amino acids in directing the selection of specific signaling pathways has not yet been identified. Sequence alignment of several G-protein coupled receptors identified a highly conserved threonine residue in the i2 loop of the 5-HT1A receptor that is a putative PK C phosphorylation consensus site and is located in a predicted amphipathic α-helical domain. To examine the role of this conserved threonine residue in 5-HT1A receptor coupling to Gi/Go proteins, this residue was mutated to alanine (T149A mutant). Wild-type and mutant 5-HT1A receptors were stably transfected into both Ltk- and GH4C1 cells to investigate receptor coupling to multiple signaling pathways. In both cell lines the T149A mutant displayed similar agonist affinities as the wild-type receptor. In Ltk- cells, the T149A 5-HT1A receptor inhibited cAMP accumulation by 30% as compared to wild-type, 83%. A 2.6-fold increase in intracellular calcium (due to phospholipase C-mediated calcium mobilization) was observed for the wild-type receptor upon addition of 100 nM 5-HT; whereas up to 10 μM 5-HT, the T149A 5-HT1A receptor failed to mediate a calcium mobilization response. When transfected in GH4C1 cells, the T149A receptor mutant fully inhibited basal cAMP and partially inhibited Gs-stimulated cAMP accumulation as compared to wild-type receptor (57±14% vs 86±2%). In contrast, the T149A 5-HT1A receptor mutant failed to block the influx of calcium induced by calcium channel agonist (±)-Bay K8644, whereas the wild-type 5-HT1A receptor inhibited the calcium influx by 40%. Thus, the threonine-149 residue is directly involved in G-protein coupling to calcium mobilization (mediated by βγ subunits) and to inhibition of calcium channel activation (mediated by Go) but plays a minor role in coupling to α_i-mediated inhibition of cAMP accumulation. The conserved i2 loop threonine may serve as a G protein contact site to direct the signaling specificity of multiple receptors.

Introduction.

A large variety of neurotransmitters, neuropeptides, and autocrine and paracrine factors mediate their biological actions by activation of receptors that are coupled to heterotrimeric G-proteins. These receptors have primary sequences that are consistent with a secondary structure composed of seven conserved α-helical transmembrane domains, three intracellular loops, and an intracellular carboxyl-terminal domain. Receptor mutational studies (1,2,3) and experiments using receptor antibodies (4) or short synthetic peptides that inhibit or mimic receptor interactions with various G-proteins (5-10) have identified the i2 loop, the N-and C- terminal domains of the i3 loop, and the membrane-proximal portion of the C-terminal tail as receptor domains that participate in receptor-G-protein interactions. However, the role of individual amino acids in determining the selectivity of receptor-mediated signals has not been addressed.

The 5-HT1A receptor is a member of a family of receptors that couple to PTX-sensitive G proteins (Gi/Go) to initiate inhibitory or stimulatory signal transduction pathways, depending on the cell type in which the receptor is expressed (11,12). When transfected in GH4C1 pituitary cells, which have characteristics of neuronal cells such as voltage-gated ion channels and regulated secretion of hormones, the 5-HT1A receptor displays an inhibitory signaling phenotype characteristic of receptors endogenously expressed in neurons (13,14). Upon activation, the 5-HT1A receptor reduces both basal cAMP and Gs-stimulated cAMP accumulation and inhibits Bay K8644-induced influx of calcium to decrease $[Ca^{2+}]_i$. These changes are associated with inhibition of secretion and inhibition of cell proliferation. However, when expressed in a variety of fibroblast-derived cells, such as Ltk-, Hela, or Balb/c 3T3, the 5-HT1A receptor enhances PI turnover, releasing intracellular calcium stores to increase $[Ca^{2+}]_i$. In fibroblast cells the 5-HT1A receptor does not alter basal cAMP levels but inhibits both forskolin- and Gs-stimulated cAMP accumulation (15-17). These responses are associated with increased DNA synthesis and ultimately with oncogenic transformation. Each response mediated by the 5-HT1A receptor is blocked by pretreatment with PTX, indicating the involvement of Gi/Go proteins. In addition to 5-HT1A receptors other receptors that couple to Gi/Go, such as the 5-HT1B and dopamine-D2 (long and short variant) receptors, reproduce this cell-specific pattern of signaling: ie., inhibitory in pituitary cells vs. stimulatory in fibroblast cells (12,18). However, the precise amino acids of the receptor that determine coupling to these pathways remain to be elucidated.

The β2-adrenergic receptor provides the most complete model of structure-function relationships for receptor-G-protein interaction. Chimeric and site-directed mutagenesis studies have shown unequivocally that the carboxyl-terminal and i3 loop domains of the β2--adrenergic receptor are essential for coupling to Gs (1-3), but the role of the i2 loop was unclear. Recent molecular studies focusing on the i2 loop of several Gq-coupled receptors have revealed the importance of this loop in coupling to PLC-linked pathways (19-21). Mutation of the Leu 131

residue to alanine in the i2 loop of the human m1 muscarinic receptor decreased PI turnover; mutation of the corresponding Leu 174 in the human m3 muscarinic receptor had the same effect (19). Mutations in the i2 loop of the angiotensin II receptor type 1 abolished Ang II-induced stimulation of inositol trisphosphate (20). Furthermore, residues 525-527 and 528-532 of the i2 loop of the TSH receptor were shown to be essential for agonist-induced cAMP and PIP_2 signaling respectively (21). These observations indicate that the i2 loop of Gq-coupled receptors is critical for activation of PLC.

Receptor mutagenesis studies have also indicated a potential role for the i2 loop in coupling to certain Gi-linked pathways. A chimeric receptor in which the i3 loop of the Gi-coupled muscarinic m2 receptor was replaced with the i3 loop of the β2–adrenergic receptor coupled to both Gs and Gi. Hence, the ability to stimulate Gi did not reside solely in the i3 loop. In the same report, a nine amino acid peptide representing the C-terminal sequence of the i2 loop stimulated high affinity GTPase activity and inhibited forskolin-stimulated adenylyl cyclase in membranes (22). Moreover, synthetic peptides corresponding to the entire i2 loop of the 5-HT1A receptor strongly inhibited forskolin-stimulated adenylyl cyclase activity (23). On the basis of the above results suggesting a role for i2 domains in Gi-mediated signaling, we examined the possibility that part of the i2 loop of the 5-HT1A receptor might be involved in Gi/o coupling to its effectors. We have therefore mutated to alanine a threonine residue located in the i2 loop of the 5-HT1A receptor that forms part of a consensus sequence that is conserved in multiple Gi/Go/Gq-coupled receptors. This site also forms part of a putative PK C consensus phosphorylation site. The wild-type and mutant 5-HT1A receptors were transfected in Ltk- fibroblasts and GH4C1 pituitary cells to investigate their potential role in receptor Gi/o-protein-coupling to cell-specific effectors.

Experimental Procedures

Materials–Restriction endonucleases and other molecular biology reagents were purchased from Boehringer Mannheim and GIBCO-BRL. Sequenase was from U.S. Biochemicals. Forskolin, IBMX, 5-HT, and VIP were from Sigma; [^{3}H]-DPAT (228 Ci/mmol) and [α–^{32}P] ATP (2,200 Ci/mmol) were obtained from Amersham Corp. Geneticin was purchased from GIBCO and Fura 2-AM from Molecular Probes, Eugene, OR.

METHODS

Cell Culture–Ltk- cells were grown as a monolayer in α-minimum Eagle's medium and GH4C1 cells were grown in F-10 medium supplemented with 8% fetal bovine serum, at 37°C in a humidified atmosphere, with 5% carbon dioxide. Media were changed 12-24 h prior to experimentation.

Construction and Expression of 5-HT1A Receptor Mutants–The *Bam*HI/*Xba*I fragment of the rat

5-HT1A receptor gene in the pZEM-3 vector (15) (containing the mouse metallothionein promoter) was subcloned into p-Select to use as a template for site-directed mutagenesis (Altered-sites mutagenesis, Promega). The putative PK C site in the second loop was mutated at T149 to an alanine using an oligonucleotide (AACAAAAGGGAGCCCCGGC) incorporating the point mutation. The mutation was confirmed by DNA sequencing. Mutated and wild-type 5-HT1A receptor cDNAs were subcloned into the eukaryotic expression vector pcDNA I (Invitrogen) and co-transfected with pSV-Neo in Ltk- cells and GH4C1 cells using calcium phosphate co-precipitation (14). Neomycin-resistant cells expressing 5-HT1A receptors were selected and grown in α–MEM or F-10 medium supplemented with 10% fetal calf serum and 700 μg/ml Geneticin. Clones were screened by Northern blot analysis.

Ligand Binding–Cell membranes were prepared from 10- or 15-cm dishes by replacing the growth medium with ice-cold hypotonic buffer (15 mM Tris, pH 7.4, 2.5 mM $MgCl_2$, 0.2 mM EDTA). After swelling for 10-15 min at 4°C , the cells were scraped from the plates, sonicated on ice and centrifuged (20,000 x g for 10 min) and resuspended in ice-cold TME (75 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, 1 mM EDTA). Aliquots of thawed and sonicated membrane preparation were added to tubes containing 200 μl TME and [^{3}H]-DPAT (Amersham) and indicated drugs. 5-HT (10 μM) was used to define nonspecific binding, which was less than 10% of total binding at concentrations of radioligand near the K_d value. Incubations with 6-8 different concentrations of [^{3}H]-DPAT (in triplicate) were initiated by the addition of 100 μg of membrane protein, carried out at room temperature for 30 minutes and stopped by filtration through GF/C (Whatman) filters and immediately washed with 3 x 4 ml of ice-cold buffer (50 mM Tris, pH 7.4). Radioactivity retained on the filter was dissolved in 5 ml of HiSafe3 (Wallac) and quantitated by liquid scintillation counting. Protein was assayed with the BIO-RAD protein assay kit using bovine serum albumin as a standard.

cAMP Assay–Measurement of cAMP was performed as described previously (11). Briefly, cells plated in six-well 35-mm dishes were washed twice with 1 ml of HBBS (118 mM NaCl, 4 mM KCl, 10 mM D-glucose, 20 mM HEPES pH 7.2) containing 100 μM IBMX and resuspended with 1 ml of buffer containing various test compounds for test incubation of 20 minutes at 37°C. The buffer was collected and stored at -20°C until assayed for cAMP by a specific radioimmunoassay (ICN). Standard curves displayed average IC_{50} of 0.5 ± 0.2 pmol using cAMP as standard. Data for cAMP assays are presented as mean ± S.E.M for triplicate wells.

Intracellular Calcium Measurement–As described previously (11) cells were harvested by incubation in HBBS + 5 mM EDTA, 0.05% trypsin (for Ltk- cells) or HBBS + EDTA (5mM) for GH4C1 cells and incubated with Fura-2, for 20-30 minutes at 37°C . The cells were centrifuged, washed twice with HBBS +1 mM $CaCl_2$ and placed in a fluorescence cuvette. Change in

fluorescence ratio was recorded on a Perkin-Elmer (Buckinghamshire, U.K.) LS-50 spectrofluorometer and analyzed by computer, based on a K_d of 227 nM for the Fura 2-Ca^{2+} complex. Calibration of R_{max} was performed by addition of 0.1% Triton X-100 and 20 mM Tris base and of R_{min} by addition of 10 mM EGTA. All experimental compounds were added directly to the cuvette from 200-fold concentrated test.

Results

For a more complete understanding of the molecular mechanisms governing the specificity of receptor-G-protein coupling the specific amino acids that are required for interactions between the receptor and G-proteins must be identified. While chimeric approaches provide insight into the function of amino acids that diverge among different receptors, we have chosen the point mutagenesis approach to address the roles of single conserved amino acids in receptor function (24). In particular, examination of the molecular structure of the 5-HT1A receptor revealed a potential PK C phosphorylation site located at T149 in the i2 loop (Fig. 1A). An alignment of peptide sequences corresponding to this region in other G-protein-coupled receptors revealed a striking conservation of the threonine residue. Furthermore, in receptors that couple to Gi/o proteins a BBTXBB (X=P/T/S, B= basic residue) consensus PK C phosphorylation sequence is well conserved. The related AATXBB (A = aliphatic residue) sequence was identified in several Gs- and Gq- coupled receptors. Based on the potential role of the i2 loop in receptor signaling, we addressed the role of this conserved threonine residue in 5-HT1A receptor function. The 5-HT1A receptor mutant T149A was generated, eliminating the hydroxyl side-chain that may serve as a contact point for G proteins or as a phosphate acceptor site for PK C (Fig. 1A). The wild-type and mutant receptors were stably transfected in receptor-negative Ltk- cells and GH4C1 cells to investigate any multiple pathways of signal transduction and G-protein coupling.

Receptor Binding. Membranes prepared from positive clones were subjected to saturation isotherm analyses using [^{3}H]-DPAT, a selective 5-HT1A agonist (15). The affinity values (K_d) calculated for the mutant receptors were in the nanomolar range (Table 1) in both Ltk- and GH4C1 cell lines and similar to the affinity of the wild-type receptor . Thus, the mutation did not greatly alter agonist affinity which is consistent with earlier reports that mutations in the cytoplasmic portions of G-protein coupled receptors have a minor influence on ligand binding (1-3). In Ltk- cells, two independent transfections yielded clones with lower receptor levels than wild-type. We included a previously-characterized T343A (i3 loop) 5-HT1A receptor mutant with a more similar receptor level for functional comparisons to the T149A receptor in these cells. The level of 5-HT1A mutant receptor expression in GH4C1 clones examined was higher than in GH4ZD10 cells expressing wild-type receptor (2.71 vs 1.10 pmol/mg of protein respectively) (24).

Receptor Coupling to the Adenylyl Cyclase Pathway. The wild-type 5-HT1A receptor

couples negatively to the adenylyl cyclase effector system. We compared the ability of wild-type and mutant 5-HT1A receptors to inhibit adenylyl cyclase by measuring cAMP accumulation in transfected clones in the absence and presence of stimulators of adenylyl cyclase: forskolin in Ltk- cells and VIP in GH4C1 cells. When expressed in Ltk- cells, neither of the T149A mutant clones, the T343A mutant, nor the wild-type receptor significantly inhibited basal (without forskolin) cAMP level. The wild-type 5-HT1A receptor inhibited the forskolin-stimulated cAMP level by greater than 80%, whereas the T343A clone with fewer receptors inhibited by 65%, lower than but not significantly different from wild-type receptor. Both of the T149A mutants in Ltk- cells reduced forskolin-stimulated cAMP accumulation by 30%, significantly less pronounced ($p< 0.001$) as compared to wild-type receptor (Fig. 2A, Table 2). This reduction in T149A receptor efficacy may have been due in part to the lower levels of receptor as compared to wild-type (Table 1). However, other threonine mutants (eg., the T343A mutant) retained receptor efficacy when expressed at similar levels (24), suggesting a partial impairment of T149A 5-HT1A receptors in coupling to inhibition of forskolin-stimulated cAMP accumulation in these cells. When expressed in GH4C1 cells, the T149A mutant inhibited basal cAMP levels to the same extent as the wild-type receptor (38±8% vs. 33±3%). The wild-type receptor markedly inhibited VIP-stimulated cAMP accumulation by 86±2% (Fig.2B and Table 2), whereas the T149A mutant decreased VIP-stimulated cAMP accumulation by 57%, not significantly different from wild-type. ($p>0.05$). These results suggest that in both Ltk- and GH4C1 cells, the T149 residue plays a partial role to mediate inhibition of Gs- and forskolin-induced enhancement of cAMP levels.

Receptor Coupling to Mobilization of Intracellular Calcium . Coupling of wild-type and mutant T149A 5-HT1A receptors to calcium mobilization in Ltk- cells was examined by monitoring $[Ca^{2+}]_i$ in cells loaded with the calcium indicator, fura-2. Upon addition of 100 nM 5-HT, an immediate 2.6-fold peak increase in $[Ca^{2+}]_i$ was induced in cells expressing the wild-type 5-HT1A receptor (Fig.3A). For comparison addition of 100 nM 5-HT to the T343A mutant clone induced a 1.7-fold increase in $[Ca^{2+}]_i$ (Fig.3B). The slight reduction in the calcium response compared to the wild-type clone may reflect the lower receptor levels in the T343A clone. On the other hand, both of the T149A mutant clones (Fig. 3C, 3D) failed to elicit any calcium response following activation with up to 100-fold higher (10 μM) 5-HT, even though inhibition of forskolin-stimulated adenylyl cyclase was observed (Fig. 2). These results indicate that the T149 residue of the i2 loop of the 5-HT1A receptor is a critical site for G-protein coupling to calcium mobilization in Ltk- cells.

Receptor Coupling to Dihydropyridine-sensitive Calcium Channels. The role of T149 in coupling the 5-HT1A receptor to inhibition of voltage-dependent calcium influx was examined in GH4C1 cells by using the dihydropyridine channel agonist (±)-Bay K8644 to activate L-type calcium channels. The addition of 1 μM of (±)-Bay K8644 to GH4C1 cells induced a 2.3-fold increase in $[Ca^{2+}]_i$ and activation of the wild-type 5-HT1A receptor with 1 μM 5-HT inhibited the

change in $[Ca^{2+}]_i$ by 40% (Fig.4A). However, the T149A mutant clone failed to respond to 1 μM of 5-HT following pretreatment with 1 μM of (±)-Bay K8644 (Fig. 4B). These results suggest that the T149 residue in the i2 loop of the receptor is a critical site inhibitory coupling to calcium channels in GH4C1 cells.

Discussion

In previous studies, we and others have identified multiple signals mediated by the coupling of receptors, including the 5-HT1A receptor, to the PTX-sensitive Gi/Go family of G proteins. We have focused on three of these pathways: 1) IP3-mediated calcium mobilization in fibroblasts; 2) inhibition of calcium channel opening in pituitary cells; and 3) inhibition of cAMP levels in both cell types. When expressed in fibroblast cells (12,15,16,17), and endogenously in lymphoid cells (26) the 5-HT1A receptor enhances PI turnover to mobilize intracellular calcium. The identification of PLC-β2 and PLC-β3 as subtypes that respond to βγ subunits derived from G protein heterotrimers (27, 28) suggests a role for release of βγ subunits in 5-HT1A receptor-induced PI turnover and calcium mobilization. The specific G protein that mediates this response is unclear although ablation of Gi2 (but not Gi1, Gi3, or Go) by over-expression of full-length antisense α subunit RNA selectively inhibited 5-HT-induced calcium mobilization in L cells (Liu, Y.F. and Albert, P.R., unpublished observations). However it appears that multiple subtypes are required since the response is not reconstituted by any single subtype of G protein (29). This is consistent with the higher levels of βγ relative to α_q subunits required to activate PLC *in vitro* (27), and the higher sensitivity of the PLC pathway to the level of receptor expression in various cell types (12). An important advantage of the Ltk- cells is that unlike other cells (eg. Hela, CHO (16), the 5-HT1A receptor induces both calcium and cAMP pathways with equal potencies (11). Thus, although the calcium mobilization pathway is often more sensitive to receptor level that the cAMP pathway, this difference is minimal in the L cells.

Inhibition of cAMP accumulation appears to be mediated by the Gi class (especially Gi2) but not by Go, in GH cells (as determined by antisense experiments (30)) and in L cells (reconstitution studies (29)). In contrast to 5-HT1A receptor-mediated calcium mobilization, inhibition of cAMP accumulation in L cells was reconstituted by a single G protein, Gi2. Thus, inhibition of cAMP accumulation appears to be mediated primarily by α_i subunits, especially the $\alpha_i 2$ subunit. Receptor-mediated inhibition of calcium channel opening in pituitary cells is mediated by Go although the specific combination of subunits is receptor dependent (30-33). Specific ablation of α_o protein using antisense strategies blocked inhibitory coupling to calcium channels, but did not alter receptor coupling to inhibition of cAMP (30). Thus, Gi/Go-coupled receptors like the 5-HT1A receptor mediate a variety of cell-specific responses via discrete G protein-effector systems: 1) in fibroblasts, the βγ subunits of multiple Gi/Go proteins appear necessary to stimulate

the isoforms of PLC that are present; 2) in pituitary cells, Go proteins (possibly via release of $\beta\gamma$ subunits (34,35)) mediate the closing of endogenously expressed calcium channels; and in both cell types, α_i proteins mediate the inhibition of basal and Gs-stimulated adenylyl cyclase activity.

The experiments presented here provide evidence that a single threonine residue in the i2 loop of the 5-HT1A receptor plays a crucial role in directing the signaling of the receptor. In particular, T149 in the i2 loop of the 5-HT1A receptor is a crucial amino acid for G-protein coupling since the mutation to alanine entirely uncouples the receptor from calcium mobilization in fibroblasts and from inhibition of calcium influx in pituitary cells, and partially attenuates the adenylyl cyclase pathway in both cell types. This is clearly different from point mutations of threonine residues in the i3 loop (eg. T343), where in L cells both calcium mobilization and cAMP inhibition pathways were maintained, although at slightly lower efficacy than wild-type receptor due to lower receptor levels (24). In contrast, the T149A (i2 loop) mutant lacked completely a calcium response despite the presence of a consistent cAMP response. The weaker cAMP response (compared to wild-type or T343A) could be due in part to lower receptor number, but the complete lack of calcium responses suggests that the receptor preferentially couples to these latter pathways via interactions involving T149. Although calcium mobilization induced by 5-HT1A receptor activation requires higher levels of agonist and receptor expression in Hela or CHO than does inhibition of cAMP (16), we have previously shown that in Ltk- cells both pathways are activated with equal potency (11). Thus, the absence of calcium mobilization in the T149A mutant reflects a selective impairment in receptor coupling.

The selective uncoupling of 5-HT1A receptor-mediated calcium mobilization but not inhibition of cAMP accumulation by acute (2 min) activation of PK C suggested that distinct coupling mechanisms may induce these signaling pathways (11). Previously we addressed whether the action of PK C to selectively uncouple the receptor from calcium mobilization required specific sites on the 5-HT1A receptor, and found that mutation of three phosphorylation sites on the i3 loop of the receptor prevented the action of PK C. Mutation of two sites had a lesser effect, whereas mutation of any single PK C site did not block PK C-induced uncoupling (24). Thus, the cumulative elimination of i3 phosphorylation sites progressively reduced PK C-induced inhibition of calcium mobilization, suggesting the involvement of these residues in mediating the 5-HT-induced PI response. These results are consistent with a requirement for multiple Gi/Go proteins in calcium mobilization, since impaired interaction of the receptor with any G protein would attenuate coupling to this pathway. However, the insensitivity of inhibition of cAMP to the same phosphorylation events indicates that phosphorylation of these i3 residues is not sufficient to uncouple all responses initiated by the receptor. The present results indicate that mutation of T149 in the i2 loop reproduces the selective inactivation of calcium mobilization observed following PK C treatment. Thus phosphorylation of this i2 loop threonine, in addition to the i3 sites, may mediate the action of PK C to block receptor-induced selective calcium mobilization.

Current scientific evidence indicates that the i3 loop is not the sole determinant of G-protein

selectivity and that other cytoplasmic domains of the receptor must also contribute. For example, using a chimeric substitution approach, residues of the i2 loop domain that lie adjacent to the T149 equivalent residue are implicated in coupling of m3 receptors to αq to stimulate PLC (36). Furthermore, the i2 loop of the gonadotropin-releasing hormone receptor was critical for coupling to signal transduction: the L147A and L147D mutants showed a significant impairment of GnRH-stimulated IP production (37). The present report complements above work by probing the role of the conserved threonine residue which was not changed in the above chimeric substitutions. We have shown that the conserved T149 residue in the second intracellular loop of the 5-HT1A receptor directs G-protein coupling in two different cell lines. In Ltk- fibroblast cells, the T149A mutant failed to mediate a calcium response and partially inhibited the cAMP accumulation as compared to wild-type (30±12 or 31±3% vs. 83±10%). The lack of coupling of T149A to calcium mobilization could reflect a loss of coupling to any of the G proteins, since multiple G proteins appear necessary to mediate this response. However, in pituitary GH4C1 cells the T149A mutant failed to inhibit calcium channel activation but did inhibit cAMP accumulation nearly as strongly as the wild-type receptor. These data suggest that the i2 loop is an important contact point for Go, which mediates calcium channel inhibition, and for activation of PLC-β2/β3 (via βγ subunits derived from multiple G proteins). However, α_i proteins (that mediate inhibition of adenylyl cyclase) do not rely as heavily on the same i2 site.

The T149 residue in the i2 loop is conserved among several Gs-, Gi- and Gq-coupled receptors (Fig. 1) and is a critical site for coupling of the 5-HT1A receptor to PI hydrolysis in Ltk- cells and for the closing of L-type voltage-gated calcium channels in GH4C1 cells, but not for inhibition of cAMP levels. Consistent with our results, Van Koppen et al., have recently shown that the equivalent Thr145 in the i2 loop of the m4 muscarinic acetylcholine receptor could be mutated to Ala without altering inhibition of adenylyl cyclase (38). In agreement with their studies, we find that the i2 loop plays a lesser role in coupling to α_i, and a more important role in coupling to PLC-linked pathways. The putative role of this i2 domain in coupling to Gi/Go proteins is supported by evidence using synthetic peptides derived from the i2 loop of Gi/Go-coupled 5-HT1A and muscarinic m2 receptors, that potently inhibited adenylyl cyclase *in vitro* (22,23). It is thus likely that multiple interactions of Gi proteins at both i2 and i3 loops contribute to coupling of the 5-HT1A receptor to inhibition of cAMP accumulation.

In this regard, Liu et al., have identified four amino acids (VTIL) in the C-terminal segment of the i3 loop of the m2 muscarinic receptor that are essential for receptor interactions with the C-terminal pentapeptide domain of Gi/o proteins (39). The threonine of the VTIL motif is conserved among several Gi/o-coupled receptors and may be a specific contact site for coupling to Gi/Go proteins, although single point mutations were not done to determine the specific amino acid coupling site. The C-terminal i3 threonine residue of the VTIL motif in the m2-muscarinic receptor lies in a predicted α-helical domain that protrudes into the cytoplasm to permit interaction with the C-terminal pentapeptide domain of α_i and α_o. Similarly, the T149 residue in the i2 loop of the 5-

HT1A receptor could be a specific contact site for determining G-protein coupling specificity in Ltk- and GH4C1 cells. The T149 residue is located at the center of a particularly hydrophilic portion of the i2 loop domain that has among the highest surface probability and antigenic indices of the receptor protein (Fig. 5). It is predicted to have an amphipathic α-helical structure that protrudes into the cytoplasmic milieu (40). Thus, T149 is well-situated to allow hydrogen-bonding interactions with intracellular proteins, G proteins, and other intracellular receptor domains. The mutation of this residue to alanine did not greatly alter the predicted secondary structural properties (flexibility, surface probability, antigenicity) of this domain (Fig. 5), and presumably acts by eliminating hydrogen bonding interactions with the threonine hydroxyl side-chain. The phosphorylation of this residue (eg. by PK C) would similarly disrupt hydrogen bonding interactions by placing a negatively-charged ionic phosphate moiety at this site.

In conclusion, the T149 defines a novel conserved hydrophilic core residue of the i2 loop amphipathic α-helical domain of the 5-HT1A receptor that directs specific interactions of the receptor which result in cell-specific coupling to calcium mobilization or inhibition of calcium entry, with a partial role in coupling to inhibition of cAMP levels. Our results further indicate that distinct receptor domains underlie coupling to α_o, α_i, and $\beta\gamma$ subunits within the Gi/Go family.

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Table 1: Binding characteristics of Ltk-/GH4C1 cells expressing transfected wild-type and mutant 5-HT1A receptors.

Membranes from clones stably transfected with wild-type or mutant 5-HT1A receptors were prepared and subjected to saturation binding analysis using [3H]-DPAT as a selective agonist (see Methods). The Kd and Bmax values were determined by nonlinear regression analysis of saturation binding data derived with the RECEPTOR FIT (Lundon program). Values shown are the mean ± S.E.M. of data from at least three independent experiments except *(n = 2). a: Data from ref. 24.

5-HT1A receptor	B_{max} (pmol/mg)	K_d (nM)
Ltk-: W.T.	**1.64 ± 0.38**	**2.74 ± 0.38**
Ltk-: T343A[a]	**0.62 ± 0.10**	**7.80 ± 2.50**
Ltk-: $T149A_1$	**0.36 ± 0.06**	**5.40 ± 0.30**
Ltk-: $T149A_2$*	**0.34 ± 0.09**	**4.30 ± 0.06**
GH_4: W.T.	**1.10 ± 0.45**	**5.62 ± 1.50**
GH_4: T149A	**2.71 ± 0.90**	**3.57 ± 1.10**

Table 2: Inhibition of cAMP accumulation in Ltk-/GH4C1 cells expressing wild-type and mutant 5-HT1A receptors.

The percent inhibition of forskolin- (in Ltk- cells) or VIP-stimulated (in GH4C1 cells) cAMP accumulation by 10 μM of 5-HT is tabulated as the mean ± S.E.M. of at least three independent experiments. Calculation for inhibition of forskolin- and VIP-stimulated cAMP accumulation: Inhibitory Activity = [(A-AS)/A] x 100, where the level of cAMP after (A) activator of adenylyl cyclase, forskolin or VIP, or activator and serotonin (AS) was measured. * pvalue< 0.001: † pvalue> 0.059

5-HT1A receptor	Adenylyl cyclase activity (% of forskolin inhibition)
Ltk-: W.T.	83 ± 10
Ltk-: T343A	65 ± 6
Ltk-: $T149A_1$	30 ± 12*
Ltk-: $T149A_2$	31 ± 3*
GH_4: W.T.	86 ± 2
GH_4: T149A	57 ± 14 †

FIGURE LEGENDS

Figure 1: A conserved threonine consensus sequence in the i2 loop of the 5-HT1A receptor. **A)** 5-HT1A receptor i2 loop. The proposed structure of the rat 5-HT1A receptor used in this study, which consists of seven hydrophobic transmembrane domains forming three intracellular loops and a short cytoplasmic carboxy-terminal tail. The location of the putative PK C consensus site in the second loop is depicted with a circle. **B)** A conserved threonine in G-protein coupled receptors. Amino acid sequence alignment of an i2 loop domain of selected receptors that have a positionally-conserved threonine or serine (boxed) in the i2 loop flanked by basic amino acid residues (outlined). Species abbreviations: r=rat, m=mouse, h=human, hm=hamster, b=bovine.

Figure 2: Inhibition of cAMP accumulation by wild-type and T149A 5-HT1A receptors in Ltk-/ GH4C1 cells. cAMP accumulation was measured in media harvested from Ltk- or GH4C1 cells transfected with wild-type (W.T.), T343A, or T149A 5-HT1A receptor cDNA's following addition of experimental compounds. Two T149A Ltk- clones from separate transfections were examined (T149A-1 and T149A-2). The data are expressed as mean ± S.E.M. of triplicate determinations from a single experiment. These results were repeated in 3-4 independent experiments. Concentrations used: **A)** Ltk- clones: 10 μM of 5-HT (serotonin) and forskolin; F/S denote co-addition of 5-HT and forskolin. **B)** GH4C1 clones: VIP, 500 nM; 5-HT, 10 μM; V/S, co-addition of VIP and 5-HT.

Figure 3: Actions of wild-type and mutated 5-HT1A receptors on $[Ca^{2+}]_i$ in Ltk- cells. **A)** Wild-type (W.T.) 5-HT1A receptor in Ltk- cells: addition of 100 nM 5-HT (indicated by arrow) induced a 2.6-fold increase in intracellular calcium when added at 60 sec. **B)** T343A mutant: 100 nM 5-HT (arrow) induced a 1.7-fold increase in $[Ca^{2+}]_i$. **C)** $T149A_1$ mutant: 10 μM 5-HT at 60 sec (arrow) failed to induce a calcium response. This result was repeated four times with similar results. **D)** $T149A_2$ mutant: 10 μM 5-HT was added (arrow) and failed to induce a change in $[Ca^{2+}]_i$. This result was repeated three times.

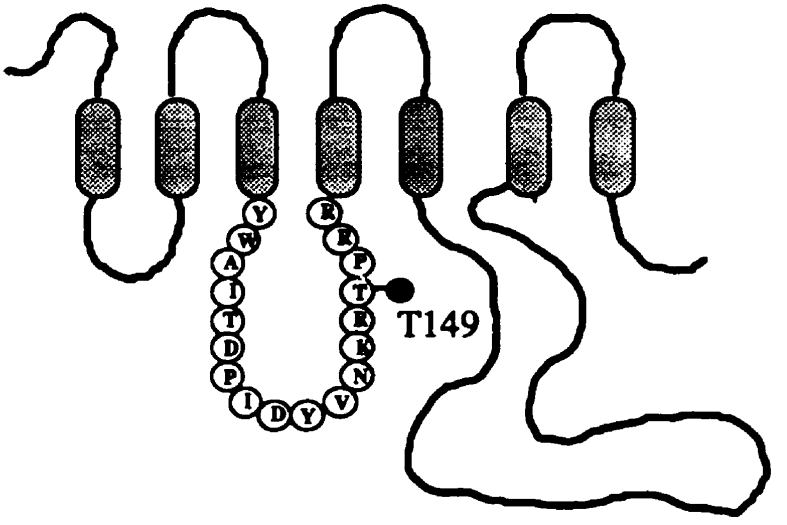
Figure 4: Actions of wild-type and T149A mutant receptors on $[Ca^{2+}]_i$ in GH4C1 cells. **A)** Wild-type (W.T.) 5-HT1A receptor in GH4C1 cells: at 60 sec, addition of 1 μM (±)-Bay K8644 induced a 2.3-fold increase in calcium influx and the addition of 1 μM 5-HT (arrow) caused a 40 % decrease in $[Ca^{2+}]_i$. **B)** T149A 5-HT1A receptor: 1 μM (±)-Bay K8644 induced a 2-fold enhancement of $[Ca^{2+}]_i$; subsequent addition of 1 μM 5-HT (arrow) failed to decrease

$[Ca^{2+}]_i$ in these cells. All curves were from a single experiment which was repeated at least three times with similar results.

Figure 5: Structural characteristics of the i2 loop of the 5-HT 1A receptor. The coding sequence of the rat 5-HT1A receptor was submitted to protein analysis using the PROTEAN program (DNASTAR Inc, Madison WI). Indices of predicted antigenicity (Jameson-Wolf plot), surface probability (Emini plot), and flexibility (Karplus-Schultz plot) are shown for the threonine-containing domain of the i2 loop. The values plotted are among the highest found in the entire receptor. **A)** Wild-type 5-HT1A receptor i2 loop. **B)** T149A 5-HT1A receptor i2 loop. Small decreases in the above parameters were restricted to the site of mutation, while the overall predicted structures were retained.

Figure 1: A conserved threonine residue in the second intracellular loop of the 5-HT1A receptor.

A)



B)

$G_{\alpha i}$	r5-HT1A	Y	V	N	K	R	T	P	R	R
	m5-HT1B	Y	S	A	K	R	T	P	K	R
	h5-HT1D	Y	S	K	R	R	T	A	G	H
	rM2	Y	P	V	K	R	T	T	K	M
	hM4	Y	P	A	R	R	T	T	K	M
	hD2	Y	N	T	R	Y	S	S	K	R
	hα-2A	Y	N	L	K	R	T	P	R	R
	hα-2B	Y	N	L	K	R	T	P	R	R
	rCannabinoid	Y	K	R	I	V	T	R	P	K
$G_{\alpha s}$	hβ1	Y	Q	S	L	L	T	R	A	R
	rβ2	Y	Q	S	L	L	T	K	N	K
	hD1	Y	E	R	M	M	T	P	K	A
	rD5	Y	K	R	K	M	T	Q	M	
$G_{\alpha q}$	hM1	Y	R	A	K	R	T	P	R	R
	rM3	Y	R	A	K	R	T	T	K	R
	hM5	Y	R	A	K	R	T	P	K	R
	hmα-1	Y	P	T	L	V	T	R	R	K
	mTRH A		Q	F	L	C	T	F	S	R
	bAngiotensin II	S	R	L	R	R	T	M	L	

FIG. 2: Inhibition of cAMP Accumulation by wild-type and mutant 5-HT1A receptors.

B) GH4C1 cells

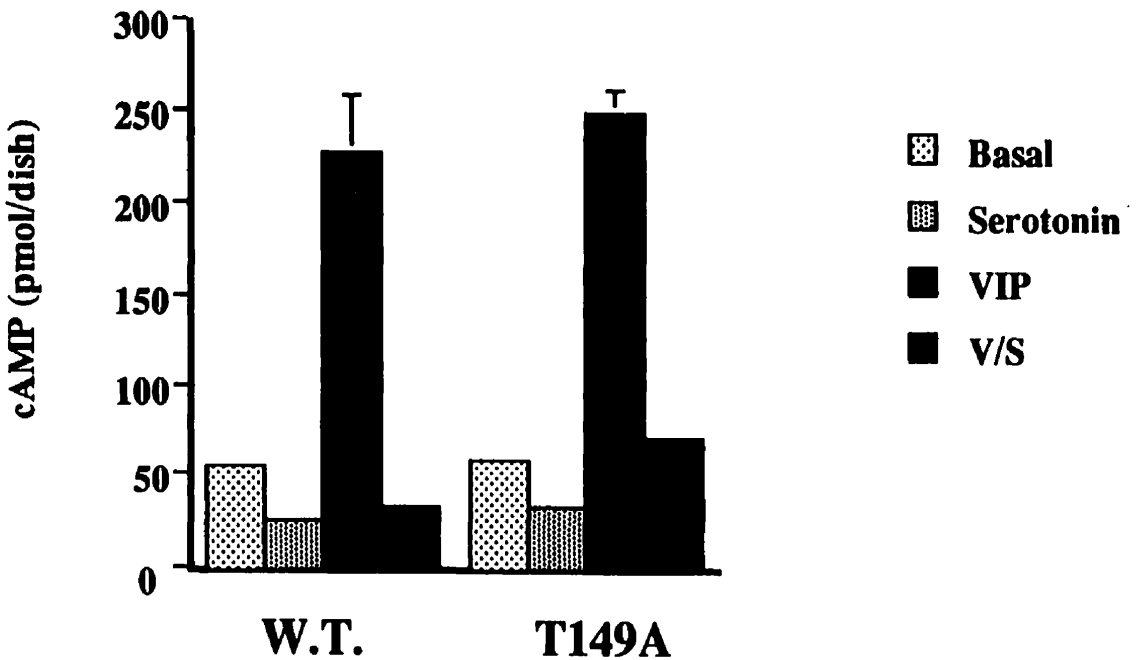


Figure 3: Effects of wild-type and mutated 5-HT 1A receptors on mobilization of $[Ca2+]_i$ in Ltk- cells.

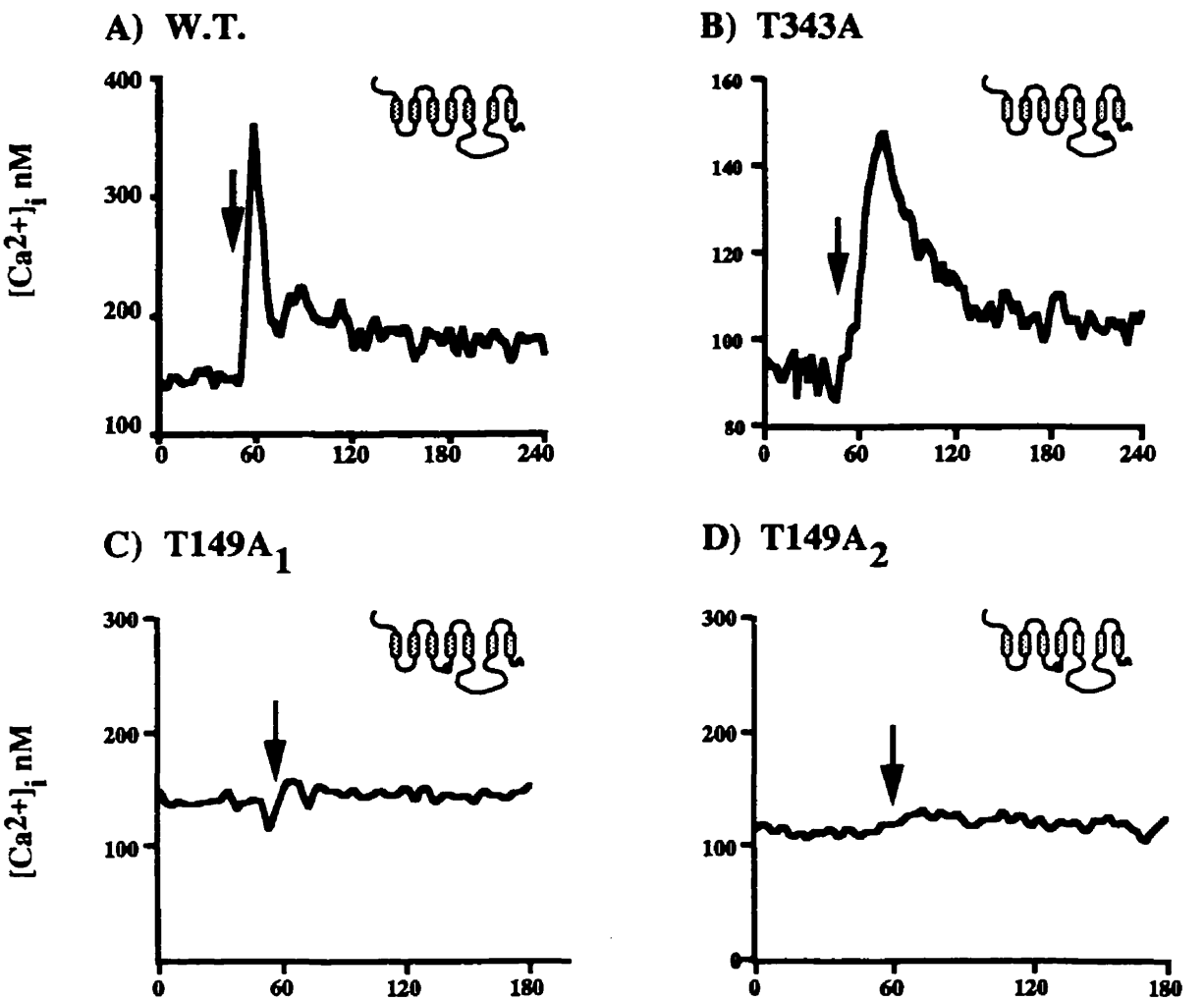


Figure 4: Actions of wild-type and T149A 5-HT 1A receptors on [Ca2+]i in GH4C1 cells.

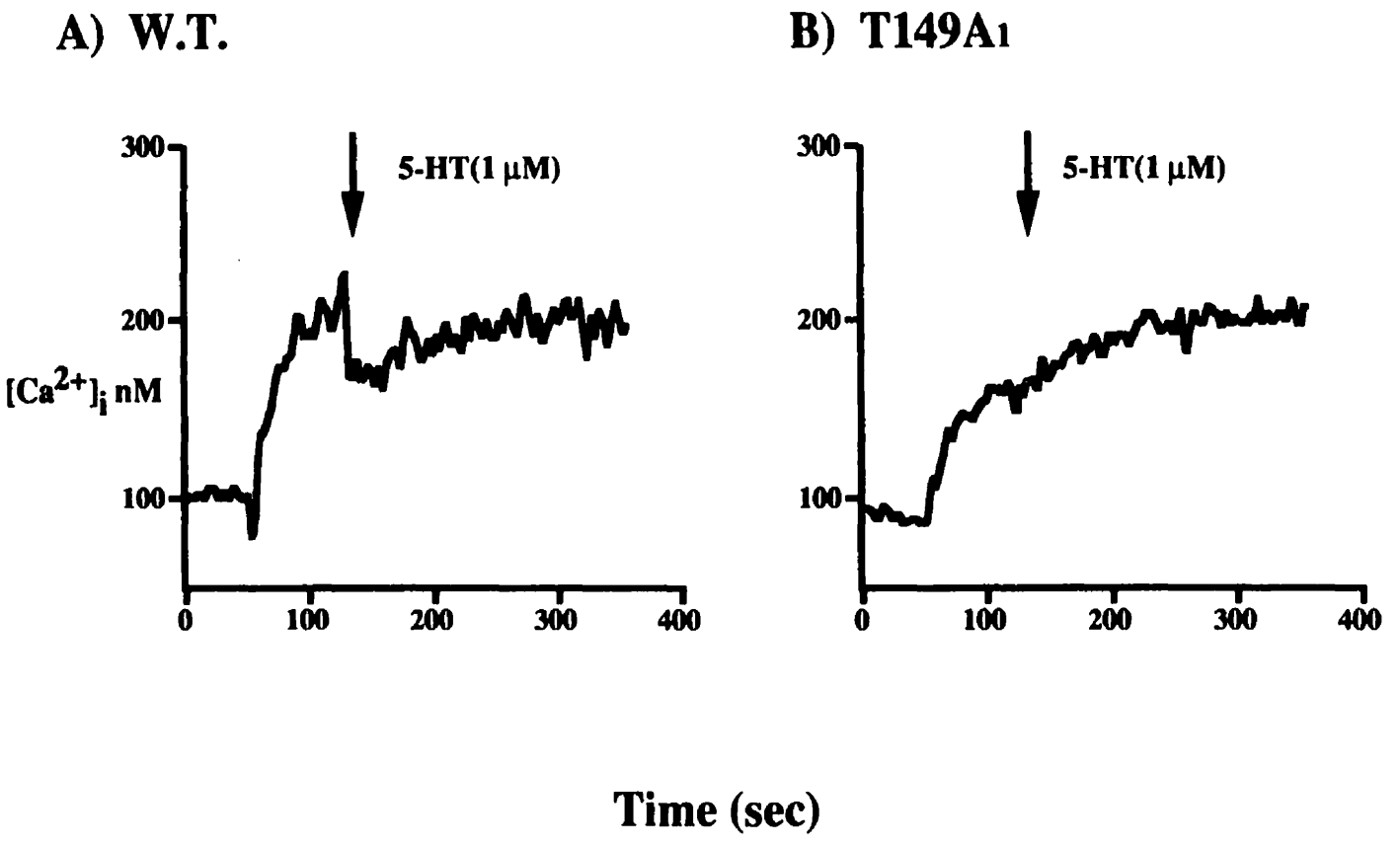
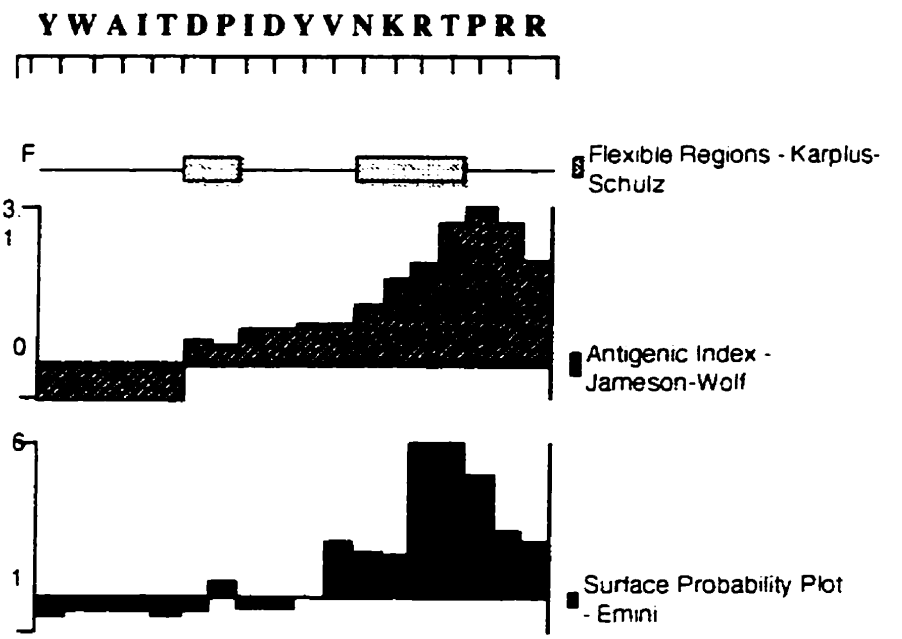
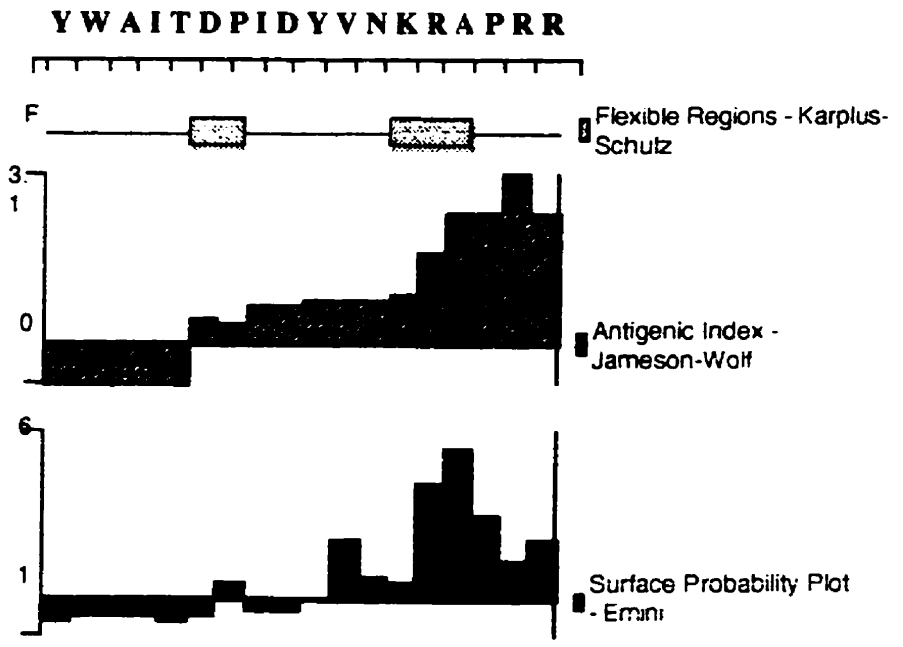


Figure 5: Structural characteristics of the second loop.

A) W.T.



B) T149A



5. Chapter 3. Cloning and characterization of the opossum GRK2: Desensitization of Gi-linked inhibition of cAMP accumulation by α2C-adrenergic but not 5-HT1B receptors in intact OK cells. P.M.C. Lembo and P.R. Albert (submitted for publication).

Classically, the regulation of G-protein coupled receptors by GRKs (G-protein-receptor kinases) has been extensively documented using mammalian expression systems (189-191). This raises the issue of physiological relevance in the modulation of receptors by these kinases. We decided to use the opossum kidney cell line (OK), an epithelial kidney cell line which endogenously expresses several Gi-protein coupled receptors including the 5-HT1B receptor and the adrenergic α-2C receptor which have been shown to undergo agonist-induced desensitization. This receptor-mediated event has been thoroughly studied and shown to involve the phosphorylation of the receptors by GRKs. Hence, this cell line potentially expresses GRKs critical for mediating this process. The objectives of this study were to determine if endogenous opossum GRKs were expressed in this cell line and if they played a role in homologously desensitizing the endogenously expressed G-protein coupled receptors? We investigated this proposal and demonstrated that the OK cell line does indeed express several GRKs. Furthermore, the OK-GRK2 enhanced the desensitization of α-2C receptor-mediated inhibition of cAMP accumulation and the kinase inactive mutant, OK-GRK2-K220R prevented this process. However, the OK-GRK2 and the kinase inactive mutant were shown to be functional in phosphorylating the 5-HT1B receptor *in vitro* but had no significant effect on the 5-HT1B receptor-mediated desensitization. Hence, the OK-GRK2 displayed Gi-linked receptor specificity. These results suggest that phosphorylation by GRK2, although feasible does not play a major role in the desensitization of the 5-HT1B receptor.

Cloning and characterization of opossum GRK2.

Desensitization of Gi-linked inhibition of cAMP accumulation by α2C-adrenergic but not 5-HT1B receptors in intact OK cells.

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Abbreviations:

GRK, G protein-coupled receptor kinase, PK C, Protein kinase C; PK A, Protein kinase A; 5-HT, serotonin; IBMX, 3-isobutyl-1-methylxanthine; [Ca++]i, intracellular free calcium concentration; PI, phosphatidylinositol; NBT, nitro blue tetrazolium chloride; BCIP, 5-bromo-4-chloro-3-indoyl phosphate;

Abstract

The GRK family of protein kinases has been isolated and primarily characterized using biochemical approaches including *in vitro* adenylyl cyclase assays in membrane preparations, and over-expression in heterologous cell systems. We have examined the function of GRK in a more physiological whole cell system, the OK opossum kidney cell line, to characterize the specificity of endogenously expressed GRK's for endogenous receptors. An OK cell cDNA library was screened using a cDNA encoding the opossum GRK-like catalytic domain. A 3.054 kb clone encoding a 688-amino acid protein was isolated that shared 92% homology with rat GRK2 (β-ARK1) at the amino acid level and was thus named OK-GRK2. Northern blot analysis revealed the presence of two mRNA transcripts of 5.0 and 3.0 kb in OK cells. The OK-GRK2 was functional since the *in vitro* transcription/translation product of the cloned cDNA phosphorylated the 70 kDa His-tagged 5-HT1B receptor transiently expressed in HEK 293 cells, whereas the kinase-inactive mutant OK-GRK2-K220R did not. Pre-incubation (45 min.) with agonist induced homologous desensitization of endogenous 5-HT1B and α2C-adrenergic receptor-mediated inhibition of cAMP accumulation measured in intact wild-type (non-transfected) OK cells. In transfected cells over-expressing OK-GRK2, agonist-induced desensitization of the α2C-adrenergic receptor, but not the 5-HT1B receptor, was enhanced. Conversely, in cells over-expressing the OK-GRK2-K220R kinase-inactive mutant, α2C-adrenergic receptor desensitization was selectively abolished. Similarly, depletion of GRK-2 protein using stable transfection of full-length antisense GRK2 cDNA, blocked desensitization of α2C-adrenergic receptors but not 5-HT1B receptors. These results imply that phosphorylation mediated by GRKs *in vitro* does not necessarily indicate functional receptor desensitization in whole cells.

Introduction

Many G protein-coupled receptor systems undergo a process of functional receptor desensitization in which the receptor's responsiveness is attenuated during prolonged exposure to stimuli such as hormones and neurotransmitters. Several mechanisms play pivotal roles in the functional desensitization of G-protein coupled receptors particularly receptor phosphorylation resulting in receptor-G protein uncoupling (1, 2). Other mechanisms that regulate receptor function include sequestration, which involves trafficking of receptors intracellularly via endosomal compartments and ligand dissociation, and down-regulation which entails a loss of receptor number due to enhanced receptor degradation or a decrease in receptor synthesis (3). Acute receptor desensitization appears to involve receptor-G protein uncoupling which occurs rapidly and results in reduced efficacy of receptors to activate target G proteins (1-3). Uncoupling has been postulated to be mediated primarily by phosphorylation of the receptor, which can preferentially uncouple the receptor from some, but not necessarily all signalling pathways (2, 4).

Acute receptor desensitization has historically been viewed as two separate mechanisms; heterologous and homologous desensitization. Heterologous desensitization is a process whereby activation of one type of receptor leads to the desensitization of non-stimulated receptors, whereas homologous desensitization refers to the loss of responsiveness of the stimulated receptor only. These rapid forms of desensitization appear to involve primarily the phosphorylation of the receptor on serine and threonine residues located in the intracellular cytoplasmic loops (1-3). There are at least two distinct classes of kinases involved in this phosphorylation process: the second-messenger kinases (eg., PKC, PKA) and second-messenger independent receptor kinases termed G-protein receptor kinases (GRKs) (2).

GRKs are a family of serine/threonine kinases that phosphorylate G-protein coupled receptors in the ligand-activated state (5,6). They belong to a family of kinases comprising six members (GRK1-GRK6) whose activities are differentially regulated by G-protein $\beta\gamma$ subunits, phospholipids, or post-translational modifications. Each kinase sequence has a centrally located catalytic domain flanked by an amino terminus that has conserved features and a carboxyl terminus that is highly variable. The C-terminal domain directs localization of the kinases to the membrane or receptor, by isoprenylation (via a *CAAX* box in GRK1), association with membrane-bound

geranylgeranylated G-βγ dimers (GRK2, GRK3), or by direct interactions with membrane phospholipids (GRK4, 5, 6) (5-9). For all of these kinases, translocation to the membrane and kinase activity appear to be essential for GRK function, since mutations that inactivate either function attenuate or block GRK-induced receptor phosphorylation and uncoupling (10, 11). The hallmark of these kinases is their specificity for activated (eg., agonist-bound) receptors. Once phosphorylated, target receptors have an increased affinity for β-arrestins, and the binding of β-arrestin to the GRK-phosphorylated receptor is thought to prevent receptor coupling to G-proteins (5-9). Thus, unlike receptor phosphorylation by PKC or PKA that directly uncouples the receptor, GRK-induced uncoupling requires both GRK and β-arrestin molecules that recognize the appropriate receptor (9).

The regulation of G-protein coupled receptors by GRKs (G-protein-receptor kinases) has been extensively documented in heterogenous expression systems using biochemical over-expression of kinase and receptor cDNAs (5-9). Phosphorylation of receptors by GRKs has been shown to occur and regulate many G-protein-coupled receptor systems such as β2- and α2A-adrenergic receptors, m2 muscarinic acetylcholine receptors, rat olfactory receptor and rhodopsin photoreceptor systems (9, 12-15). While some substrate specificity is observed in these studies, the question remains whether the endogenous levels of GRK/β-arrestin are sufficient to mediate agonist-induced homologous desensitization in non-transfected cells. Recently, GRK2 (β-ARK1) was shown to selectively regulate and desensitize thrombin-mediated calcium mobilization in *Xenopus* oocytes (16) whereas GRK3 (β-ARK2) was demonstrated to desensitize the inhibition of voltage-dependent calcium channels mediated by the α2-adrenergic receptor in isolated intact chick sensory neurons (17). In addition, Shih and Malbon elegantly demonstrated that depending on the heterologous expression system used, either the second messenger-dependent kinases (PKC or PKA) or the GRKs played a predominant role in promoting agonist-induced uncoupling of the β2-adrenergic receptor (18). This raises the issue of physiological relevance in the modulation of receptors by these kinases. Hence, we decided to examine the influence of endogenous GRK on the homologous desensitization of 5-HT1B and α2C adrenergic receptors endogenously in the opossum kidney cell line (OK) (19-22).

OK cells are an epithelial kidney cell line derived from the North American opossum,

Didelphys virginiana (23). These cells endogenously express several G-protein coupled receptors including 5-HT1B, α2C adrenergic, dopamine-D1, and PTH receptors (19, 21, 24, 25). These endogenously-expressed G protein-coupled receptors undergo agonist-promoted desensitization with respect to both inhibitory and stimulatory actions (20, 22, 26, 27). For example, both 5-HT1B and α2C-adrenergic receptors mediate inhibition of adenylyl cyclase and undergo acute homologous desensitization of this response following exposure to agonist (20, 22). However, the above studies were biochemical in nature, assessing the extent of desensitization by a prelabeling technique. We have addressed whether the desensitization observed can be detected in intact cell preparations by specifically measuring cAMP generation in whole cells.

The objectives of the present study were to determine whether: A) endogenous opossum GRKs were expressed in this cell line and B) whether they play a role in the homologous desensitization process of the endogenously expressed G-protein coupled receptors. We have identified in OK cells a GRK-2 homologue, OK-GRK2, and shown that the kinase phosphorylates the 5-HT1B receptor *in vitro*. In addition, the OK-GRK2 displayed Gi-receptor specificity in enhancing the attenuation response of the α2C receptor-mediated inhibition of cAMP accumulation and the kinase-inactive mutant OK-GRK2-K220R abrogated this process. The action of the OK-GRK2 was selective for the α2C adrenergic receptor responsiveness and had no significant effect on the 5-HT1B receptor-mediated event..

Experimental Procedures

Materials- Restriction endonucleases and other molecular biology reagents were purchased from Boehringer Mannheim except as indicated. Sequenase was from Pharmacia, [α-^{32}P]ATP and [γ-^{32}P]ATP were purchased from Amersham. Serotonin, IBMX, and forskolin were from Sigma. UK 14304 was purchased from RBI chemicals. Tissue culture media and sera were from Gibco-BRL. OK cells were purchased from ATCC. The human 5-HT1B receptor cDNA clone (28) was a generous gift of Dr. Brian K. O'Dowd, University of Toronto.

Cell Culture- OK cells were grown to 80-90 % confluency with D-MEM (Hi-glucose) medium and supplemented with 8% fetal bovine serum, at 37°C in a humidified atmosphere, with 5% carbon dioxide. Media were changed 12-24 h prior to experimentation.

PCR- Random hexamers were used as primers to reverse transcribe total RNA (1 µg) isolated from the OK cell line using Superscript reverse-transcriptase (Gibco-BRL). The cDNAs were amplified by PCR [1 min 95 °C, 2 min 54 °C, 2 min 72 °C, 40 cycles: (Hot Tub), Amersham] using degenerate oligonucleotides, sense (5'-GGCAAGATGTA(T/C)GC(A/T/C/G)ATGAA-3') and antisense (3'-AC(C/T)TCGGG(A/C/G/T)GCCATGTACCC-5') designed to highly conserved regions of the catalytic domain of GRK2 (drosophila, rat and bovine (29)). The PCR reactions contained 1 µg of reverse-transcribed OK cell mRNA, 1.5 mM $MgCl_2$, and 5 x Hot Tub buffer. Two distinct cDNAs of 380 bp were isolated (10-1 and A-1) and subcloned into the *Eco* R1 site of pBluescript (KS+, Stratagene). DNA sequence analysis using the Sanger method with a T7 polymerase-based DNA sequencing kit (Pharmacia) revealed that they shared 81% and 66% identity at the nucleotide level to the rat GRK2, respectively.

Construction of OK cDNA library- Poly-A+ RNA was isolated from the OK cell line using the QuickPrep Micro mRNA Purification kit (Pharmacia). For cDNA synthesis, 5 µg of Poly-A+ RNA was reverse transcribed using Superscript reverse-transcriptase and the complete synthesis of double-stranded DNA was performed using the oligo-dT Riboclone cDNA Synthesis kit (Promega). The blunt-ended cDNAs were fractionated and *Eco* R1 adapters ligated followed by ligation into *Eco* R1 pre-digested Lambda ZAP II arms (Stratagene). Gigapack II Gold

(Strategene) extracts were used to package recombinant lambda phage extracts.

cDNA Cloning- The oligo-dT-primed OK cell cDNA library was amplified and 10^6 plaques were plated, transferred to Hybond N+ membranes and screened with the 350 bp A-1 and 10-1 PCR fragments labeled using a randomly-primed labeling kit (Boeringer-Mannheim) with the following hybridizations conditions (Solution A): 50% formamide, 50% dextran-sulphate, 1% SDS, 5 x SSC, 5 x Denhardt's, 10 mM Tris and 100 µg/ml sonicated salmon sperm DNA at 42°C overnight. The filters were washed with 2 x SSC/1% SDS at room temperature followed by a high stringency wash with 0.1% SDS/0.2 x SSC at 65 °C. The ten clones identified by this method were isolated by repeated plating and screening with the labeled PCR fragments. The isolated clones were rescued with helper phage to yield the cDNAs as inserts in pBluescript SK vector. All ten clones were restriction-mapped and were found to be identical. A clone was subjected to sequencing using T7 polymerase as an enzyme and oligonucleotides to regions of the sequence, and also reverse and universal primers were used. The isolated clone was 3.054 kb clone and highly homologous to the rat GRK2, and was therefore named OK-GRK2.

Northern Blot Analysis- Poly-A+ RNA was isolated from the OK cell line using the QuickPrep Micro mRNA Purification kit (Pharmacia). Seven micrograms of poly-A^+ OK RNA and three micrograms of 0.24-9.5 kb RNA ladder (Gibco-BRL) were fractionated on a 1.3 % agarose-formaldehyde gel and transferred to Hybond-N membrane (Amersham). The 2.1 kb KpnI fragment of OK-GRK2 was labeled and hybridized to the membrane in Solution A overnight with 100 µg/ml sheared, sonicated salmon sperm at 42°C. The filter was washed with 0.1% SDS /0.2 x SSC at 65 °C and exposed for 4 days at -70°C to Kodak XAR film with an intensifier.

Construction of K220R Mutant and Stable Transfections of sense, antisense, and K220R mutant in OK cells- The *Eco* R1 fragment of the OK-GRK2 gene was subcloned into p-Select to use as a template for site-directed mutagenesis (Altered-sites mutagenesis, Promega). The oligonucleotide 5'-ATCAAGACACCTCATGGCATA-3' was used to incorporate the point mutation. The mutation was confirmed by DNA sequencing using T7 polymerase (Pharmacia). The K220R mutant, antisense, and wild-type OK–GRK2 cDNAs were subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen) and stably transfected into OK cells using the calcium phosphate co-

precipitation method. Neomycin-resistant clones were selected and grown in D-MEM medium supplemented with 10% fetal calf serum and 1.5 mg/ml Geneticin. Positive clones were identified by Northern and Southern blot analyses.

Western Analyses- Western blot analyses were performed using cytosolic fractions from the RNA-positive transfected clones. Cytosolic fractions were isolated as follows; the various transfected OK cells were trypsinized and resuspended with 150 µl of lysis extraction buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.8), 0.01 % sodium azide, 10 mM NaF, 30 mM sodium pyrophosphate) followed by addition of 150 µl of lysis extraction buffer containing 4% NP-40, 5 mM Na_3VO_4, 10 ug/ml leupeptin, 10 mM benzamidine, 2 µg/ml soybean trypsin inhibitor, 1mg/ml aprotinin, 1 mM PMSF, 10 mM TPCK, and 20 mM iodoacetamide. This was incubated on ice for 30 minutes and the supernatant was recovered after spinning at 4 °C 15 minutes at 12000 X*g* . Using BSA as a standard (BIO-RAD) for protein concentration, 50 µg of total protein were loaded in SDS-PAGE sample buffer, resolved by a 10% SDS-PAGE and electroblotted onto Hybond nitrocellulose membranes (Western blotting kit ECL, Amersham) for 2 hours at 250 mA constant current. Blots were pre-blocked in TBS-Blotto (150 mM NaCl, 20 mM Tris-HCl pH 8.0, 5% non-fat dried milk) at room temperature. The recombinant bovine β–ARK1 polyclonal antibody (1:1000) was hybridized to the membranes in TBS-Blotto for one hour at room temperature. Blots were washed in TBS-Blotto-0.5% Tween20 (4 x 15 min, 3 x 5min for high stringency) or TBS-Blotto-0.05% Tween20 (4 x 15 min, 3 x 5min for low stringency) and incubated with secondary antibody (anti rabbit IgG) 1:1000 for one hour at room temperature. Blots were washed in TBS-Blotto-Tween20 as described above and incubated with the Amersham ECL detection reagents and developed for up to 10 minutes.

Receptor Kinase Assay His_6-5-HT1B receptor was constructed as follows. The human 5-HT1B receptor was excised from pCM plasmid using the *Nco* I site and the His epitope oligonucleotide fragment[(5'-GCCGCCACCATGGGA(CAC)6-3)'and(3'CGGCGGTGGTACCCT(GTG)6TAC-5')] was ligated in frame and subcloned in *Bam* H1/*Xba* I sites of pcDNA3. The His_6-tagged 5-HT1B receptor was transiently transfected in HEK 293 cells by calcium-phosphate coprecipitation method. Forty-eight hours post-transfection the His-tagged 5-HT1B receptor membranes were assayed for biological activity using a cAMP assay as previously described (30). Moreover, crude

membranes were isolated and subjected to western analysis using Ni-NTA conjugate (according to Qiagen protocol). To visualize the tagged receptor the blot was hybridized with 1:1000 Ni-NTA conjugate for one hour at room temperature followed by 3X10 minutes washing with TBST (500 mM NaCl, 0.05% Tween-20, 20 mM Tris-HCl, pH 7, 3% BSA in TBS buffer) and developed with 5% NBT/5% BCIP. The wild-type and kinase-inactive OK-GRK2-K220R were *in vitro*-translated using the TNT T7 polymerase-coupled reticulocyte lysate system (Promega). For the phosphorylation reaction, 100 µg of crude membranes transiently expressing the His_6-tagged 5-HT1B receptor were incubated with the reticulocyte-translated proteins (wild-type and kinase inactive mutant OK–GRK2) for 1 hour at 30°C. The phosphorylation reaction contained 20 mM Tris-HCl pH 7.2, 100 µM [γ-^{32}P]ATP (3200 mCi/mmol), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM sodium phosphate, 10 µM 5-HT, with 10 mM NaF, 10 mM disodium pyrophosphate as phosphatase inhibitors and 10 µg/ml leupeptin, 10 mM benzamidine, 1mg/ml aprotinin, 1 mM PMSF as protease inhibitors in a total volume of 100 µl . The reactions were quenched with 0.5 ml of 0.1 M cold sodium phosphate pH 7.2 buffer. His-tagged receptors were eluted from the Pro-Bond resin using denaturing conditions according to the protocol (Invitrogen). The samples were resuspended in SDS-sample buffer, resolved by SDS-PAGE and visualized by autoradiography.

cAMP Assay- Measurement of cAMP was performed as described previously with some modifications (30). Briefly, cells plated in 24-well dishes were washed twice with 1 ml of DMEM/Hepes (serum-free DMEM medium + 20 mM Hepes (pH 7.2))Buffer A (serum-free D-MEM medium containing 4 mM $MgCl_2$, 0.3 mM ascorbic acid and) and incubated with 0.5 ml/well of DMEM/Hepes for 45 minutes containing either no addition, 100 nM/1 µM 5-HT, or 100 nM/1 µM UK 14304. Following incubation, medium was quickly decanted, and replaced with warm assay medium (DMEM/Hepes + 100 µM IBMX) with agents (10 µM forskolin, 1µM 5-HT or 1 µM UK14304) and incubated for 15 minutes at 37°C. In control experiments, the cells were washed twice with DMEM/Hepes prior to addition of assay medium: it was determined that the 5-HT pretreatment did not alter subsequent basal or forskolin-stimulated cAMP levels. The reactions were stopped by removing the medium. The supernatants were collected and assayed for cAMP

by a specific radioimmunoassay (ICN) as described (30). Standard curves displayed average IC_{50} of 0.5 ± 0.2 pmol using cAMP as standard. Data for cAMP assays are presented as mean ± S.E.M for triplicate wells. The % desensitization was calculated as follows {1 - [(F - XFS)/(F-FS)]} X 100.

Results

Cloning of the opossum GRK2 (OK–GRK2)- Degenerate oligonucleotides derived from the highly-conserved catalytic region of GRKs were used to identify novel GRK cDNAs from the OK cell line (29). Two distinct 380-bp fragments (A1 and 10-1) of distinct nucleotide sequence were isolated that shared >92% predicted amino acid identity to the catalytic domains of rat β-ARK1 and β-ARK2 (GRK2 & 3). These cDNA fragments were used to clone full-length GRK cDNA's from a λ-ZAP II cDNA library constructed using OK cell RNA. A 3.054-kb cDNA clone (named OK-GRK2) that shared 81% nucleotide identity with rat GRK2 (β-ARK1) cDNA sequence (Fig. 1) was identified in 10/10 positive clones. The OK-GRK2 cDNA contains a predicted open reading frame that encodes a 688-amino acid protein with 92% and 80% amino acid sequence identity with rat GRK2 and GRK3, respectively. The amino acid sequence of OK-GRK2 and rat β-ARK1(GRK2) share 95% identity in the catalytic domain (Fig.2, shaded), 86% in the amino-terminus, 93% in the carboxyl-terminus (Fig. 2, shaded); hence the clone was designated OK-GRK2.

Northern Blot Analysis of OK-GRK2- In order to verify the expression of OK-GRK2 RNA in this cell line, poly-A+ RNA isolated from OK cells was subjected to Northern blot analysis and probed with OK-GRK2 cDNA. As shown in Fig. 3, two distinct GRK-2-related mRNA species with molecular sizes of approximately 5.0 kb and 3.0 kb were detected. The intense band at 5.0 kb may correspond to the predominant OK-GRK2 cDNA clone in Fig. 1, whereas the smaller species may represent an alternatively-spliced variant or a less abundant, closely-related GRK mRNA variant present in the OK cell line, as suggested by the distinct RT-PCR products identified using GRK-directed oligonucleotides.

Kinase Activity of OK-GRK2- The function of the OK-GRK2 clone was examined using a receptor kinase assay of the *in vitro*-translated cloned OK-GRK2. The 5-HT1B receptor was selected as a potential substrate since it is expressed in OK cells and has been found to desensitize in the presence of the agonist, 5-HT (20, 31). In order to examine 5-HT1B receptor phosphorylation, a His_6-epitope tagged variant of the human 5-HT1B receptor was constructed and expressed transiently in HEK 293 cells. Western analysis using a nickel conjugate to detect

the His_6 sequence revealed a single species with molecular weight of approximately 70 kDa that was present in membranes from HEK 293 cells transfected with the His_6-receptor construct, but absent in mock-transfected HEK 293 cells (data not shown). The molecular weight is consistent with the 70 kDa size observed for the non-tagged 5-HT1B receptor in transfected cell lines (32). The function of the His_6-5-HT1B receptor was examined in HEK-293 cells by measuring receptor-mediated inhibition of forskolin-induced cAMP accumulation (19). As depicted in Fig. 4A, 10 μM 5-HT mediated a 40% inhibition of forskolin-stimulated cAMP accumulation in HEK-293 cells transfected with His_6-5-HT1B receptor cDNA. No inhibition was observed in non-transfected HEK 293 cells (data not shown), suggesting that HEK-293 cells lack functional 5-HT1 receptors. Thus, insertion of the His_6 epitope at the amino-terminus of the 5-HT1B receptor is permissive for coupling to inhibition of cAMP levels. Site-directed mutagenesis was used to construct OK-GRK2 K220R, a mutant that lacks the catalytic lysine responsible for the phosphotransferase reaction (10). Both wild-type and mutant GRK proteins were synthesized using *in vitro* transcription/translation reagents. As assessed by Western analysis using a polyclonal anti-bovine GRK2 antibody (10), *in vitro* translated wild-type and mutant OK-GRK2 both had the same electrophoretic mobility as control purified bovine brain GRK2 (80 kDa) (Fig. 4B). To assay the function of the wild-type and mutant kinases, *in vitro* kinase reactions were performed using 100 μg of crude HEK 293 membrane proteins expressing the His-tagged 5-HT1B receptor as a substrate. Equal amounts of membranes and *in vitro*- translated kinases were added and the mixture was incubated in the presence of 10 μM 5-HT to activate the 5-HT1B receptor. The phosphorylated species observed at 70 kDa (Fig. 4C) corresponded exactly in size to the His_6-5-HT1B receptor identified by Western blot analysis. The lower molecular weight species may represent a 5-HT1B receptor degradation fragment produced during the assay. There was a basal level of phosphorylation detected in the control (left lane), probably due to endogenous membrane-associated GRK activity, since no phosphorylation was observed in the absence of agonist. The wild-type kinase (middle lane) phosphorylated the transfected 5-HT1B receptor, as identified by product at 70 kD (arrow). When corrected for background (Fig. 4D), receptor phosphorylation in the presence OK-GRK2 K220R was similar to the control (right lane). These results show that the

cloned wild-type OK-GRK2 phosphorylates the 5-HT1B receptor *in vitro*, whereas the K220R mutation abrogates the G-protein receptor kinase activity.

Expression of sense, antisense, kinase-inactive OK-GRK2. The role of endogenous OK-GRK2 in receptor desensitization of G-protein coupled receptors expressed endogenously in OK cells was addressed using three complementary approaches: a) overexpressing wild-type OK-GRK2 to examine whether receptor attenuation is enhanced; b) constructing and overexpressing a kinase-inactive mutant to quench free $\beta\gamma$ subunits, rendering the endogenous GRK2 non-functional (11) and finally c) using a full-length antisense GRK2 cDNA construct to specifically deplete endogenous GRK2 protein expression (33).

The full-length OK-GRK2 cDNA (sense and antisense) and the kinase-inactive K220R mutant (sense) were subcloned in the pcDNA3 vector and transfected into OK cells. Stable transfectant colonies were selected by growth in G418-containing media and clones with the highest level of RNA expression for each construct were isolated for Western analyses using the recombinant bovine GRK2 polyclonal antibody (Fig. 5A and 5B). In the non-transfected OK cells, OK-GRK2 had the same mobility as purified bovine GRK2 (Mr=80 kDa), but was weakly-detected suggesting that the endogenous level of OK-GRK2 is relatively low. Two clones for each construct were isolated for further characterization, for the over-expressed sense construct clones GRK:3 and GRK:18 and the kinase-inactive clones KI:5, KI:19 and KI:28. These clones over-express OK-GRK2 protein as compared to the endogenous protein (Fig. 5A and 5B). To assess the decrease in endogenous OK-GRK2 protein for the antisense construct, the stringency of hybridization and washing was reduced (see Methods). As depicted in Fig. 5B, the endogenous protein is seen at 80 kDa and clones expressing the antisense construct AS:4 and AS:8 show a decrease in OK-GRK2 protein levels as compared to the endogenous protein.

Alteration of receptor desensitization. In order to establish a whole cell assay for receptor desensitization, the ability of endogenous 5-HT1B receptors to undergo agonist-induced desensitization in wild-type OK cells was examined (Fig. 6). Following examination of the time-course of desensitization, cells were pretreated for 45 min. using 0.1 to 1000 nM 5-HT, followed by a 15 min. assay for receptor-mediated inhibition of forskolin-stimulated cAMP accumulation (Fig. 6). A concentration-dependent attenuation of the ability of the 5-HT1B receptor to inhibit

cAMP accumulation was observed, indicating an agonist-induced functional desensitization of the receptor. The endogenous α2C receptors were also shown to undergo homologous desensitization when pretreated with either 100 nM or 1 μM UK14304 in the parental cell line (Fig.7). This assay was used to examine agonist-induced desensitization in the GRK clones. A representative experiment using OK (non-transfected), GRK:3 and 18, KI:5 and 19 and AS:4 clones is shown (Fig. 7). The agonist-dependent desensitization of the 5-HT1B receptor was different in the KI and AS clones only, whereas there were clear differences in α2C-adrenergic receptor desensitization measured in parallel. In particular, both the KI:5 and AS:4 clones in which GRK2 activity should be blocked, lacked evident desensitization of the α2C-receptor, whereas the GRK2 overexpression produced an enhancement in α2C-adrenergic receptor desensitization.

The above experiments were repeated multiple times with several clones and the data are averaged as the relative desensitization as compared to parental OK cells (Fig. 8). Sense clones GRK:3 and GRK:18 which over-express the OK-GRK2 were analyzed to determine if homologous desensitization of the 5-HT1B and α2C-adrenergic receptors was enhanced. Overexpression of OK-GRK in the OK cell line enhanced the desensitization of the α2C-adrenergic receptors (*$p < 0.05$ for GRK:3) but did not augment the agonist-specific desensitization of the 5-HT1B receptor as compared to the parental cell line. A similar profile was observed with the GRK:18 (n=1) clone at enhancing the desensitization response of the α2C-adrenergic receptors. This suggests that the α2C receptors in the OK cell line are regulated by OK-GRK2 while the 5-HT1B receptors are not, even though the 5-HT1B receptors can be phosphorylated by GRK2 *in vitro*. The kinase-inactive mutant KI: 5 (n=3) completely abrogated the desensitization process of the α2C receptors at 100 nM and 1 μM UK 14304 (*p value < 0.05 and **p value < 0.005, respectively). The KI:19 mutant (n=2) had a similar profile as KI:5 at both concentrations of agonist, it precluded the desensitization of the α2C receptors by 50% as compared to the parental cell line. These results suggest that the inactive kinase competes to render the endogenous OK-GRK2 non-functional. Interestingly, the KI:19 and KI:28 clones in three independent experiments were shown to consistently enhance the 5-HT1B receptor-mediated attenuation as depicted in Figs. 7 and 8 (* p value < 0.05 and † p value < 0.08). The AS:4 clone showed a decrease in endogenous OK-GRK2 protein (Fig. 5B) and significantly precluded the agonist-mediated

desensitization of the α2C-adrenergic receptor at 100 nM and 1 μM UK14304 (*p value < 0.05 and **p value < 0.005, respectively). Surprisingly, AS:4 clone significantly enhanced the desensitization of the 5-HT1B receptors at 1 μM 5-HT only (*p value < 0.05). These observations indicate that the α2C receptors are regulated in whole cells by OK-GRK2, whereas regulation of 5-HT1B receptor desensitization involves other kinases or processes. In these experiments, no significant differences were observed among the clones compared to wild-type OK cells in the stimulation (-fold basal) by forskolin or the inhibition (% forskolin stimulated cAMP level) by 5-HT1B or α2C receptor agonists. Thus the action of OK-GRK2 was selective for regulation of agonist-induced receptor desensitization.

Discussion

The opossum kidney cell line, OK cell line endogenously expresses several G-protein coupled receptors that have been shown to undergo homologous desensitization (20, 22, 24, 25, 31). Homologous desensitization of several adrenergic receptors is a well-studied agonist-specific receptor event involving receptor phosphorylation by G-protein receptor kinases (GRK), followed by the binding of a cofactor named arrestin which results in the attenuation of a receptor-mediated signal-transducing event (6-9). The aim of the present study was to clone GRKs from the OK cell line and investigate if the GRKs can specifically regulate the desensitization process of the endogenously expressed Gi-protein coupled receptors using three different approaches: a) overexpressing the GRK b) constructing a kinase-inactive mutant and c) using an antisense construct to the endogenous GRK.

A novel opossum GRK: The opossum GRK clone we describe here is most similar to the previously-identified bovine and rat GRK2 (β-ARK1) (34, 35), hence we have designated it opossum GRK2. Computer alignment of the nucleotide sequences from the coding regions of the OK-GRK2 revealed 81% and 80% identity to the coding sequences of the rat and bovine GRK2 messages, respectively. For comparison, the rat and bovine nucleotide sequences are 88% identical. The level of amino acid conservation is quite high among the three sequences. The deduced amino acid sequence of OK-GRK2 shares 91.7% and 92.2% homology with the rat and bovine sequences, respectively, reflecting the evolutionary distance between marsupial and

mammal species. By contrast, the rat and bovine species share 98.3% homology. Important features identified in the rat and bovine GRK2 such as the catalytic domain responsible for activation: amino acid identity in the catalytic domain amongst these three species of GRK2 is > 99%. In addition, the C-terminal region containing the pleckstrin homology domain that is responsible for the docking of βγ subunits is highly conserved (36,37). Thus, the clone has a structural characteristics of a functional GRK.

Northern analysis revealed two mRNA transcripts of approximately 5.0 and 3.0 kb. Similarly the message size we observe was approximately the same as found in the bovine brain (35). The presence of the second smaller band at 3.0 kb might represent alternative splicing of a large intron in the untranslated regions of the message. The gene for the human GRK2 was recently cloned and the structural organization of this gene was found to be composed of 21 exons interrupted by 20 introns (38). This may explain the smaller message RNA seen at 3.0 kb.

Phosphorylation in vitro vs. desensitization: A His_6-tagged 5-HT1B receptor was constructed to investigate if the wild-type OK-GRK2 kinase was functional. The OK-GRK2 phosphorylated functional His_6-tagged 5-HT1B receptor *in vitro* in an agonist-dependent manner whereas the OK-GRK2 K220R mutant did not. A precise consensus sequence for GRK-mediated phosphorylation of G-protein-coupled receptors has not yet been delineated. Recently, four consecutive serines in the third intracellular loop of the α2A-adrenergic receptor were found to be critical sites in rapid agonist-promoted desensitization and also specific sites for GRK2-mediated phosphorylation (39). A list of potential sites for GRK-mediated phosphorylation was compiled where several members of the G-protein coupled receptor superfamily contained amino acid sequences in the third loop or carboxyl terminus which shared high degree of homology to the site found on the α-2A-adrenergic receptor (EESSSS where serine and/or threonine residues are flanked by acidic amino acid residues). A closer examination of the molecular structure of the 5-HT1B receptor revealed a conserved DSXXSSSS sequence in the third intracellular loop (19, 28) that resembles the GRK phosphorylation consensus site, and could serve as the site of phosphorylation by OK-GRK2.

The present findings demonstrate a discrepancy between the *in vitro* phosphorylation of 5-HT1B receptors by OK-GRK2 and the importance of OK-GRK2 in agonist-induced desensitization of this receptor in intact cells. Bylund and co-workers have shown that

homologous desensitization for the 5-HT1B and α2C-adrenergic receptors endogenously expressed in the OK cell line occurs using a [^{3}H]-adenine prelabeling technique to measure cAMP production in intact OK cells (20, 21, 31). We also detected agonist-induced desensitization of the 5-HT1B and α2C-adrenergic receptors in non-transfected intact cells (parental cell line) using a specific cAMP radioimmunoassay. Overexpression of OK-GRK2 enhanced agonist-induced desensitization of the α2C-adrenergic receptors at 1 μM UK14304, whereas no significant effect on desensitization of 5-HT1B receptors was observed. Thus, OK-GRK2 displayed Gi-coupled receptor specificity in intact cells. Although *in vitro* phosphorylation experiments demonstrated that the 5-HT1B receptor can act as a substrate for OK-GRK2, these results imply that the *in vitro* conditions for phosphorylation may be favorable but do not necessarily mimic a physiological environment for inducing 5-HT1B receptor desensitization. Thus, although OK-GRK2-mediated 5-HT1B receptor phosphorylation is observed *in vitro*, its importance in 5-HT1B receptor desensitization is not favored in intact cells. These results suggest that factors (eg., localization or accessibility, other regulatory proteins) other than suitability as a phosphorylation substrate can determine whether a receptor will be regulated by a given GRK subtype.

Protein kinases other than OK-GRK2 may mediate the 5-HT1B receptor desensitization event. Recently, it was shown that the dopamine D1A receptor was differentially regulated in terms of agonist-mediated desenstization by various GRKs when transfected in HEK 293 cells (40). The notion that another member of the GRK family might be responsible for mediating agonist-promoted attenuation of the 5-HT1B receptor is conceivable. A GRK3-related cDNA fragment of 380 bps was identified in the OK cell line using PCR with degenerate oligonucleotides and could correspond to a second GRK subtype. This suggests that other GRKs might be expressed in the cell line that may be responsible for promoting homologous desensitization of the 5-HT1B receptor. This could be investigated using specific GRK monoclonal antibodies to determine GRK specificity as elegantly demonstrated by Oppermann and colleagues (41). Another possibility is that the attenuation response for the 5-HT1B receptors may not necessarily be mediated by GRK-induced phosphorylation, but by a second messenger kinase such as PKC. Interestingly, the 5-HT1B-receptor mediated calcium response was shown to be uncoupled by acute pre-activation of PKC in OK cells (42).

Receptor specificity of OK-GRK2: Our observations demonstrate that OK-GRK2 exhibits Gi-coupled receptor specificity in the OK cell line using three complementary approaches: over-expression of the OK-GRK2 enhanced the attenuation response mediated by the α2C-adrenergic receptors (but not of 5-HT1B receptors), whereas expression of the kinase inactive mutant or the antisense construct abrogated this process. The latter two approaches demonstrate the importance of the relatively low level of endogenous OK-GRK2 in α2C-adrenergic receptor desensitization. The kinase-inactive OK-GRK2-K220R mutant prevented the attenuation response mediated by the α2C-adrenergic receptor. Similarly, this kinase-deficient GRK2 mutant was previously shown to preclude agonist-induced desensitization of the endogenously expressed β2-adrenergic receptor but not for PGE2 receptors in the same bronchial cell line (10). The inactive kinase also prevented the acute desensitization of the m2-muscarinic receptor, α1B-adrenergic receptor-mediated PI response and the angiotensin II receptor in transfected HEK cells (13,43,44). Our results also demonstrate that depletion of OK-GRK2 using antisense RNA expression resulted in complete attenuation of agonist-mediated desensitization of α2C-adrenergic receptors. In contrast, the antisense clone had a minor enhancing effect on the 5-HT1B receptor desensitization response at 1μM 5-HT only. This suggests that the specific decrease in the expression level of endogenous OK-GRK2 can significantly and selectively alter the α2C-adrenergic receptor-mediated event. The results imply that the α2C-adrenergic receptor is a potential substrate for the OK-GRK2 in whole cells since it inherently expresses several GRK-phosphoryable sites in the third loop (21). Interestingly, the opossum α2C adrenergic receptor is a homologue of the human α2C4 subtype which has never been shown to undergo homologous desensitization (13, 45). Hence, we have demonstrated that the α2C receptors can be physiologically modulated by the discriminating activity of OK-GRK2.

Surprisingly, in OK cells over-expressing the kinase-inactive mutant OK-GRK2-K220R, the efficacy of agonist-induced desensitization of the 5-HT1B receptor was enhanced (ie., at 100 nM and 1 μM 5-HT). This observation is supported by the recent finding that the rat GRK2-K220R also enhanced agonist-mediated attenuation of the α1B-adrenergic receptor-mediated PI response (43). One possible explanation for enhanced potency of agonist-induced receptor desensitization by OK-GRK2-K220R might be the interaction of GRK with signalling molecules downstream of the receptor. Current scientific knowledge supports this hypothesis since GRK2

has been shown to bind to βγ subunits of G-proteins and also phosphatidylinositol bisphosphate via its pleckstrin homology domain (36,37,46). It is possible that OK-GRK2-K220R might sequester βγ subunits or unknown proteins critical for inactivation of certain adenylyl cyclase subtypes in the OK cell line, leading to impaired 5-HT1B receptor signalling. In contrast, for the α2C-adrenergic receptors competition with active GRK2 for receptor recognition appears to exert the dominant effect to block desensitization. Thus the kinase-inactive mutant GRK2, while a useful tool, appears to have multiple actions that can impair signalling of receptors that are insensitive to GRK2.

Interestingly, the role of GRK *in vivo* may supercede its role in receptor desensitization. Recently, transgenic mice overexpressing GRK2 (β-ARK1) specifically in cardiac tissue were generated and these mice not only exhibited reduced cardiac contractility in response to isoproterenol but the myocardial adenylyl cyclase activity was attenuated due to the reduced functional coupling of the β2-adrenergic receptors (48). Whereas, mice overexpressing a β-ARK inhibitor showed increased cardiac contractility. In addition, mice with a disruption of the GRK2 gene were shown to display severe cardiac malformations hence suggesting a role in fetal development (49). These results suggest the pivotal role played by GRK2 physiologically.

To conclude, we have cloned a functional GRK2 from the opossum kidney cell line and have shown that in intact cells OK-GRK2 displays Gi-coupled receptor specificity in mediating the homologous desensitization process of the α2C-adrenergic receptors, but in contrast to results *in vitro* was inactive at the 5-HT1B receptors.

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Figure Legends:

Figure 1. Nucleotide and deduced amino acid sequences of the OK-GRK2. The OK-GRK2 was isolated with a randomly labeled PCR fragment of OK-GRK2 cDNA (380 bp). The clone was 3.056 kb in length. The amino acid sequence of OK-GRK2 begins with the first ATG in the nucleotide sequence and is numbered on the right hand side of the sequence.

Figure 2. Alignment of the deduced amino acid sequences of OK-GRK2 with the rat (R) and bovine (B) GRK2. Overall amino acid identities of OK-GRK2 with the bovine GRK2 is 92.2% and 91.7% identity with the rat homologue. The sequences of the rat and bovine GRK2 were derived from *GenBank*. The amino acids in outline represent nonconserved residues. The amino acid sequences were aligned using the CLUSTAL algorithm program from *DNASTAR INC*. (Madison, WI). The shaded regions of the alignment represent the catalytic and C-terminal regions. The outlined residues represent amino acid substitutions in the opossum, rat and bovine GRK2 homologues.

Figure 3. Northern blot analysis of OK cell mRNA. Poly A^+ RNA (7 μg) from the OK cell line was isolated and separated on a 1.2% formaldehyde agarose gel, transferred to Hybond N membrane and probed with the OK-GRK2 *Kpn* I cDNA fragment (2.1kb). Two bands of OK-GRK2 mRNA with estimated sizes of 5.0 and 3.0 kb were present after the autoradiogram was exposed for 4 days at -70 C. The hybridization and washing conditions were performed as described under "Experimental Procedures".

Figure 4. Kinase activity of OK-GRK2 and OK-GRK2-K220R. A) His_6-5-HT1B receptor-mediated inhibition of cAMP accumulation 48 hours post-transfection. HEK 293 cells transiently transfected with the His_6-5-HT1B receptor were plated in 24 well plates and assayed 48 hours post-transfection. The His_6-5-HT1B receptors mediated 40% inhibition of forskolin-stimulated cAMP accumulation. The percent inhibition of (10 μM) forskolin-stimulated cAMP

accumulation by 5-HT (10 μM) is tabulated as the mean ± S.E.M. Calculation for inhibition of forskolin-stimulated cAMP accumulation [(F-FS)/F] x 100, where the level of cAMP after forskolin (F) or forskolin and serotonin (FS) was measured. **B)** Western analysis of *in vitro* translated OK-GRK2 and OK-GRK2-K220R mutant using 2 ng of purified bovine β-ARK1 (GRK2) as a positive control. The bands at 80 kDa represent GRK. **C)** Phosphorylation of His_6-5-HT1B receptors *in vitro* by wild-type OK-GRK2. The migration of a 70 kDa band is indicated on the ordinate (arrow) which corresponds to the 5-HT1B receptor as identified by Western analysis of a replicate gel (see Experimental Procedures). **Left lane**, control: membranes in the absence of added kinase ; **middle lane**, membranes with added *in vitro*-transcribed OK-GRK2; **right lane**, membranes with added OK-GRK2-K220R. Crude HEK 293 membranes (100 μg) transiently expressing His_6-tagged receptor were incubated with either the wild-type or mutant kinase in the presence of 10 μM 5-HT and γ^{32}-ATP at 30 °C for 1 hour followed by the isolation of the receptors on a nickel-conjugated column. The samples were subjected to 10% SDS-polyacrylamide gel electrophoresis, dried and exposed to XAR film for 4 days at -70 °C. Shown is a representative experiment of two performed. **D)** Quantitative densitometry (with background subtracted) of the 70 kDa band representing the His_6-5-HT1B receptor phosphorylated by kinases. The ordinate represents arbitrary units of optical density.

Figure 5. Expression of OK-GRK2 in transfected OK cells. A and B) Western analyses of OK-GRK2 in OK cells. The wild-type, the kinase-inactive mutant K220R, and the antisense GRK2 cDNA constructs were subcloned in the mammalian expression vector pcDNA3 and transfected stably in OK cells. Western blot analyses were performed using sonicated membranes from transfected clones; 50 μg of total protein were loaded in each lane and Westerns were probed using the recombinant bovine β-ARK1 polyclonal antibody (1:1000). Western blots were developed using the ECL method (Amersham). The arrows indicate the purified bovine β-ARK1 (2 ng) which migrated at Mr 80 KDa. Sense clones (sense:3 and 18) and kinase-inactive clones (KI:5, 19 and 28) over-express OK-GRK2 as compared to parental cell line (W.T.). In Figure 5B, decreased amounts of OK-GRK2 were found in antisense clones (AS:4 and AS:8) as

compared to the parental non-transfected cell line.

Figure 6. Agonist-induced desensitization of the 5-HT1B receptor. Concentration dependence of 5-HT preincubation on inhibition of forskolin-stimulated cAMP accumulation. Cells were incubated with indicated concentrations 5-HT for 45 minutes and assayed as previously described (30) using 1 μM 5-HT and 10 μM forskolin. Values are means ± S.E.M.

Figure 7. Desensitization of 5-HT1B or α2C-adrenergic receptor-mediated inhibition of cAMP accumulation in OK-GRK2 cell clones. cAMP accumulation was measured in media harvested from OK cells following 45 minutes preincubation with 100 nM or 1 μM of 5-HT or α2 agonist UK 14304 (UK), and processed as previously described using 1 μM 5-HT or UK 14304 and 10 μM forskolin (F). The data are expressed as mean ± S.E.M. of triplicate determinations from a single experiment. The cell lines tested include parental (OK) or OK clones transfected with antisense OK-GRK2 (AS:4), sense OK-GRK2 (GRK:3 and 18), or OK-GRK2-K220R (KI:5 and 19) cDNA's. The % desensitization for OK, AS:4, GRK:3 and 18, or KI:5 and 19 at 1 μM was 14%, 20%, 5%, 15%, 1% and 10% for 5-HT, respectively; 20%, 7%, 60%, 45%, 0% and 17% for 1 μM UK. These assays were repeated with similar results for various clones in multiple independent experiments.

Figure 8. Differential modulation of α2C-adrenergic and 5-HT1B receptor desensitization by GRK2. Experiments were performed as described in Fig. 7, and the % desensitization was calculated for each clone and condition, and normalized to the value for OK cells in the same experiment. The dashed line at unity (1) represents normalized receptor desensitization calculated in non-transfected OK cells (control). Values greater than unity indicate enhancement of desensitization, while values less than unity indicate abrogation of desensitization. The cell lines tested include OK clones transfected with antisense OK-GRK2 (AS:4), sense OK-GRK2 (GRK:3), or OK-GRK2-K220R (KI:5, KI:19, KI:28) cDNA's. **A) α2C receptor desensitization:** Cells were pretreated with [UK]=100 nM or 1 μM, and subsequent receptor-mediated inhibition of cAMP assessed. **B) 5-HT1B receptor desensitization:** Cells were

pretreated with 100 nM or 1 μM 5-HT.† p value < 0.08; *p value < 0.05; **p value < 0.005; ¶ range of duplicate samples for KI:19 at 1 μM and 100 nM are 5% and 7%, respectively.

Figure 1: Nucleotide and deduced amino acid sequences of OK-GRK2..

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GTTGCTGTCGGGGGCGCGGAGGTCTGCGAGCTCCGGGACAGCGGCGGCAAAAGAGGCGTAGGGGGCTGCAAGATGGCGGACCTAGAGGCCGTCCTGGCCG                100
                                                                  Met Ala Asp Leu Glu Ala Val Leu Ala

ACGTGAGCTACCTGATGGCCATGGAGAAGAGCAAGGCCACGCCGGCTGGCCGGGCCAGCAAGAAGATCCTGCTGCCAGAGCCCACGATCCGAGCGTCTAT                200
Asp Val Ser Tyr Leu Met Ala Met Glu Lys Ser Lys Ala Thr Pro Ala Gly Arg Ala Ser Lys Lys Ile Leu Leu Pro Glu Pro Thr Ile Arg Ala Ser Met

GCAGAAGTACCTTGAAGACCGTGGGGAGGTGACCTTCGAGAAGATTTTCTCTCAGAAGCTCGGCTACCTGCTCTTCAGAGAGTTCTGCCTCAACCACATG                300
Gln Lys Tyr Leu Glu Asp Arg Gly Glu Val Thr Phe Glu Lys Ile Phe Ser Gln Lys Leu Gly Tyr Leu Leu Phe Arg Glu Phe Cys Leu Asn His Met

GAGGAGGCCAAGCCCCTGGTGGAGTTCTATGACGAGATCAAGAAATACGAGAAGCTGGACTCGGAGGAGGAGCGCACCGTCAAAAGCCGAGAGATCTTTG                400
Glu Glu Ala Lys Pro Leu Val Glu Phe Tyr Asp Glu Ile Lys Lys Tyr Glu Lys Leu Asp Ser Glu Glu Glu Arg Thr Val Lys Ser Arg Glu Ile Phe

ACTTGTACATCATGAAGGAGCTGCTGTCCTGCTCGCACCTTTTCTCAAAGAGTGCCACCGAGCACGTGCAGAGCCGGCTCCTCAAGAAGCAGGTGCCCAC                500
Asp Leu Tyr Ile Met Lys Glu Leu Leu Ser Cys Ser His Leu Phe Ser Lys Ser Ala Thr Glu His Val Gln Ser Arg Leu Leu Lys Lys Gln Val Pro Thr

AGACCTGTTCCAGCCTTACATCGAGGAGATCTGCCAGCGGTTCCGGGATGACGTGTTCCAGAAATTCATCGAGAGTGAGAAGTTCACTCGGTTCTGCCAA                600
Asp Leu Phe Gln Pro Tyr Ile Glu Glu Ile Cys Gln Arg Phe Arg Asp Asp Val Phe Gln Lys Phe Ile Glu Ser Glu Lys Phe Thr Arg Phe Cys Gln

TGGAAGAACGTGGAGCTCAACATMCATCTGACCATGAATGACTTCAGTGTGCATCGGATCATTGGCCGAGGTGGCTTTGGCGAGGTCTACGGCTGCCGCA                700
Trp Lys Asn Val Glu Leu Asn Ile His Leu Thr Met Asn Asp Phe Ser Val His Arg Ile Ile Gly Arg Gly Gly Phe Gly Glu Val Tyr Gly Cys Arg

AAGCCGACACGGGCAAGATGTATGCCATGAAGTGTCTTGATAAAAAGCGTATCAAGATGAAGCAGGGGGAGACGCTGGCTCTGAATGAACGGATCATGCT                800
Lys Ala Asp Thr Gly Lys Met Tyr Ala Met Lys Cys Leu Asp Lys Lys Arg Ile Lys Met Lys Gln Gly Glu Thr Leu Ala Leu Asn Glu Arg Ile Met Leu

TTCCCTTGTCAGCACTGGGGACTGTCCCTTCATCGTCTGCATGTCCTATGCATTCCACACTCCAGACAAGCTCAGCTTCATCCTAGATCTCATGAATGGG                900
Ser Leu Val Ser Thr Gly Asp Cys Pro Phe Ile Val Cys Met Ser Tyr Ala Phe His Thr Pro Asp Lys Leu Ser Phe Ile Leu Asp Leu Met Asn Gly

GGGGACCTTCACTATCACCTGTCCCAGCACGGCGTCTTCTCGGAGTCAGACATGCGCTTCTATGCGGCCGAGATCATCCTGGGCCTGGAGCACATGCACA                1000
Gly Asp Leu His Tyr His Leu Ser Gln His Gly Val Phe Ser Glu Ser Asp Met Arg Phe Tyr Ala Ala Glu Ile Ile Leu Gly Leu Glu His Met His

GTCGATTTGTGGTCTACCGAGACCTGAAGCCCGCTAACATCCTTCTGCACGAGTTTGGCCACGTCCGCATCTCTGACCTGGGCCTGGCCTGTGACTTCTC                1100
Ser Arg Phe Val Val Tyr Arg Asp Leu Lys Pro Ala Asn Ile Leu Leu His Glu Phe Gly His Val Arg Ile Ser Asp Leu Gly Leu Ala Cys Asp Phe Ser

CAAGAAGAACGCCCACGCCAGCGTGGGTACCCATGGCTACATGGCTCCCGAGGTCCTGCAGAAGGGGGTGGCCTATGACAGCAGCGCCGATTGGTTCTCC                1200
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Leu Gly Cys Met Leu Phe Lys Leu Leu Arg Gly His Ser Pro Phe Arg Gln His Lys Thr Lys Asp Lys His Glu Ile Asp Arg Met Thr Leu Thr Met

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Val Asn Ala Ala Asp Ala Phe Asp Ile Gly Ser Phe Asp Glu Glu Asp Thr Lys Gly Ile Lys Leu Leu Asp Ser Asp Gln Glu Leu Tyr Arg Asn Phe

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Lys Met Arg Asp Ala Ala Gly Ser Ala Asn Gly Leu  •

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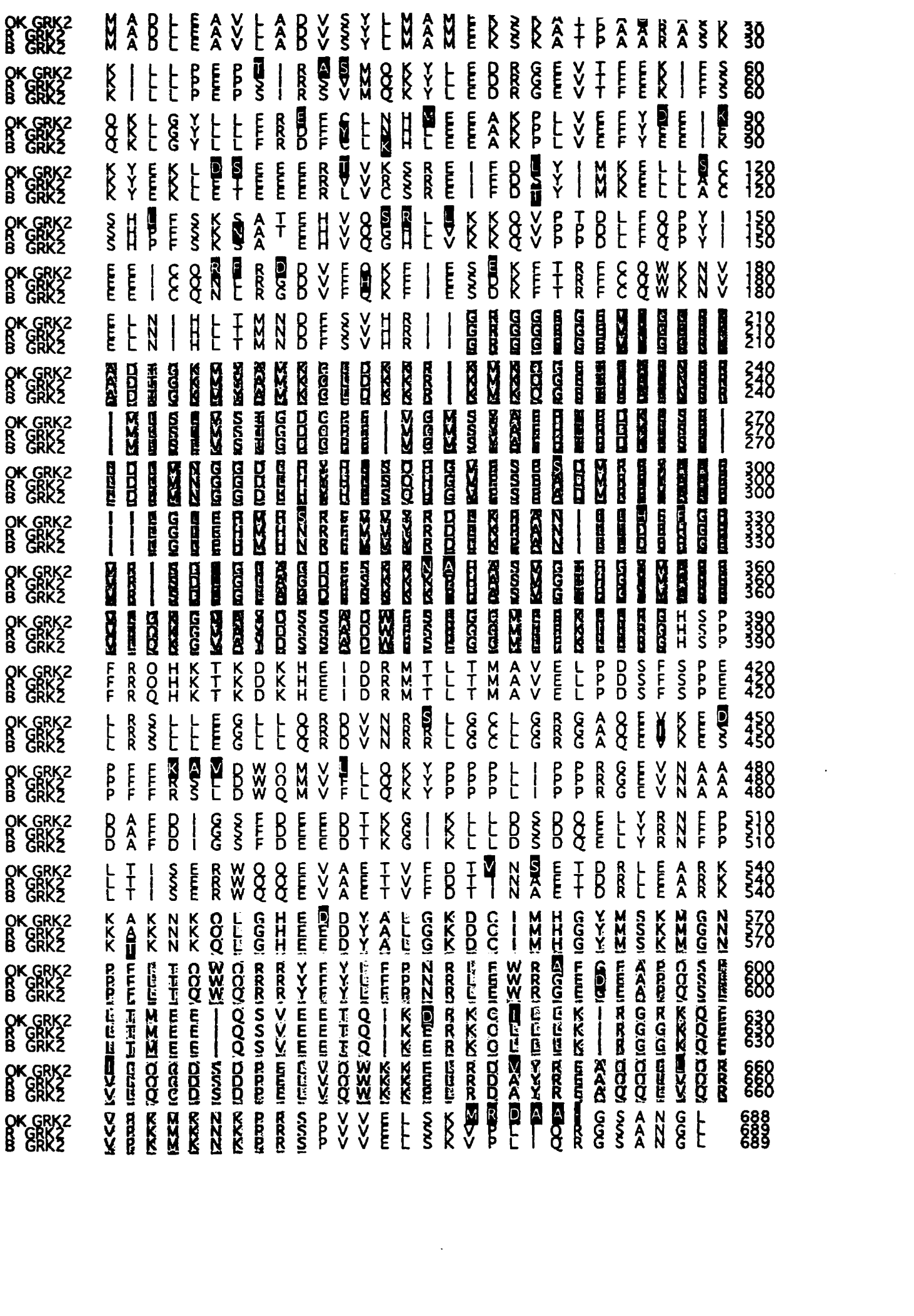
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R GRK2
B GRK2
30
60
90
120
150
180
210
240
270
300
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510
540
570
600
630
660
688
689

Figure 3. Northern analysis of OK-GRK2.

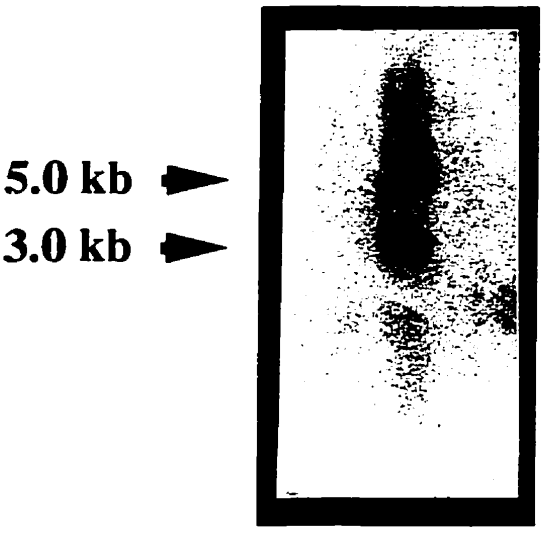


Figure 4. Kinase activity of OK-GRK2 and OK-GRK2-K220R.

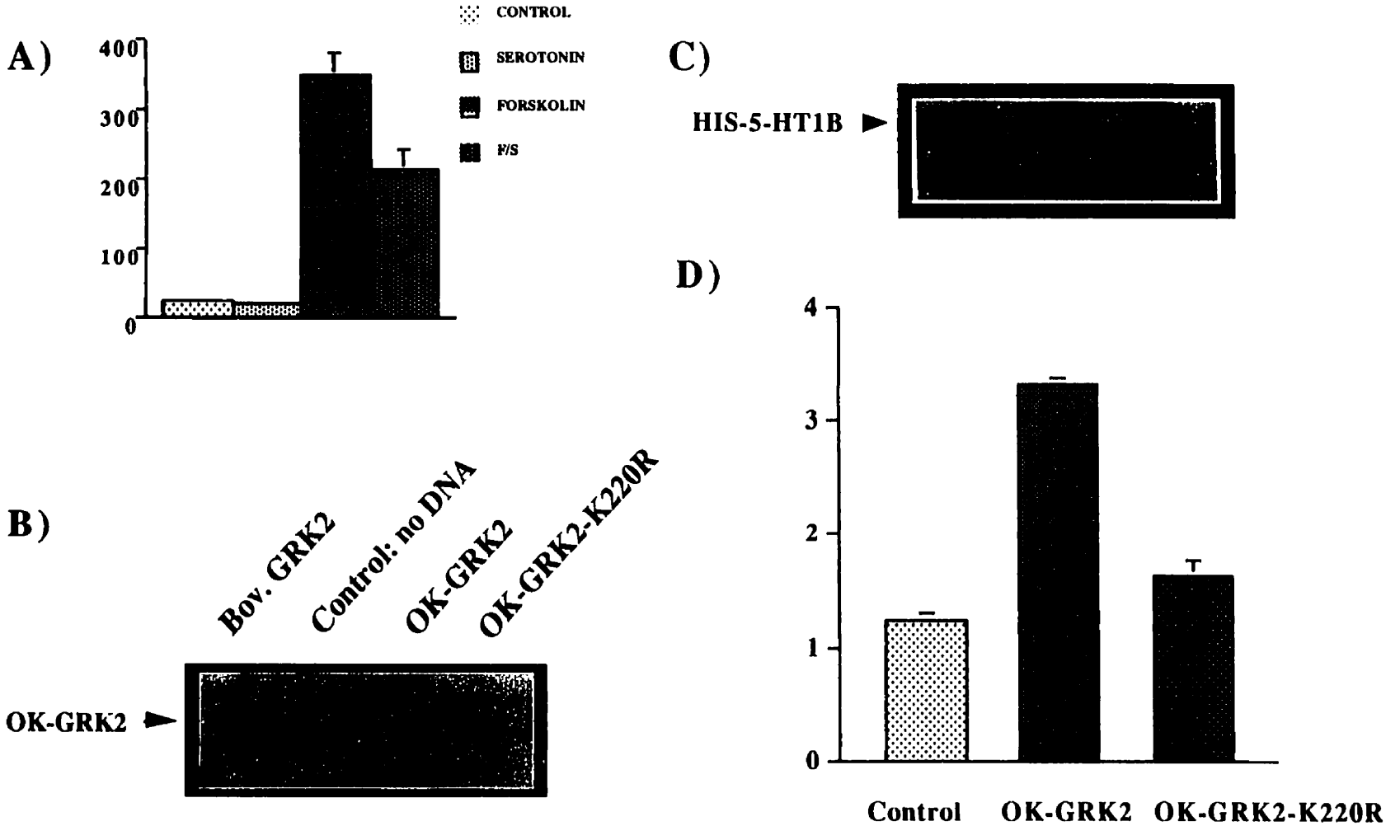


Figure 5. Western analyses of stably transfected OK-GRK2 clones.

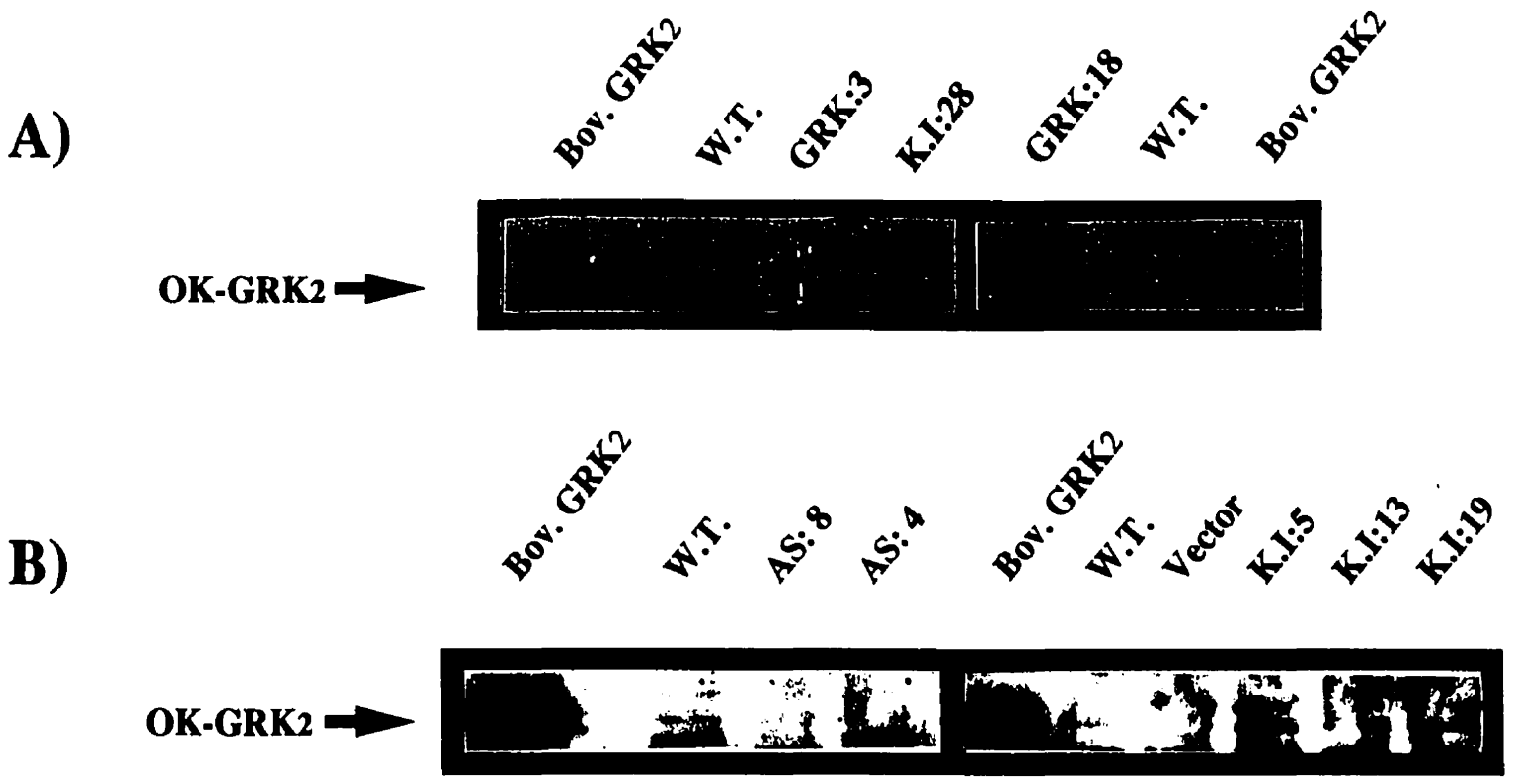


Figure 6: Agonist-induced desensitization of the 5-HT1B receptors.

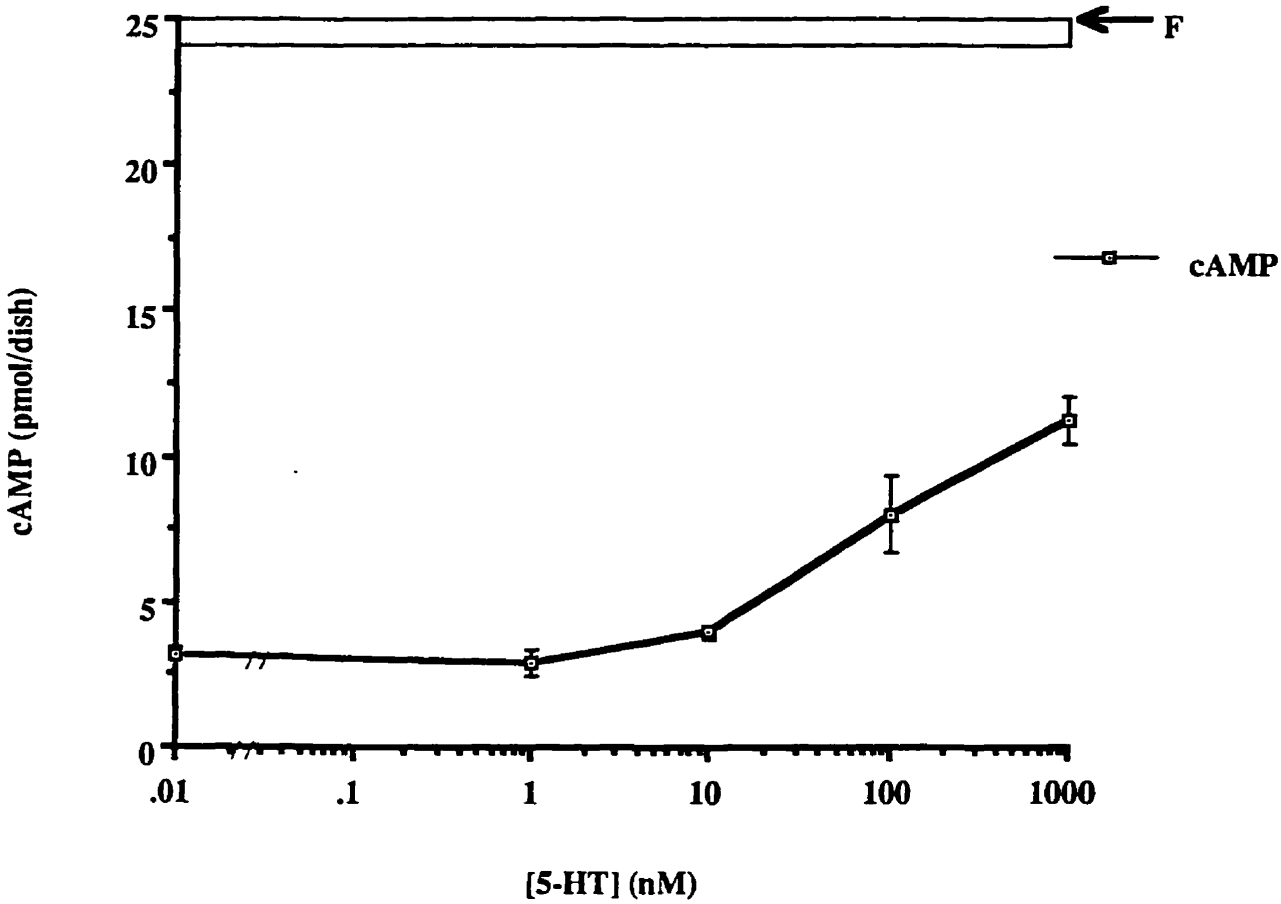


Figure 7. Inhibition of cAMP accumulation mediated by 5-HT1B receptors and a2C receptors.

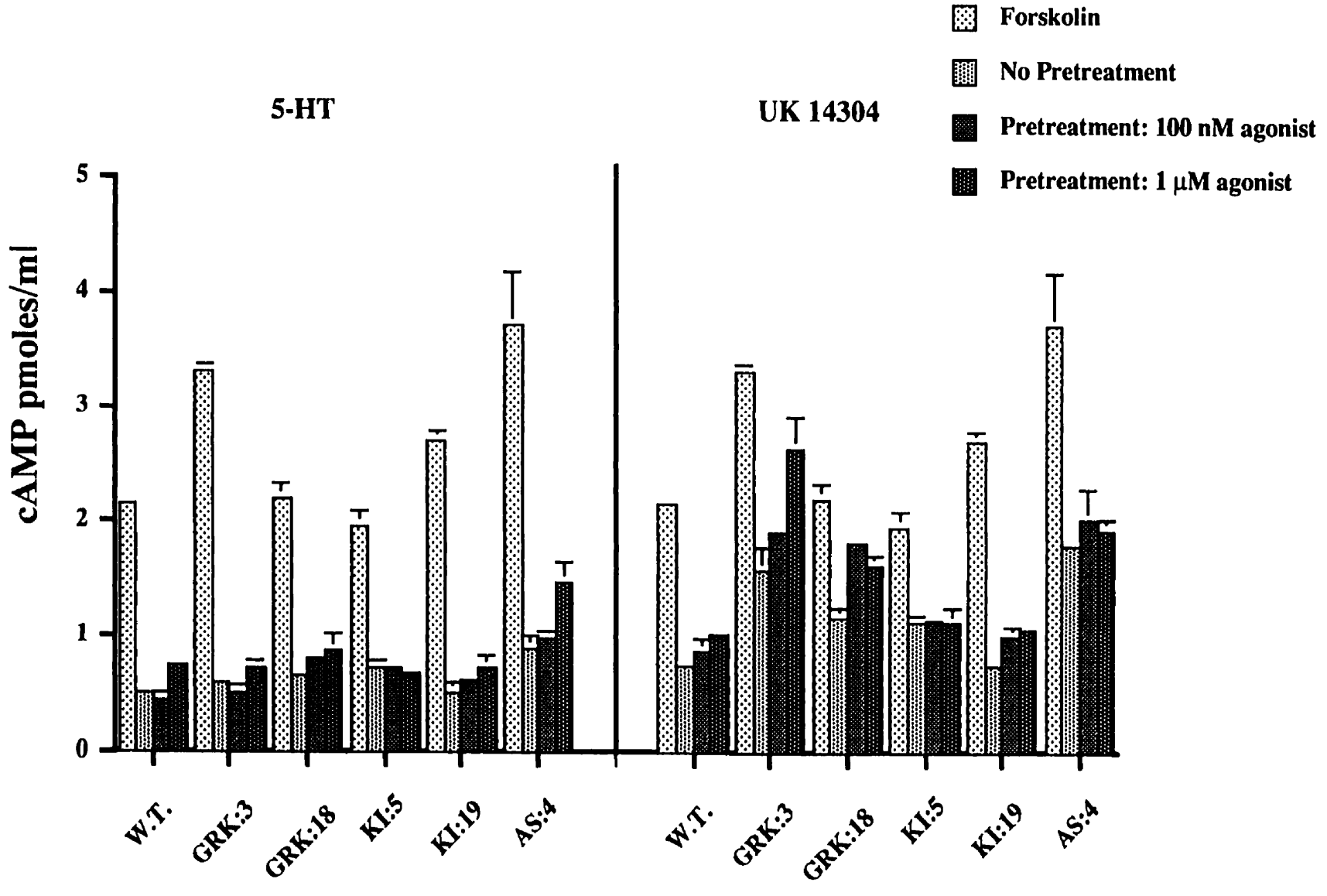
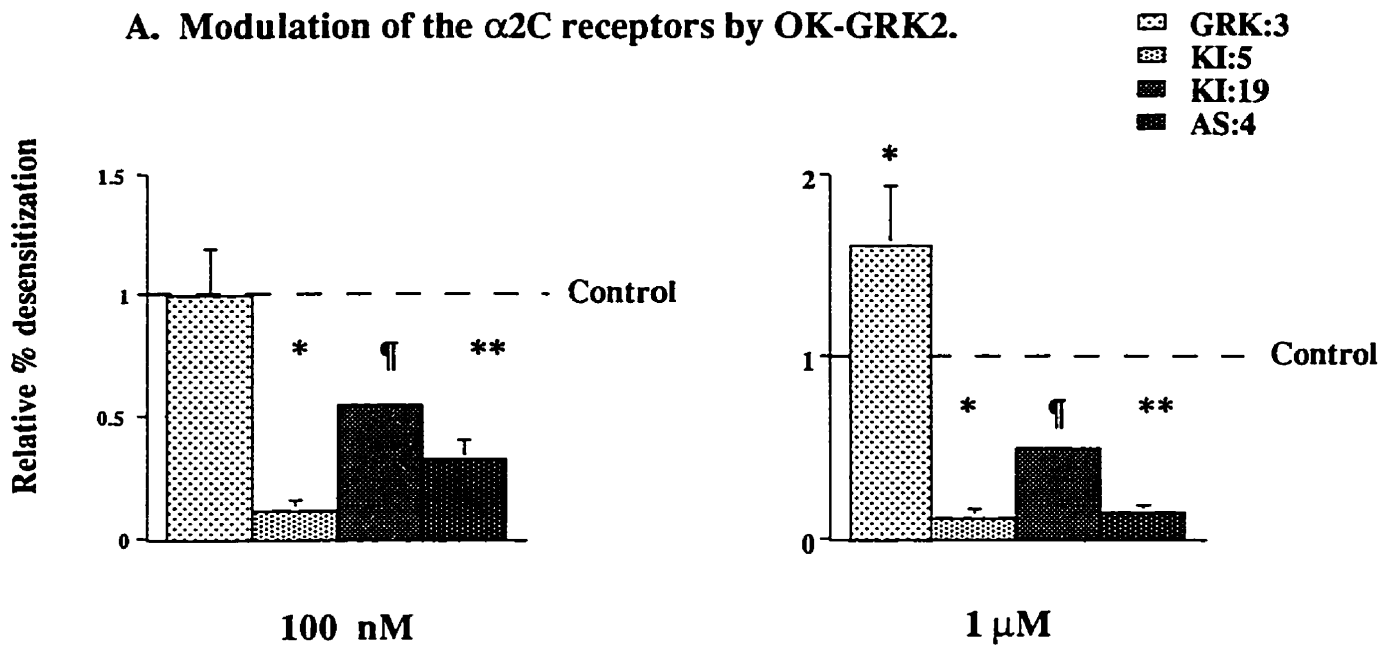
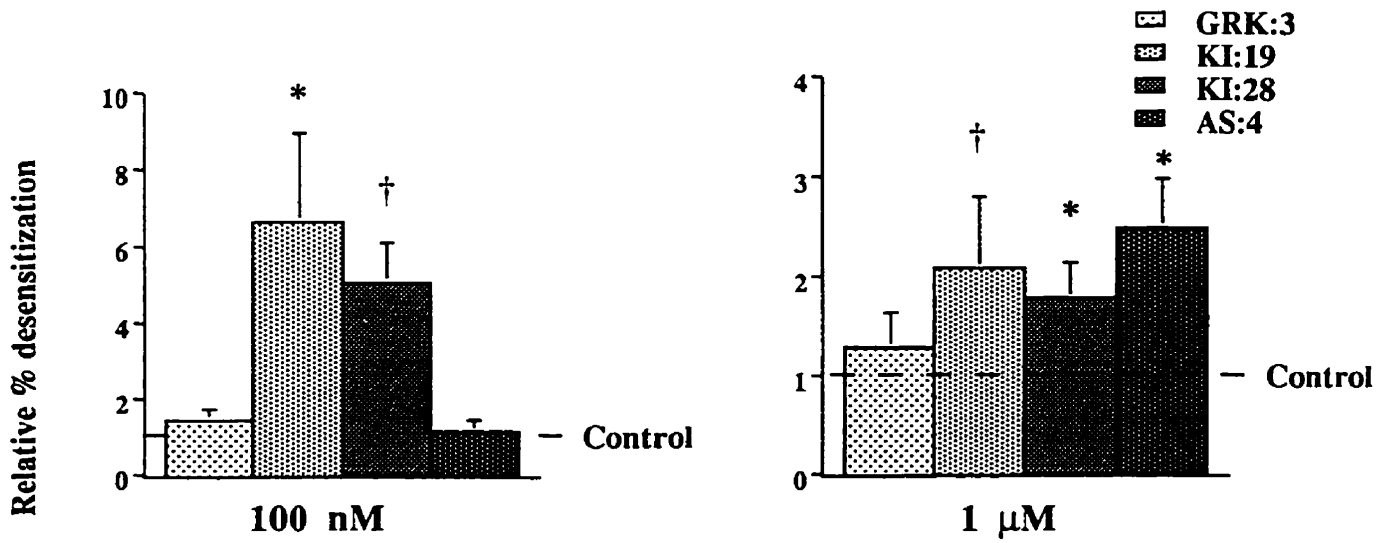


Figure 8. Relative % desensitization of the α2C and 5-HT1B receptors





Summary and General Discussion

Clinical evidence points to a critical involvement of the serotonergic system in the pathophysiology of depression and anxiety (231-233). Biochemical, electrophysiological and behavioural studies have all implicated serotonin receptor-mediated events as significant contributions in the onset of depression and anxiety (231-235). As previously mentioned, sustained administration of SSRIs only exert their full antidepressant effects after a few weeks of treatment (231). The same clinical profile has been observed with 5-HT1A receptor agonists involved in alleviating symptoms of anxiety (233). Blier and de Montigny hypothesized in the late eighties that the delay in onset for the antidepressive actions of SSRIs was due to the progressive desensitization of the presynaptic 5-HT1A receptors (or somatodendritic autoreceptors). This eventually results in the normal firing activity of serotonergic neurotransmission due to the desensitization process of the 5-HT1A autoreceptor. Interestingly, it has also been postulated that terminal receptors, known to inhibit the release of 5-HT in the synaptic cleft are of the 5-HT1B subtype (5-HT1D α and β in humans). These autoterminal receptors have been shown to desensitize following treatment with SSRIs (231-235). This type of desensitization allows more 5-HT to be released in the synaptic cleft and enhances serotonergic neurotransmission. These adaptive responses by some serotonin receptor subtypes may explain the delayed enhancement of serotonergic neurotransmission in alleviating certain types of major affective disorders. Hence, understanding the regulation and structure-function relationships in short-term desensitization of serotonin receptors is quite important. Deciphering their regulation at a molecular level might not only provide insight as to why this delay exists but might also help to elucidate more effective serotonergic drugs.

Therefore, we were interested in the regulation and structure-function relationships of serotonin receptors particularly the heterologous desensitization process and the G-protein coupling domains of 5-HT1A receptor in fibroblasts and neuron-like cells. Furthermore, we also decided to examine and investigate the mechanism of agonist-induced desensitization of the 5-HT1B receptor endogenously expressed in the opossum kidney cell line (OK). The use of molecular techniques will allow us to isolate the functions and critical responses of serotonin receptors within a model system and hopefully apply these novel findings to the serotonergic biological system.

Summary of Results, Implications and Future Directions.

Chapter 1. Multiple phosphorylation sites are required for pathway-selective uncoupling of the 5-HT1A receptor by protein kinase C. *Mol. Pharmacol.* 48:1024-1029 (1995).

As previously described functional desensitization is defined as a decrease in receptor responsiveness due to prolonged exposure to agonist (149). Desensitization of G protein-coupled receptors requires receptor-uncoupling. This immediate phase of receptor-G-protein uncoupling is characterized by the activation of protein kinases which phosphorylate the receptor. Activation of some protein kinases requires the presence of second messengers. Several G-protein-coupled receptors are known to be positively coupled to PI hydrolysis, this results in the generation of two second messengers, IP3 and DAG (154). IP3 is known to mobilize intracellular calcium stores whereas DAG activates PKC. PKC is believed to be a key player in signal transduction. Pre-activation of PKC, a second messenger kinase, results in a loss of receptor-mediated PI turnover and calcium mobilization. Thus, PKC is able to act in a negative feedback loop such that PLC activation generates a product (DAG) that inhibits phospholipase activation via PKC. Several receptors are regulated by this mechanism including Gs, Gq- and Gi/Go-coupled receptors (161-170).

In 1991, Raymond demonstrated that acute activation of PKC by PMA could phosphorylate and desensitize the 5-HT1A receptor suggesting receptor phosphorylation as the prime mechanism of PKC-mediated heterologous desensitization (163). This was followed by Liu *et al.* observations that acute pretreatment with TPA abolished the 5-HT1A-receptor-mediated mobilization of intracellular calcium response but did not reduce nor alter the receptor-mediated inhibition of cAMP accumulation (19). These biochemical experiments only demonstrated a correlation between phosphorylation and PKC-mediated desensitization. More direct evidence was needed to establish the role of phosphorylation by PKC in uncoupling the 5-HT1A receptor.

We were interested in determining at what level PKC acted upon to inhibit receptor coupling to the PLC effector pathway: was it at the receptor, the G-protein or the effector system?

Previously, biochemical studies had demonstrated that each signalling component of the receptor-G protein-PLC-β signalling system including the G-protein and the effector pathway could be phosphorylated by PKC (255, 256). Unfortunately, these studies did not address which constituent was the principal target and which one played the prominent role in PKC-mediated heterologous desensitization.

Previous evidence strongly suggested that the 5-HT1A receptor could be the target for PKC action. The approach we decided to use to address and identify the site of PKC was site-directed mutagenesis of consensus PKC phosphorylation sites of the receptor. We believe that this is the most direct way because it not only eliminates the phosphate acceptor hydroxyl side-chain (serine/threonine residues) by converting the residues to structurally similar aliphatic amino acids such as alanine or glycine, but it also precludes the phosphorylation reaction without altering the integrity of the receptor's structure. This method has the advantage of selectively modifying an individual target site of PKC action without altering the widespread actions of PKC (161).

We identified four consensus phosphorylation sites on the receptor, three sites located in the i3 loop and one in the i2 loop domain. These putative PKC phosphorylation sites are highly conserved in the human, rat and murine 5-HT1A receptors. Site-directed mutagenesis was used to generate the following mutant 5-HT1A receptors in the third intracellular loop; three single point mutants T229A, S253G, and T343A, which substituted alanine and glycine for the phosphate acceptors threonine and serine, respectively, a double mutant T229A/S253G and a triple mutant T229A/S253G/T343A. The mutants were stably transfected into receptor-negative Ltk- cells to investigate PKC's actions. This mutational analysis combined with other experimental approaches demonstrated the following;

1) All of the i3 loop 5-HT1A receptor mutants were functional at inhibiting forskolin-stimulated cAMP accumulation and 5-HT-mediated calcium mobilization responses. The calcium and cAMP responses mediated by T229A mutant were reduced as compared to wild-type.
2) Pretreating the **single point mutants** with 100 nM TPA, completely abrogated the 5-HT-mediated calcium mobilization response but had no effect on the adenylyl cyclase pathway.
3) Receptor phosphorylation uncoupled the receptor selectively from activation of PLC but still

allowed coupling to inhibition of adenylyl cyclase as assessed by the EC50 values. These values were indistinguishable for the 5-HT-induced inhibition of cAMP accumulation in the presence or absence of TPA for both the wild-type and triple mutant receptors.

4) The 5-HT-induced calcium response was still present following acute pretreatment of the double and triple mutants with TPA ($54 \pm 6\%$ and $74 \pm 7\%$, respectively).

These observations provided the first direct evidence that eliminating multiple PKC phosphorylation sites on the receptor precluded the complete uncoupling of the 5-HT1A receptor from the PLC signalling pathway in Ltk- cells. This study establishes not only A) a causal link between receptor phosphorylation and PKC-mediated uncoupling of the 5-HT1A receptor from the stimulation of PI hydrolysis but also B) that receptor phosphorylation by PKC plays the predominant role in heterologously desensitizing the 5-HT1A receptor from the PLC pathway in Ltk- cells. Although there is still some residual inhibitory effect of PKC activation on the triple mutant which could be mediated by phosphorylating other sites on the receptor, G proteins, or effector, the evidence strongly indicates that PKC-mediated phosphorylation of the 5-HT1A receptor is the principal mechanism for heterologous desensitization. In addition, site-directed mutagenesis and chimeric studies were also used as techniques to demonstrate that certain PKC sites played critical roles in modulating the regulation process of the β2-adrenergic receptor, the GABA-A receptor, the neurokinin 2 receptor, the gastrin-releasing peptide receptor and the voltage-gated calcium channels (164, 257-260). Recently, the glucagon-like peptide-1 receptor in COS cells and Chinese hamster lung fibroblasts was also shown to require at least three PKC sites in the C-terminal tail for PKC-mediated heterologous desensitization (261).

This novel "pathway-selective" view of desensitization contrasts with β-adrenergic receptor uncoupling where one signalling pathway, stimulation of adenylyl cyclase has been investigated thoroughly but other pathways have not been examined. We have demonstrated that the pathway-selective modulation of the 5-HT1A receptor by PKC involves phosphorylating multiple sites on the receptor and this implies that receptor phosphorylation by PKC can sculpt the signalling properties of the 5-HT1A receptor.

The structural basis for the specificity of PKC to selectively block receptor-mediated

calcium mobilization but not cAMP accumulation was not specifically addressed in this study. The third cytoplasmic loop has clearly been shown to be important for G protein coupling and subtle modifications in this domain, such as phosphorylation or point mutation, may modify the signalling ability of the receptor. Point mutations in the C-terminal region of the third loop of several G-protein coupled receptors have resulted in constitutive activation of receptors (137-144). All of these studies demonstrate that side-chain modifications at a single amino acids can drastically alter the selection of receptor signalling pathways.

Does suppression of receptor-mediated calcium mobilization by acute activation of PKC occur *in vivo* ? The opossum kidney cell line which endogenously expresses the 5-HT1B receptor, a close relative of the 5-HT1A receptor, not only induced a 5-HT-mediated calcium mobilization response but when acutely treated with TPA, the calcium response was completely inhibited without significantly blocking receptor-mediated inhibition of cAMP accumulation (39). The same profile was observed with the transfected human 5-HT1B receptor in Ltk- cells (38). Since the PKC sites we have mutated in the 5-HT1A receptor are conserved in the human and opossum 5-HT1B receptors, those sites may also mediate PKC-induced uncoupling of the 5-HT1B receptor from PLC pathway in both cell lines. This study implies that the pathway-selective modulation by PKC is not only observed with receptors such as the 5-HT1A receptor expressed exogenously but also with endogenously expressed 5-HT1B receptor. A neuronal cell-line endogenously expressing the 5-HT1A receptor has been characterized, it remains to be established if acute activation of PKC can differentially regulate this receptor in this cell line (262). The selective modulation mediated by PKC has been observed with the dopamine D2 receptors and the olfactory epithelium where preactivation of PKC blocks the PI turnover mediated by receptors without affecting the receptor-mediated cAMP signal (162, 263).

Since the 5-HT1A receptor has never been detected to positively couple to the PLC pathway in the CNS, what possible role could PKC-mediated uncoupling play in the brain? Recently, a study was published demonstrating that PKC activation induced pathway-selective modulation in neurons. Acute (min) pretreatment with TPA showed that the 5-HT1A receptor was partially uncoupled from inhibition of calcium channel opening but remained coupled to the opening of potassium channels (264). This suggests that 5-HT1A receptor signalling can be selectively

modulated *in vivo* by preactivation of PKC. What does this selective channel blockade of 5-HT1A receptor mediated events by PKC mean with respect to antidepressants? The presynaptic 5-HT1A receptors are known to stimulate the opening of K+ channels causing hyperpolarization and inhibition of neuronal firing (265). Long-term treatment with antidepressants results in the desensitization of presynaptic 5-HT1A receptors followed by a decrease in the inhibition of firing and an enhancing effect on the release of 5-HT in the synaptic cleft. Selective modulation of the 5-HT1A receptor by PKC *in vivo* would the open the K+ channels resulting in the hyperpolarization of membranes and a decrease in not only neuronal firing but also in neurotransmitter release. Hence, PKC could theoretically potentiate the activity of antidepressants by decreasing "the delayed onset" of the therapeutic effects of these agents by setting in motion the desensitization mechanism for the presynaptic 5-HT1A receptors.

Future Directions.

Several questions still remain unanswered. For example which PKC isoform is responsible for mediating the pathway-selective uncoupling of the 5-HT1A receptor ? One approach is to initially find out which PKC isoforms are expressed in this cell line, followed by sequentially eliminating the isoforms using either antisense technology or using a selective irreversible binding agent to each isoform in order to determine which PKC is responsible for mediating this pathway-selective modulation of the 5-HT1A receptor in Ltk- cells.

It still unclear why the PLC pathway and not the adenylyl cyclase pathway is selectively blocked by PKC-mediated receptor phosphorylation. Evidence from our laboratory suggest that multiple Gi/Go proteins couple the receptor to PLC whereas a single G-protein subtype couples to cAMP. The inhibition of cAMP accumulation was demonstrated to be mediated by the Gi class particularly the Gi2 subtype as assessed by antisense experiments and reconstitution studies (245-246). Whereas, multiple G protein subtypes have been shown to be required for the PLC activation since the response was not reconstituted by any single subtype of G protein (244). One possible explanation for the differential PKC-mediated desensitization is that since multiple Gi/Go proteins couple the receptor to PLC and a single subtype couples the receptor to the adenylyl cyclase, an incomplete uncoupling of receptor-G protein interaction may be sufficient to block the 5-HT1A receptor from coupling to calcium mobilization. One approach to use in order to determine if

multiple G-proteins are indeed require for 5-HT1A receptor-PLC coupling is to use the insect Sf9 system. This model system was used to express the 5-HT1A receptor and the signalling properties have been characterized (266). In addition, the insect system has recently been utilized to explore the functional interaction and coupling efficacy between, several G-proteins and a variety of βγ combinations, and the 5-HT1A receptor (267). These observations suggest that this cell system can confer proper functional interactions between the 5-HT1A receptor and G-proteins. Hence, it can probably be used to determine and investigate specific G-protein(s) uncoupling induced by selective PKC isoforms. How does phosphorylating the receptor occlude receptor–G-protein coupling? The transfer of phosphate moieties on serine and threonine residues creates a negatively-charged milieu which probably causes the receptor to undergo some conformational change such that the G-proteins are sterically hindered from making potential contact sites with the receptor and the receptor system becomes uncoupled.

Furthermore, future studies in the laboratory will be directed at determining the signalling properties and characteristics of the double and triple 5-HT1A receptor mutants in neuronal-like cells. Current work in our laboratory is underway to determine the signal transduction features of these mutants and the possible regulation by PKC in neuronal-like cells.

To conclude, Chapter 1 demonstrated that multiple PKC consensus phosphorylation sites located in the third cytoplasmic loop of the 5-HT1A receptor mediates pathway-selective uncoupling of the receptor from calcium mobilization by acute PKC activation in Ltk- cells.

Chapter 2. A conserved threonine residue in the second intracellular loop of the 5-HT1A receptor directs signalling specificity. P. M. C. Lembo, M. H. Ghahremani, and P. R. Albert (*under revision*).

G-protein coupled receptors transmit their binding signal to effector molecules via heterotrimeric G proteins. Understanding the binding interface or contact sites for the receptor and G-proteins has been a major goal in the field of signal transduction. Most of the research in deciphering coupling regions has focused on the areas of receptors and G-proteins required for

activation. Current scientific investigations using anti-receptor antibodies, synthetic peptides and mutational analysis have all revealed that several cytosolic regions of G-protein coupled receptors particularly residues in close proximity to the transmembrane domains participate in the functional coupling and interaction of receptors and G-proteins (68, 69, 92).

To fully understand the molecular mechanisms governing the specificity of receptor-G-protein coupling, specific amino acids required for interactions between the receptor and G-proteins must be identified. The role of individual receptor amino acids in directing the selection of specific signalling pathways must be determined. A close examination of the molecular structure of the 5-HT1A receptor revealed a potential PKC phosphorylation site located at T149 in the i2 loop, located in a predicted amphipathic α-helical domain, a region that has been postulated to be critical in G-protein coupling (68, 69, 79, 92). Sequence alignment of peptide sequences corresponding to this region in other G-protein-coupled receptors also revealed a striking homology of this threonine residue. Furthermore, receptors known to couple to Gi/o proteins possess a BBTXBB motif (X=P/T/S, B= basic residue) which corresponds to a consensus PKC phosphorylation sequence (158, 159). The akin AATXBB (A = aliphatic residue) sequence has been identified in several Gs- and Gq- coupled receptors as well (268).

Receptor mutagenesis studies have indicated a potential role for the i2 loop in coupling to certain Gi-linked pathways (68, 69). A chimeric receptor in which the i3 loop of the Gi-coupled muscarinic m2 receptor was replaced with the i3 loop of the β2–adrenergic receptor coupled to both Gs and Gi. Thus, indicating that the ability to stimulate Gi did not reside solely in the i3 loop (99). Varrault and colleagues demonstrated the importance and potential role of the i2 loop in 5-HT1A receptor-mediated cAMP signalling response using synthetic peptides. Peptides corresponding to the entire i2 loop were shown to strongly inhibit forskolin-stimulated adenylyl cyclase activity (100). These studies clearly suggested a pivotal role for the i2 domain in Gi-mediated signalling. We therefore examined the possibility that part of the i2 loop of the 5-HT1A receptor might be involved in Gi/o coupling to its effectors. Since this threonine is conserved in a multitude of Gi/Go/Gq-coupled receptors we postulated that it may play a role in receptor-G-protein interactions. We addressed the potential role of this conserved threonine residue could have on the functional and signalling properties of the 5-HT1A receptor. The point mutagenesis approach was

used to generate the 5-HT1A receptor mutant T149A, eliminating the hydroxyl side-chain which could theoretically serve as a contact point for receptor-G-protein interactions. The wild-type and mutant receptors were stably transfected in receptor-negative Ltk- cells and GH4C1 cells to investigate if multiple pathways of 5-HT1A receptor were affected. Using site-directed mutagenesis and several biological assays we demonstrated the following:

1) When transfected in GH4C1 cells, the T149A 5-HT1A receptor mutant had the following signalling characteristics:

A) Inhibition of basal and Gs-stimulated cAMP formation was still present.

B) The mutant receptor failed to couple to the inhibition of calcium channel activation.

2) When expressed in Ltk- cells, the T149A 5-HT1A receptor mutant displayed the following signalling features:

A) The receptor was completely uncoupled from the calcium mobilization pathway.

B) The T149A mutant still retained the ability to inhibit cAMP accumulation.

These results suggest that the threonine residue located at position 149 in the second loop of the 5-HT1A receptor is a critical site for receptor-G-protein coupling to the PLC pathway and the inhibition of calcium influx via calcium channels. The role of the second loop in PKC-mediated desensitization could not be addressed since this point mutation completely uncoupled the receptor from the PLC signalling pathway. This also suggests that a modification such as phosphorylation at this residue (T149) may mediate the selective uncoupling of this receptor.

Our observations are in agreement with the results of Van Koppen *et al.*, where the equivalent proximal threonine residue in the second loop of the muscarinic m4 receptor was mutated to an alanine residue and found not to alter the inhibition of adenylyl cyclase but to affect agonist-induced receptor internalization (269). Our results clearly demonstrate the importance of the second loop having a role in proper PLC coupling for the 5-HT1A receptor. Interestingly, a similar B-B-X-X-B motif present in the second loop of the m1 muscarinic receptor was recently mutated (only the basic amino acids next to the threonine) and found not to be required for PI

hydrolysis (270). Although, preliminary mutagenesis data from the same laboratory suggest that this region participates in agonist-induced desensitization and receptor internalization. It remains to be established what the exact role of this invariant threonine in the C-terminal region of the second loop in the m1 muscarinic receptor is responsible for. This particular motif B-B-X-X-B located at the C-terminal region of the third loop of the α1B-adrenergic receptor was recently shown to specifically activate Gα14 in COS-7 cells (271). This not only suggests the importance of this motif in receptor-G-protein coupling but also implies that this region may be a specific contact site for Gi/o protein.

Blin and colleagues recently demonstrated using chimeric and mutational analyses that the second loop of the m3 receptor greatly contributed in stimulating PI hydrolysis as well as proper Gq/11 recognition for the m2 receptor. Furthermore, mutational analysis enabled them to pinpoint four critical amino acid residues in the second loop responsible for conferring the ability of the m3 receptor to couple to Gq/11 protein (122). These amino acid residues lie in the same region as the threonine 149 of the 5-HT1A receptor which confers PLC pathway-selectivity. These results are consistent with the earlier report of Wong *et al.* where the second loop in the m1 muscarinic receptor was replaced with the equivalent loop corresponding to the β1-adrenergic receptor and the resultant mutant was less efficient at stimulating the PI pathway (102).

Recently, a specific G-protein contact site was identified in the m2 muscarinic receptor. A four amino acid epitope (VTIL) located at the C-terminal region of the third cytoplasmic loop of this receptor was shown to be responsible for interacting with Gi/o proteins. They also identified that this threonine residue was a highly conserved residue among Gi/o-coupled receptors (132). The similar threonine residue in the 5-HT1A receptor, T343 was found not to significantly contribute to the G-protein coupling on its own (176). In this regard, the threonine at position 149 in the second loop of the 5-HT1A receptor and other Gs/i/q coupled receptors may play the equivalent role in being an essential elemental site in receptor-G-protein interactions.

Why was inhibition of cAMP accumulation by the mutant 5-HT1A receptor still present in both Ltk- and GH4C1 cells? One possible explanation is that the i2 loop threonine residue alone, when mutated to an alanine residue, is not sufficient to hinder or mask critical sites for receptor-G-protein coupling to the adenylyl cyclase effector. This could explain why synthetic peptides

corresponding to the entire second loop of the 5-HT1A receptor strongly inhibited cAMP production (100). Synthetic peptides corresponding to nine amino acids in the C-terminal region of the second loop of the m2 muscarinic receptor were also shown to be crucial in mediating the inhibition of adenylyl cyclase (99).

What potential role can these regions play in conferring G-protein selectivity and what possible feature do they all have in common that enables these domains to be critical components in receptor-G-protein interactions? When examining secondary-structure prediction algorithms they reveal that these regions of receptors as well as synthetic peptides form a hydrophilic amphipathic α-helical structure which protrudes in the cytoplasmic milieu. This protrusion probably facilitates the contact sites between receptor and G-proteins by properly arranging contact and recognition sites. Interestingly, T149 is located in a hydrophilic area and probably makes contact with potential G-proteins via hydrogen bonding. Once mutated to an alanine residue the loss of the hydroxyl group side chain abrogates the possibility for hydrogen interactions and the contact site between receptor and G-protein is no longer feasible. Hence, phosphorylation of this residue (T149) by PKC could have similar consequences, disrupt the H-bonds by the transfer of a phosphate moiety and interfere with receptor-G-protein-interactions.

Future Directions. Obviously, one issue to address is the question which we were not able to directly investigate: is the PKC consensus site in the second loop of the 5-HT1A receptor critical for PKC-mediated heterologous desensitization? One way to unravel this is again to use site-directed mutagenesis to mutate one or several of the basic amino acid residues flanking the threonine which form part of the PKC consensus site and investigate if these mutants when acutely pretreated with TPA occlude the PLC pathway (provided that they do not have a similar signalling profile as T149A 5-HT1A receptor). This is an issue which definitely requires further testing since this particular domain has been implicated in coupling to both the adenylyl cyclase and PLC pathways in many receptors including the 5-HT1A receptor, the TSH receptor and the m3 muscarinic receptor (99, 100, 123). It was recently shown that synthetic peptides corresponding to this region of the i2 loop in the muscarinic receptor was indeed a substrate for PKC-mediated phosphorylation (272). This implies that phosphorylation of this residue may mediate the complete uncoupling of the receptor. The action of PKC was also previously addressed demonstrating that

multiple phosphorylation sites in the i3 loop of the receptor selectively uncoupled the receptor from the calcium mobilization pathway without affecting the cAMP pathway. However, mutation of two sites had a lesser effect and a single mutated PKC site had a similar functional profile as the wild-type receptor (176). These observations suggest and reinforce our hypothesis that coupling to the calcium mobilization pathway requires multiple Gi/Go proteins and that the same phosphorylation events are not sufficient to uncouple all responses initiated by the receptor. The T149 residue located in the i2 loop may recreate the selective inactivation of calcium mobilization observed following PKC treatment. Thus, phosphorylation of threonine in the i2 loop, in addition to the i3 sites, may mediate the action of PKC to selectively block receptor-induced calcium mobilization. Alternatively, another hypothesis could be that the i2 loop may be the only critical G-protein contact site for the 5-HT1A receptor. One way to test this would be to create pseudosubstrate domains of the putative PKC sites in the i3 loop, acting as intramolecular inhibitors of PKC and this would allow us to investigate if the i2 loop is the sole contact site.

Since our results imply that distinct 5-HT1A receptor domains may couple differentially to α_o, α_i and $\beta\gamma$ subunits within the Gi/Go family one question to address is which G-proteins are responsible for mediating the signalling specificities of the 5-HT1A receptor in Ltk- and GH4C1 cells. As previously mentioned, our laboratory has clearly demonstrated that the Gαi2 is responsible for the inhibition of cAMP accumulation mediated by the 5-HT1A receptor in both Ltk- and GH4C1 cells whereas multiple G-proteins and high levels of $\beta\gamma$ subunits are required for 5-HT1A receptor-mediated calcium mobilization. Whereas, inhibition of calcium channel opening mediated by G-protein coupled receptors seems to require Gαo and is also dependent on specific combinations of $\beta\gamma$ subunits (236-239, 249). Another unresolved issue is concerning the selective G-protein coupling in GH4C1 cells. The mutant T149A 5-HT1A receptor failed to block the influx of calcium via calcium channels but inhibition of cAMP accumulation was present in GH4C1 cells. What is the reason for this selectivity? Expressing the T149A 5-HT1A receptor in cells with different combinations of $\beta\gamma$ subunits and G-proteins may elucidate which G-protein or $\beta\gamma$ subunits are needed for coupling.

To conclude, this chapter provides the first evidence in receptor-G-protein coupling that a single amino acid residue, threonine 149 located at the C-terminal region in the second intracellular

loop of the 5-HT1A receptor, plays a central and pivotal role in directing specific receptor-G-protein interactions resulting in cell-specific coupling to calcium mobilization or inhibition of calcium influx and but still capable of coupling to the inhibition of adenylyl cyclase.

Physiological Relevance.

Both chapters have addressed the mechanism of PKC-mediated receptor desensitization and PKC-mediated receptor signalling. We have demonstrated that PKC can selectively modulate the functional activities of the 5-HT1A receptor *in vitro*. We have confirmed that the pathway selective uncoupling of the 5-HT1A receptor involves phosphorylation of multiple serine and/or threonine residues located in close proximity to receptor regions previously shown to be pivotal domains in receptor-G-protein interactions. In essence, acute activation of PKC selectively uncouples the 5-HT1A receptor via receptor phosphorylation.

As previously mentioned, Penington and coworkers have recently shown a pathway-selectivity modulation by 5-HT1A receptor in raphe neurons. Activating PKC acutely was shown to uncouple the 5-HT1A receptor from inhibition of calcium channel opening but the receptor could still partially couple to the opening of potassium channels. It is quite tempting to speculate that the threonine residue 149 in the second loop of the 5-HT1A and the PKC sites located in the third loop of the receptor might have similar mechanisms in neuronal cells: that is when phosphorylated it differentially modulates the function of the 5-HT1A receptor in cells and allows for pathway selectivity to occur.

Chapter 3. Cloning and characterization of the opossum GRK2: Desensitization of Gi-linked inhibition of cAMP accumulation by α2C-adrenergic but not 5-HT1B receptors in intact OK cells. P. M. C. Lembo and P. R. Albert (submitted for publication).

The first two chapters dealt with the modulation of the 5-HT1A receptor by PKC. We

established that preactivation of PKC desensitizes the 5-HT1A receptor in a heterologous manner in Ltk-cells and that the second loop of the 5-HT1A receptor plays a crucial role in receptor-G-protein coupling. We were also interested in determining if the 5-HT1A receptor was subject to agonist-induced desensitization or homologous desensitization. Furthermore, we decided to characterize the molecular basis for homologous desensitization occurring in a physiological system. At the time these studies were initiated a cell line endogenously expressing the 5-HT1A receptor had not yet been established. But the opossum kidney cell line (OK) had not only been characterized but it expressed several Gi-linked receptors including the 5-HT1B receptor and α-2C receptor which had previously been shown to undergo agonist-induced homologous desensitization (273, 274). Hence, chapter 3 investigates the process of homologous desensitization with respect to endogenously expressed Gi-coupled receptors.

Homologous desensitization is defined as the loss of receptor responsiveness in the presence of agonist (149). This process has extensively been studied with the β2-adrenergic-adenylyl cyclase system and the light-sensitive receptor rhodopsin (149, 189-191). This mechanism involves several independent and sequential events: a) the binding of agonist followed by receptor-G-protein uncoupling b) activation of a serine/threonine GRK-mediated phosphorylation reaction of the receptor and d) the binding of arrestin which is believed to uncouple the receptor-G-protein system (189-191).

Agonist-induced receptor attenuation is a fundamental characteristic in receptor regulation. The GRKs and arrestins have mainly been characterized and studied *in vitro* using biochemical approaches and over-expression in heterologous cell systems. Shih and Malbon demonstrated using an antisense approach that homologous desensitization mediated by either second messenger-kinases (PKC or PKA) and/or second-messenger independent kinases (GRKs) was cell-specific (226). These observations not only raise the issue of physiological relevance in the regulation of receptors by these kinases but also suggest that agonist-induced receptor desensitization should be studied in a cell system endogenously expressing the receptor of interest. Thus, we decided to use the opossum kidney cell line (OK) to study receptor desensitization since it endogenously expresses the 5-HT1B and α-2C receptors. Both receptors have been shown to undergo

homologous desensitization and thus must express endogenous GRKs and arrestins which regulate the receptors. We therefore attempted to clone opossum GRKs and arrestins to investigate their potential mechanism for agonist-induced receptor desensitization and also to assess if GRK receptor-specificity exists "*in vivo* ".

Using cloning techniques and a physiological cAMP specific radioimmunoassay we demonstrated the following:

1) Two GRKs were identified by RT-PCR amplification of OK cell RNA: a full-length 3.054 kb cDNA was cloned, sharing 92% homology at the amino acid level with the rat GRK2 and named OK-GRK2. The other PCR fragment shared 93% with GRK3.
2) Northern analysis revealed the presence of two mRNA transcripts of 5.0 and 3.0 kb.
3) A kinase-inactive mutant OK-GRK2-K220R was generated and both the mutant and wild-type kinases were functional based on *in vitro* phosphorylation assay of the 70-kDa His-tagged 5-HT1B receptor.
4) OK-GRK2 displayed receptor specificity: no significant effect was observed with the 5-HT1B receptor whereas the overexpressed kinase significantly enhanced the desensitization response of the α2C-receptors as compared to wild-type.
5) The kinase inactive mutant OK-GRK2-K220R and antisense significantly diminished the agonist-induced desensitization of the α2C-receptor while having in some cases an enhanced effect on the 5-HT1B receptor-mediated desensitization response.

These results suggest that the OK-GRK2 demonstrates receptor-specificity in the OK cell line: no effect is observed with the 5-HT1B receptor-mediated desensitization but OK-GRK2 significantly enhances the agonist-induced desensitization of the α2C-receptor *in whole cells* .

The first question is why does the OK-GRK2 phosphorylate the 5-HT1B receptor in *vitro* and not induce the loss of receptor response? In this report, we demonstrate for the first time that the 5-HT1B receptor is a substrate for GRK2-mediated phosphorylation *in vitro*. Recently, elegant mutagenesis experiments were performed to determine potential sequence motifs that would be

targeted for GRK-mediated phosphorylation (217, 218). Interestingly, the opossum 5-HT1B receptor bares such motifs in the third intracellular loop (275). Although, these experiments suggest that the 5-HT1B receptor is a substrate for GRK2 *in vitro,* these results imply that GRK-mediated phosphorylation of receptors in *vitro* does not necessarily mimic receptor desensitization *in vivo*. Hence, it is imperative that agonist-induced desensitization mediated by the phosphorylating activities of GRKs be investigated in a physiological context. Shih and Malbon elegantly demonstrated this concept using antisense RNA/DNA technology where the contributions of several kinases including PKA, PKC and GRK2 were found to be cell specific in mediating agonist-induced desensitization of the β2-adrenergic receptors (226).

One possible explanation as to why phosphorylation by OK-GRK2 is not sufficient to enhance agonist-promoted desensitization of the 5-HT1B receptor as compared to the parental cell line, is that phosphorylation by GRK2 occurs *in vitro* but not *in vivo* . It may be that other endogenously expressed GRKs are responsible for fully phosphorylating the receptor and mediating homologous desensitization. Recently, GRK-specific monoclonal antibodies were generated and used to demonstrate receptor-specificity mediated by endogenous GRK phosphorylation (276). This is an approach which can be used in the future to investigate and determine if endogenously expressed GRKs are capable of phosphorylating the opossum 5-HT1B receptor. Or alternatively, phosphorylation is not sufficient to necessarily promote desensitization and may require the appropriate arrestins which may or may not be expressed in this particular cell line. Thus, the *in vitro* assay conditions for the phosphorylation may not mimic the *in vivo* desensitization situation.

Our results demonstrate that OK-GRK2 mediates Gi-linked receptor-specificity within the OK cell line. We have demonstrated that the OK-GRK2 enhances agonist-promoted desensitization of the α-2C receptors compared to the parental cell line. Although not addressed in this study, these results suggests that the opossum α-2C receptor is a potential substrate for OK-GRK2. Interestingly, the opossum α-2C receptor has been cloned and shown to be the α-2C4 subtype (277). The third intracellular loop has several serines and threonines flanked by acidic amino acid residues, regions known to be potential GRK phosphorylation sites (189, 217, 218). The human equivalent receptor subtype has never been shown to undergo functional

desensitization (198, 213, 278). These observations are clearly in conflict with our results and earlier reports demonstrating that the opossum α-2C4 receptor does indeed undergo agonist-induced desensitization (274).

A consistent observation in this study was that the kinase-inactive mutant (OK-GRK2) enhanced the 5-HT1B receptor desensitization. This observation is quite peculiar since it has been shown with other receptors to block this event. This kinase-deficient GRK2 mutant was previously shown to block agonist-induced desensitization of the endogenously expressed β2-adrenergic receptor and not PGE2 receptors when overexpressed in bronchial cells (210). The same mutant kinase also prevented the acute-desensitization of the m2 muscarinic receptor and the type 1A angiotensin II receptor in HEK 293 cells (167, 213). Interestingly, Divani *et al.* recently demonstrated that the kinase inactive mutant of GRK2 when overexpressed impaired the α1B-receptor-mediated PI hydrolysis (203). These observations are in agreement with our results demonstrating that the OK-GRK2 K220R mutant enhances the attenuation response of the 5-HT1B receptor-mediated inhibition of cAMP accumulation. Why would the inactive kinase enhance receptor desensitization rather than block this process? There are two possible explanations a) mutation of this conserved lysine residue in the catalytic domain could have altered the association and dissociation constants of the enzyme such that it associates or binds to the receptor via specific βγ subunits with higher affinity and dissociates more slowly, thus acting as an arrestin-like protein or b) the K220R mutation causes the OK-GRK2 to sequester unknown critical downstream components of the signalling system and compete with activation of a particular pathway. Evidence for the latter possibility exists. Recently, PIP2, a component of the PLC pathway was shown to bind to the pleckstrin domain and inhibit the functional activity of GRKs suggesting maybe that this mutation increases the affinity of GRK2 for PIP2 (227-229). An analogous situation could also be occurring with the 5-HT1B receptor-adenylyl cyclase system in the OK cells where this inactive mutant might be sequestering either specific combinations of βγ subunits or specific opossum adenylyl cyclase subtypes or maybe an unknown component downstream essential for this pathway. The antisense clone had a similar effect on the 5-HT1B-mediated receptor attenuation response at 1μM 5-HT only. This could represent a possible negative feedback loop where altering the level of a specific endogenous GRK may nonselectively increase

the activity of another GRK. In contrast, the kinase inactive and the antisense clones both significantly abrogated the desensitization mechanism of the α2C receptors.

Future Directions

In 1991, Cassill *et al.* isolated several GRKs from *Drosophila.* Based on the sequence alignment of several species of GRKs we designed degenerate oligonucleotides corresponding to the catalytic domain, the most highly conserved region of GRKs (280). Using RT-PCR we isolated two fragments of 380 bps from the OK cell line corresponding to GRK2 and GRK3. Only the full length GRK2 was cloned and characterized. The possibility of other GRKs being expressed in the OK cell line exists and might be responsible for regulating the agonist-induced desensitization process of other endogenously expressed G-protein coupled receptors in the OK cell line in a substrate-specific manner. This is one aspect that should be further investigated since several G-protein coupled receptors endogenously expressed in the OK cell line have been shown to undergo agonist-induced desensitization including the PTH receptor and the dopamine D1-receptor (281, 282).

The endogenously expressed 5-HT1B receptor in the OK cell line has been shown to couple to both the adenylyl cyclase and PI hydrolysis via pertussis-toxin sensitive G-proteins (38). We have demonstrated that the kinase inactive mutant may play a minor role in enhancing the desensitization process of adenylyl cyclase mediated by 5-HT1B receptor. Another unresolved issue is can the OK-GRK2 impair the calcium mobilization response mediated by the opossum 5-HT1B receptor? Furthermore, there also exists cross-talk between PKC and GRK. Recently, it was shown that PKC could phosphorylate GRK2 *in vitro* (226-228). It has been established in our laboratory that the opossum 5-HT1B-mediated calcium response is negatively regulated by PKC (38, 39). What happens to the PI-response mediated by the 5-HT1B receptor when PKC is acutely preactivated in cell lines overexpressing OK-GRK2 and the kinase inactive mutant since there is now evidence linking GRKs to the regulation of the PLC pathway (203)? These are issues which will require further investigation.

Are there arrestin proteins expressed in this cell line? The same strategy was used, degenerate oligonucleotides to conserved regions were designed and used to RT-PCR total RNA derived from this cell line. A fragment of 380 bp corresponding to arrestin 2 was isolated.

Unfortunately, a full-length clone was not isolated from the cDNA library. Cloning full-length arrestins from the cell line and overexpressing these proteins will enable us to determine if desensitization is enhanced. This is another matter which necessitates further investigation.

One intriguing issue to resolve is does the kinase inactive mutant bind to an unknown downstream component of the signalling system or does it bind with increased affinity to the receptor-$\beta\gamma$ complex? One approach to address this is to perform cross-linking experiments in the cell lines to establish if indeed the kinase inactive mutant is acting as an arrestin.

Physiological Relevance

This thesis suggests that the 5-HT1A receptors in Ltk- cells and the endogenously expressed 5-HT1B receptors in OK cells may be regulated and modulated by PKC rather than by specific GRKs. This regulatory situation may completely differ in neuronal cells. The simplest approach to address if these serotonin receptor subtypes undergo agonist-induced desensitization in neuronal cells via GRKs is to use a characterized neuronal raphe cell line or isolate and create a specific line to answer this question. The initial step would be to determine if the endogenous serotonin receptors are functional and undergo desensitization. Once this is determined RNA would be isolated from this cell line, followed by RT-PCR using degenerate oligonucleotides to the catalytic domain of several GRKs, identification of potential GRKs and a functional desensitization experiment in a cell line not expressing these factors such as the Sf9 cell system.

Summary and Conclusions

In the first chapter of the thesis we have demonstrated that acute activation of PKC can selectively modulate the 5-HT1A receptor by phosphorylating multiple sites in the third intracellular loop and have established that phosphorylation of the receptor is the prime mechanism for PKC-mediated heterologous desensitization. We have also shown in the second chapter that the highly conserved threonine which forms part of a PKC consensus phosphorylation site in the i2 domain of this receptor is a critical receptor-G-protein contact site in both Ltk- and GH4C1 cells. This suggest that phosphorylation events can potentially result in a selective uncoupling of the receptor

from one or several subtypes of G-proteins. In the last chapter we have suggested that the OK-GRK2 displays substrate-specificity and enhances agonist-induced desensitization of the α2C receptors and not the 5-HT1B receptors.

The clinical evidence suggests that several serotonin receptors such as the 5-HT1A and 5-HT1B receptors are desensitized following the longterm treatment of antidepressive therapies. Since the clinical effects of antidepressants coincide with the desensitization of these receptors: the above observations might provide insights as to how to promote desensitization of these receptors more quickly and effectively. For instance activating a nearby PLC-linked receptor would generate DAG, the second messenger which could activate PKC and selectively modulate the activities of the 5-HT1A receptor. It may be that these initial steps are needed to set in motion the down-regulation of these receptors so that the clinical effects of antidepressants can occur more rapidly.

Implications

Is there any direct evidence suggesting that desensitization plays a critical role *in vivo* ? Many studies using either reconstituted vesicles or over expression in heterologous systems have demonstrated that individual GRKs are not only capable of phosphorylating certain receptors but also induce homologous desensitization of receptors including the α1B, α2C and β1-adrenergic receptors, the δ-opioid receptor, dopamine D1A and the angiotensin type 1A receptor (167, 183, 198, 201-202, 213). But one intriguing question still remains, do endogenously expressed kinases truly mediate receptor phosphorylation and desensitization *in vivo* ? Recent findings have suggested a critical and vital role for GRK2 *in vivo*. Firstly, the levels of GRK2 are elevated during chronic heart failure suggesting an imbalance in receptor function (206). Secondly, transgenic mice expressing GRK2 were generated and shown to exhibit a significant attenuation in isoproterenol-mediated contractility of the heart. This observation indicated that the β-adrenergic receptors are *in vivo* substrates for GRK2 as assessed by binding isotherms indicating that a large percentage of the receptors were in the uncoupled low-affinity state (208). Thirdly, mice overexpressing the C-terminal region of GRK2 (region where the βγ subunits bind, hence quench endogenous GRK2), displayed a marked enhancement in inotropy (208). Lastly, "knockout" mice with a disruption in the GRK2 gene manifested severe cardiac malformations suggesting an

essential and integral role for GRK2 in fetal development (283). Interestingly, other members in the GRK family, namely GRK3, shares a high homology with GRK2 but the critical role of GRK2 could not be compensated for by other constituents further supporting the conclusion that GRKs are critical and specific factors *in vivo*. In addition, other components in the cascade leading to G-protein coupled-receptor desensitization such as arrestins have also been shown to be important *in vivo* . In 1992, Richard and colleagues demonstrated that activated rhodopsin was functionally inactivated within milliseconds *in vivo* (284). This was followed by elegant experiments showing that photoreceptor-specific arrestin derived from *Drosophila* were essential elements in mediating the termination response of rhodopsin activation in the photosignal transduction pathway (222). This study also demonstrated that in the absence of functional arrestins, the photoreceptor cells degenerated due to the continuous activation of the phototransduction cascade. Recently, arrestin binding was found to determine the kinetics of rhodopsin inactivation *in vivo* suggesting that the rate-limiting step in receptor inactivation is arrestin binding (285). This illustrates the need for regulatory mechanisms to inactivate and regenerate receptors for the maintenance of biological equilibrium. All of these observations support the hypothesis that receptor desensitization by GRKs and arrestin proteins not only occur *in vitro* but also in *vivo*.

Is receptor desensitization a phenomenon observed *in vivo* following long term treatment with drugs? In 1968, it was hypothesized that the β-receptors could be dysfunctional in asthma (286). The issue of tolerance has become an important aspect concerning the therapeutic efficacies of long-acting β-agonists, evidence regarding receptor desensitization and the use of β-agonists exists, but remains controversial. Several studies suggest that the clinical significance in the modest reduction in the density of β-adrenergic receptors with longterm treatment may be critical while others postulate that a decrease in receptor responsiveness is a minor component with little clinical consequence due to the large receptor reserve in the airway (287). In contrast, longterm treatment with SSRIs have unequivocally shown that the 5-HT1A and 5-HT1B receptors are desensitized, suggesting that receptor desensitization does indeed occur pharmacologically (231-234, 288). Although physiological desensitization has never been reported *in vivo*, the data derived from transgenic mice or *Drosophila* and knockout experiments do indeed suggest that these factors are crucial and vital for normal physiologic regulation.

My research endeavours will contribute to understanding the molecular mechanisms governing receptor desensitization, which is necessary if we are to investigate the regulation of receptor function *in vivo* and may even lead to possible therapeutic manipulations. More work is still needed to fully investigate the mechanisms and roles of desensitization *in vivo*, which as cited can vary depending on the receptor and the site of expression.

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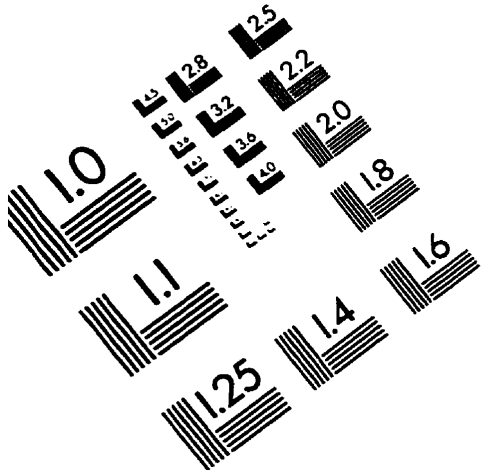

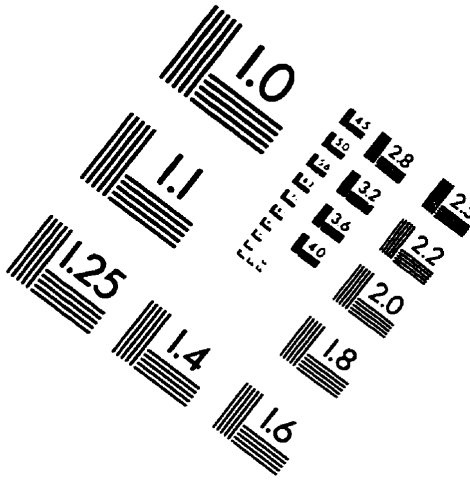

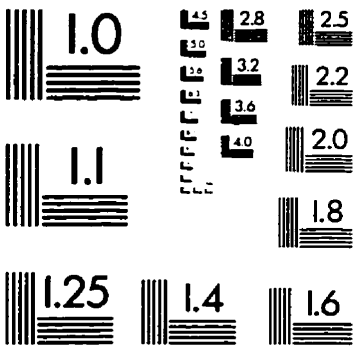

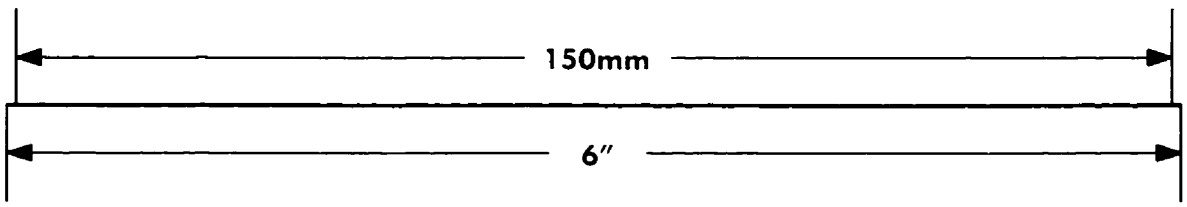

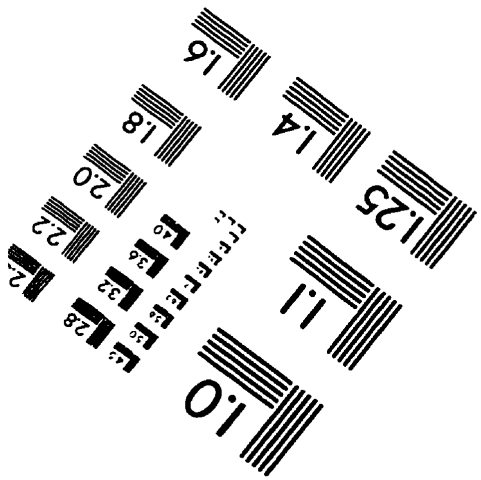

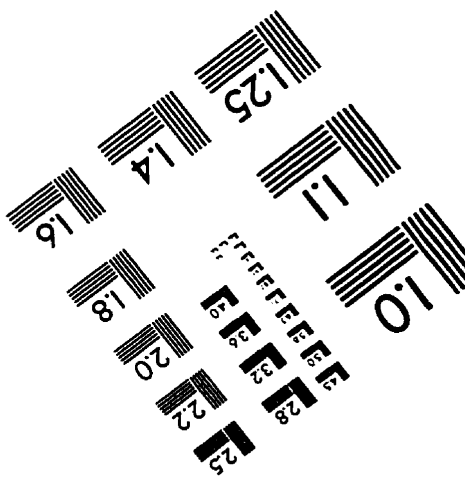

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