Role of Specific Amino Acid Residues in the Intracellular Domains of Human D1 Dopamine Receptors

by

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List of Abbreviations

$\alpha$2AR: $\alpha$2-adrenergic receptor;
$\beta$2AR: $\beta$2-adrenergic receptor;
BHK cells: baby hamster kidney cells;
CCKBR: cholecystokinin B receptor;
CHO cells: Chinese Hamster Ovary cells;
CRFR: Corticotropin releasing factor receptor;
D1DR: D1 dopamine receptor;
D2DR: D2 dopamine receptor;
DA: dopamine;
DMEM: Dulbecco's Modified Essential Medium;
eNOS: endothelial nitric oxide synthase;
ETAR: endothelin A receptor;
ETBR: endothelin B receptor;
GPCR: G protein-coupled receptor;
GRK: G protein-coupled receptor kinase;
5-HT1AR: 5-hydroxytryptamine1A receptor;
5-HT1BR: 5-hydroxytryptamine1B receptor;
Kd: dissociation constant;
LH/CGR: luteinizing hormone/chorionic gonadotropin receptor;
M2AChR: m2 acetylcholine receptor;
M3AchR: m3 acetylcholine receptor;
mGluR4R: metabotropic glutamate receptor subtype 4;
MOR: μ opioid receptor;
PKA: cAMP-dependent protein kinase;
PKC: protein kinase C;
SEM: standard error of mean;
SSTR5: somatostatin receptor type 5;
TM: transmembrane domain;
TRHR: thyrotropin releasing hormone receptor;
TSHR: thyrotropin receptor;
TxA2-R: thromboxane A2 receptor;
V2R: V2 vasopressin receptor;
VIP: vasointestinal peptide;
VTA: ventral tegmental area.
Role of Specific Amino Acid Residues in the Intracellular Domains of Human D1 Dopamine Receptors

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Abstract

In the past decade, over 300 G protein-coupled receptors (GPCR) have been cloned. Much has been learned in the structure-function relationship of these receptors. However, many questions still remain to be solved.

D1 dopamine receptor (D1DR) plays an important role in central nervous system and in periphery. The role of specific amino acid residues in the intracellular domains of D1DR was investigated. I demonstrated that the D1DR was palmitoylated at the Cys347 and Cys351. These two cysteines were replaced with alanines both individually and together. The data revealed that palmitoylation was not involved in G coupling.

A consensus PKA sequence into the carboxyl tail of D1DR. The data and analysis using the extended ternary complex model demonstrated that the PKA sequence close to the palmitoylation site conferred constitutive desensitization to D1DR.

A spontaneous serine mutation of the first intracellular loop caused a constitutive activation of MSH receptor. It was shown that the serine to leucine mutation in the corresponding region of D1DR did not elicit constitutive activation or enhanced receptor-G protein coupling.
The significance of the cdc2 phosphorylation site in the second intracellular loop of D1DR was investigated. It was shown that removal of the cdc2 site could abolish D1 receptor internalization, suggesting that this phosphorylation site, in addition to PKA/PKC and GRK sites, might also be involved in agonist-induced receptor internalization.

The relationship between receptor density and maximal adenylyl cyclase response was examined. The data revealed that efficacy as measured by the response (maximal activation of adenylyl cyclase) varied with receptor density and reached a saturated state with receptor overpression.

All the studies taken together signify that each GPCR may have unique features and should be studied individually. Conclusions obtained from one receptor or a 'prototype' should not be generalized freely to others only on the basis of homologous sequence or common topography.
Chapter 1

General Introduction

Part of this chapter was modified from the following review:

1.1 G Protein-Coupled Receptors in General

Many functions of cells and organs are controlled through a process known as signal transduction that involves the conversion of a variety of extracellular hormones or neurotransmitters (first messengers) into intracellular signals (second or third messengers) that ultimately modulate the activity of multitudinous effectors. A common pathway involves the binding of the first messenger to a cell surface receptor, which activates various types of G proteins. The activated G proteins, in turn, modulate a number of intracellular enzymes (adenylyl cyclases and phospholipases) or ion channels.

Inherited and acquired defects that alter the function of the first messenger or other transducing components (G protein coupled receptor, G protein, etc.) can lead to defective signal transduction that causes diseases. Clinical examples of defects of those signaling components have been described (reviewed by Raymond, 1994).

The G protein-coupled receptor (GPCR) superfamily consists of over a thousand members that interact with numerous hormones, autacoids, light, gustatory and olfactory signals, neurotransmitters, and paracrine substances. The cloning of genes encoding many of the GPCRs has allowed the delineation of the structure-function relationships for this class of receptors. Different regions of GPCRs play different roles in receptor function. The relationship between the structure and functions of G protein-coupled receptors has been extensively investigated and reviewed (Dohlman et al, 1991; Strader et al, 1994). GPCRs are single protein molecules that span the plasma membrane seven times, with the amino terminus being in the extracellular space and the carboxyl terminus being in the cytoplasm. The amino terminus and three extracellular loops, typically contain N-glycosylation sites, the functions of which are unknown, and highly conserved.
cysteine residues which may be critical for maintaining the proper tertiary structure of the receptor (Dohlman et al, 1990). For small endogenous ligands such as epinephrine, norepinephrine, acetylcholine, dopamine, or serotonin, the seven transmembrane stretches appear to be critical in the formation of the ligand binding pocket. For peptide hormones, the amino terminus and the extracellular loops may also participate in ligand binding by forming docking sites for the hormone. The carboxyl terminus and three intracellular loops, contain binding sites for G proteins and phosphorylation sites for protein kinase A (PKA), protein kinase C (PKC), and G protein-coupled receptor kinases (GRKs) (Lefkowitz, 1993a), which are implicated in heterologous (PKA and PKC) and homologous (GRK) forms of receptor desensitization.

Mutations, natural or engineered, of receptors might lead to an array of defective receptor functions, or the production of overly sensitive or constitutively active receptor molecules. The clinical manifestations of those defects or constitutive activities would depend on the severity of receptor dysfunction, the distribution of the receptor population, and the functions which the particular receptor subserves. Critical mutations of widely distributed receptors could be lethal even in utero. Dysfunction of receptors with highly specific distributions or functions might be expected to have highly distinctive clinical manifestations. Minor mutations of receptor genotype may lead to minor variations in phenotypes that have few clinical consequences.

Both inherited and acquired mutations causing defects or constitutive activation in the function of the G protein-coupled receptors have been described. For example, in familial nephrogenic diabetes insipidus, the ability of V2 receptors to bind to vasopressin is impaired by several mutations (Raymond 1994), leading to loss of renal urine
concentrating ability. Other examples of mutations causing defective receptors and
diseases are autosomal dominant and autosomal recessive retinitis pigmentosa
(rhodopsin), color blindness (blue opsin), steroid-dependent asthma (β2-AR), etc.
(reviewed by Raymond, 1994).

Similarly, the constitutive activation of many receptors has been widely described
and reviewed (reviewed by Lefkowitz et al, 1993b and Raymond, 1994), some of which
have been associated with disease. For example, a single amino acid change, resulting in
constitutive activation of the gonadotropin LH receptor, was described in individuals with
familial male precocious puberty (Shenker et al, 1993). The Jansen type of metaphyseal
chondrodysplasia is another example, in which a constitutively active PTH-PTHr
receptor causes a profound hypercalcemia and short-limbed dwarfism (Schipani et al,
1995). Many other receptors with constitutive activities have been described, e.g., α2-
adrenoceptor (Ren et al, 1993), melanocyte-stimulating hormone receptor (Robbins et al,
1993), D5 (D1B) dopamine receptor (Tiberi, 1994), vasoactive intestinal peptide 1
receptor (Gaudin et al, 1998), and δ opioid receptor (Befort et al. 1999).

Therefore, with the burgeoning investigations into the structure and functions of
GPCRs, a full understanding of these receptors and their role in pathogenesis of diseases
will be gained. Those developments undoubtedly presage an exciting era of discovery of
how defective receptors could lead to diseases. On the other hand, the abundance of
available information and the increasing number of cloned GPCRs offer a great
opportunity for development of therapeutic agents for many diseases.

1.1.1 Ligand Binding of GPCRs
In recent years, we have seen an explosion of primary sequence information due to the widespread application of molecular cloning techniques. GPCRs bind a variety of ligands ranging from small biogenic amines to peptides, small proteins, and large glycoprotein hormones. Despite the wide range of ligands that activate these receptors, the overall structural features of the GPCR family are highly conserved (Dohlman et al, 1991; Strader et al, 1994; Ji et al, 1998) as described above. The majority of the primary sequence homology resides within the hydrophobic transmembrane domains, whereas the hydrophilic N-terminus, C-terminus, and extracellular and intracellular loops are more divergent. The seven transmembrane (TM) regions are arranged as a closed loop in the counterclockwise direction from TM1 to TM7 when viewed from the extracellular surface. The primary sequences of GPCRs share 85-95% homology for species homologues, 60-80% homology for related subtypes, 35-45% for other members of the same family and 20-25% for unrelated GPCRs (Strader et al, 1994).

Upon detailed analysis of their sequences, GPCRs can be further classified into three subfamilies, known as the rhodopsin (or Family A), secretin/vasointestinal peptide (VIP) (or Family B), and metabotropic glutamate (mGlu) receptor families. Members of the rhodopsin/β-adrenergic subfamily (or Family A) constitute the majority of GPCRs identified to date. In addition to rhodopsin and the β2-adrenergic receptors, this family includes receptors for other small molecules such as dopamine and serotonin, as well as peptides such as substance P, bradykinin, angiotensin, and somatostatin, and glycoproteins such as luteinizing hormone. The structure-function relationships between ligand binding to the receptor and signal transduction to G-proteins have been extensively
studied for several members of this family. The secretin/VIP and mGlu families share little primary sequence homology with each other or with the rhodopsin/β-adrenergic family of receptors, although their overall predicted tertiary structure is similar.

Because of the paucity of tertiary structural information on the family of GPCRs, investigators are combining biochemical, pharmacological, and genetic approaches with molecular modeling and biophysical analysis to identify key elements in the signaling mechanism of these receptors.

Unfortunately, obtaining high-quality crystals for structural determination of membrane proteins remains extremely difficult. Until the structure of at least one GPCR is determined at atomic resolution, the structural insights can only be inferred from mutagenesis studies combined with computer modeling analyses. The current 3-dimensional models of GPCRs will continue to be modified as additional experimental data become available. Meanwhile, receptor modeling can provide a visualization tool with which to formulate testable hypothesis that can be verified by receptor mutagenesis and/or new ligand design.

The binding domains of GPCRs for small molecules of ligands were determined mainly through mutagenesis studies of adrenergic receptors. Initial deletion mutagenesis experiments in β2-AR indicated that the hydrophilic loop regions of the receptor were not important for agonist or antagonist binding, suggesting that the ligand binding sites of the β2-AR must involve residues within the hydrophobic transmembrane domain. More detailed analysis of the binding site relied on mutagenesis within the TM regions of the β2-AR. All adrenergic agonists and antagonists were basic amines, suggesting that there was an acidic residue in the receptor that can provide the counter-ion for the amine on the
ligand. Systematic mutagenesis of the negatively charged residues in the transmembrane domain of the β2-AR implicated Asp113 in TM 3 as the counter-ion for the basic amines in both agonists and antagonists. Subsequent analysis indicated that whereas this ionic interaction is a major source of binding energy between the ligand and the receptor, it is not critical for receptor activation. In fact, both agonists and antagonists interact with the side chain of Asp113 in the receptor (Strader et al, 1994). In contrast, agonist activation of the β-AR was determined to involve an interaction of the catechol ring of the agonist with residues in TM 5 and TM 6. The catechol-containing aromatic ring appears to interact with the side chain of Phe290 in TM 6, whereas the catechol hydroxyl groups of the agonist form hydrogen bonds with the side chains of two Ser residues, at positions 204 and 207 in TM 5. The specific interactions between the ligand and the receptors at these positions were elucidated using 2-dimensional mutagenesis, in which the activation of these mutant receptors by analogs of isoproterenol having each of the hydroxyl groups replaced with a hydrogen were analyzed. The studies indicated that there is a specific hydrogen bond linking the side chain of Ser204 to the meta-hydroxyl group of the ligand and a second specific hydrogen bond linking Ser207 in the receptor to the para-hydroxyl group of the agonist (Strader et al, 1989). Molecular modeling predicts that these two Ser residues would be located one helical turn apart in TM5 of the receptor, suggesting that the simultaneous interaction of the catechol ring with these two Ser hydroxyls and Phe290 in TM6 would serve to orient the agonist very specifically in the binding pocket of the receptor. Further analysis of several GPCRs in various laboratories has indicated that the third intracellular loop connecting TM 5 and TM 6 plays a role in receptor-G protein interactions (Dohlman et al, 1991).
Examination of the related GPCRs reveals that the binding points of the 2 Ser in TM5 and the Phe in TM6 for the catechol ring of β2AR agonists are representative of a similar binding pocket for all biogenic amine receptors and that the ionic receptor-ligand interaction at Asp113 (or Asp at equivalent position) of the TM3 is conserved across the family of receptors (Wess et al, 1991; Dohlman et al, 1991; Strader et al, 1994).

Now that about 40 different biogenic amine GPCRs have been cloned, it is possible to determine, by simple inspection of the primary sequence of a GPCR, whether the natural ligand for that receptor is a biogenic amine. The presence of an Asp at the position analogous to 113 in transmembrane helix 3, along with the pattern of residues in TM 5 and TM 6 described above, is diagnostic of a biogenic amine receptor and can be used to identify such a receptor from a group of orphan receptors whose ligands are not known.

The endogenous ligands for many GPCRs are peptides, ranging from 3 (thyrotropin-releasing hormone) to 40 amino acid residues (glucagon) in length. Conceivably, the ligand binding domains of these receptors may differ from those of receptors for small molecules. Site-directed mutagenesis experiments carried out mainly in neurokinin (NK) receptors, have indicated that the peptide agonist-binding domain for the peptide receptor is much more diffuse than that of biogenic amine receptors, and may involve the extracellular domains (the N-terminus and the extracellular loops) as well as the TM regions (reviewed by Strader et al, 1994 and Ji et al, 1998).

The discovery of morphine as a non-peptide agonist of the opiate receptor has long been a proof that it is possible to develop nonpeptide ligands for peptide receptors. Since the nonpeptide ligands are substantially smaller than their natural peptide ligands
and approach the size of biogenic amines, it seemed likely that the nonpeptide ligands bind to the TM regions. Extensive mutagenesis studies showing that the extracellular domains of the NK1 receptor play a minor role in nonpeptide antagonist binding (Strader et al, 1994) confirms the above hypothesis, suggesting that pharmacologically defined competitive relationships arise from a mutual exclusion effect and do not require that the peptides and the antagonists make the same intermolecular contacts with the receptor.

Glycoprotein hormones, LH, FSH, hCG and TSH, are the largest GPCR agonists, for which receptors consist of two domains of equal length, a 300-400 residue N-terminus and a membrane-traversing portion of about 300 amino acids (Ji et al, 1998). These hormones bind exclusively to the N-terminus, which undergoes conformational changes and elicit secondary changes in the membrane-traversing domain. Both the α and β subunits of the glycoprotein hormones bind with the N-terminal segment, and the liganded N-terminus secondarily contact with the three extracellular loops to generate a signal. In fact, mutations in the three extracellular loops have been reported to result in constitutive activation of the glycoprotein hormone receptors.

It is obvious that there exist diverse modes of ligand binding for GPCRs of small molecules, peptides and glycoproteins. The characterization of detailed structures of G protein-coupled receptors has been greatly facilitated by mutagenesis (as well as biophysical analyses and modeling). Modeling and mutagenesis data suggest the existence of a general small-molecule-binding site in GPCRs for peptides, perhaps a residual biogenic amine or retinal site, which can potentially be exploited for the design of small-molecule antagonists for this class of receptors. Also, the discovery of a multitude of "new" receptor subtypes that are homologous but distinct from those
previously characterized pharmacologically, has opened possibilities for therapeutic specificity by permitting the targeting of specific receptor subtypes in tissues of interest. The integration of genetic analysis with molecular modeling will ultimately provide higher-resolution structural data and lead to the design of more potent and specific medications that act through members of the GPCR superfamily.

1.1.2 The Activation of G Proteins by GPCRs

The GPCRs rely on a large family of G proteins to mediate signal transduction to the effectors. The original notion that a receptor activates a single effector pathway within a particular cell has been changed to the current concept of great promiscuity among individual receptors, G proteins and effectors. It is now clear that a single receptor can activate several different pathways in a given cell, although the predominant pathway may vary depending on cell types. Much of the research into the interaction between receptors and G proteins has focused on localizing the sites of contact, the sequences required for activation, sequence elements controlling specificity, and elements involved in desensitization and uncoupling from G protein. GPCRs are embedded in the membrane, exposing only a small surface to the cytoplasm. This surface is composed predominantly of the ends of the transmembrane helices and the connecting loops. It is now clear from several lines of evidence that much of this surface is involved in the interaction with the G proteins (Gether and Kobilka et al, 1998). In particular, mutational studies have implicated residues at the cytoplasmic ends of all helices and the portions of the loops closest to the membrane as critical for overall interaction with G protein and
specificity. Initial studies with the β2AR demonstrated that removal of residues from either end of the third intracellular domain uncoupled the receptor from G protein (Dohlman et al., 1991). In addition, mutations within the second intracellular domain and at the proximal end of the C-terminal tail also reduced the efficiency of coupling. Subsequently, point mutations and chimeric substitutions within these same regions continued to point to their involvement in direct interaction with the G protein (Hein and Kobilka, 1995).

While specific residues and sequences are important for mediating the interaction, the secondary structure of the region is also of great importance. Although there has been much speculation as to how the receptor transmits its signal to the G protein, the exact mechanisms are still unknown. Recent data have suggested that the receptor exists in several dynamic states. When agonists bind, the receptor shifts to a state with higher affinity for agonists and induces a conformational change in the G protein, triggering its activation (Gether and Kobilka, 1998). Some mutations (reviewed by Lefkowitz et al., 1993b and by Hein and Kobilka, 1995) demonstrate an increase in the basal (ligand-independent) activation of the G protein and an enhanced sensitivity to agonists, suggesting that these mutant receptors are constitutively activated. In all of these cases, the data imply that the native receptor is conformationally constrained in the unliganded state and that agonists induce a change that results in a higher affinity for both the ligand and for the G protein. The nature of these conformational changes remains to be determined. Taken together, these results support a model in which the intracellular ends of the transmembrane domains and the intracellular loops connecting them are held together in an inactive conformation in the resting state of the receptor. Upon binding
agonist, a conformational change occurs in the receptor that "opens" the intracellular surface, allowing the G protein to interact with portions of the receptor, most frequently involving the amino- and carboxyl-terminal ends of the third intracellular loop and the C terminus (Hein and Kobilka, 1995; Carlson et al., 1998). However, other intracellular parts of some GPCRs are also involved in G protein activation, e.g., calcitonin receptor (the first intracellular loop, Nussenzveig et al., 1994), gonadotropin-releasing hormone receptor (the first intracellular loop, Arora et al., 1998), vasopressin receptor (the second intracellular loop, Liu and Wess, 1996), m5 muscarinic receptor (the second intracellular loop, Burstein et al., 1998) and metabotropic glutamate receptor 1 (mGluR1) (the second and third intracellular loops and the carboxyl tail, Francesconi and Duvoisin, 1998).

The discovery of (natural and mutant) receptors being able to activate G proteins in the absence of agonist and the finding of the phenomenon that the affinity for the uncoupled state of the constitutively active mutant receptor was linked to the efficacy, which could not be explained by the conventional model, has led to the formerly most widely accepted model, the ternary complex model (De Lean et al., 1980), being modified to the extended ternary complex model (Samama et al., 1993). The conventional ternary complex model could be used accommodate constitutive activity, full agonist, partial agonist, neutral antagonist, partial and full inverse agonist (negative antagonist) (Costa et al., 1992), but failed to explain the observation that the affinity of a variety of ligands for constitutively active mutant receptors, in the absence of G protein, varied in parallel with their efficacy. To explain this phenomenon, the extended ternary complex model proposes that the receptors exist in an equilibrium of two distinct states: the inactive (R) and active (R*) states. In the absence of agonists, the basal level of receptor activity is
determined by the relative proportion of the active $R^*$. The efficacy of ligands is a reflection of their ability to alter the equilibrium between the two states. This model not only reconciles the concept of constitutive activity of receptors and different classes of drugs (full agonists, partial agonists, neutral antagonists and inverse agonists) on receptor activity, but also offers explanation for the efficacy-related change in affinity for the uncoupled state of the constitutively active receptor (Samama et al, 1993).

Both biophysical analyses and mutagenesis studies of several GPCRs have demonstrated that the formation of the $R^*$ state involves the movements of TM3 and TM6 (reviewed by Gether and Kobilka, 1998). However, in the absence of high resolution structural analysis and more detailed information about the conformational changes following ligand binding, the molecular mechanism of GPCR activation is not completely understood.

### 1.1.3 Regulation of GPCR Activities

The signal transduction by GPCRs is regulated by changes in the functional state of the receptor (desensitization by phosphorylation) as well as the number of receptors on the surface of the cell (internalization and down-regulation) (reviewed by Lohse, 1993 and Lefkowitz, 1998). Receptor phosphorylation and internalization occur relatively quickly (seconds to minutes) and receptor down-regulation occurs more slowly (over hours). The processes of receptor desensitization have been most extensively characterized for the β2 adrenergic receptor (β2AR), and the above mechanisms
operative for β2AR are also being observed for other GPCRs, often with significant variations.

In β2AR, there are two consensus sites for PKA phosphorylation, one in the third intracellular loop and the other in the carboxyl terminus. Phosphorylation of the PKA site in the third intracellular loop is more important (Clark et al., 1989). Phosphorylation of GPCRs by PKA or PKC can occur in the absence of agonist and can be mediated indirectly by other receptors coupled to Gs or Gq, respectively. Phosphorylation directly alters receptor conformation such that interaction with the G protein is impaired. Such a process is known as heterologous desensitization, because any stimulant that elevates cAMP (for PKA) or diacylglycerol (in the case of PKC) has the potential to cause the phosphorylation and desensitization of any GPCRs containing appropriate PKA and/or PKC consensus phosphorylation sites. Another form of desensitization is homologous desensitization, by which prolonged stimulation of a receptor often results in reduced responsiveness to subsequent challenges by the same agonist. Homologous desensitization is mediated by a variety of receptor-specific GPCR kinases (GRKs) (reviewed by Lefkowitz, 1993a), which phosphorylate receptors at serine or threonine residues in the intracellular domains. Then the GRK-phosphorylated receptors bind an accessory protein known as arrestin, which sterically interferes with the interaction with G protein (Lohse, 1993). The two types of receptor desensitization by PKA/PKC and by GRKs differ in three important ways. First, GRKs only phosphorylate agonist occupied receptors whereas PKA/PKC can phosphorylate the receptors in the absence of agonist. Second, phosphorylation of the PKA-modified receptors impairs receptor function directly, whereas the disruption of receptor-G protein coupling by GRK phosphorylation
requires arrestin. Currently, six GRKs and at least six arrestins (some with alternative splicing) have been cloned (Lefkowitz, 1998). Third, βARK-mediated regulatory mechanisms would be especially important in the presence of high level catecholamines, such as in neural synapses (Lohse et al., 1990). On the contrary, the slower PKA-mediated modulation would predominate at lower agonist concentrations, i.e., in response to normal levels of circulating catecholamines (Roth et al., 1991).

The roles of arrestin and the GRKs in regulating the interaction of the receptor with G protein, beyond a simple blocking function in homologous desensitization, is now beginning to be realized. Interestingly, several lines of evidence suggest that binding of arrestin is the initial step of receptor internalization (Lefkowitz, 1998).

It has been known for a long time that signal transduction can also be regulated by altering the density of functional receptors at the cell surface (Lohse, 1993), which is effected by translocation of receptors intracellularly, a process known as receptor internalization. Receptor internalization of β2AR has been shown by immunocytochemical methods (confocal microscopy), which has demonstrated that agonist-induced removal of plasma membrane receptors is mediated by internalization of receptors into the same endosomal sorting system used by transferrin receptors (vonZastrow and Kobilka, 1992). The subtypes within a receptor group may vary in the extent of receptor internalization. For instance, β2AR displays pronounced receptor internalization, but β1 and β3 subtypes do not (Lohse, 1993). Internalized receptors in the endosomes may either end in degradation in lysosomes (to enter into down-regulation) or be resensitized by removal of phosphates (Yu et al., 1993) and recycled to the cell surface.
The process of down-regulation is less well investigated, but as in the case of other receptor functions, receptor down-regulation has been studied more extensively in adrenergic receptors (reviewed by Lohse, 1993). PKA phosphorylation of β2AR seems to facilitate down-regulation, since mutant receptors lacking the PKA sites are down-regulated slower (Bouvier et al, 1989); whereas GRK phosphorylation does not appear to play a part, as studies show that mutant β2AR without the βARK (GRK2) sites down-regulate normally (Lohse, 1993). Down-regulation may also be mediated via reduction of the receptor mRNA (Bouvier et al, 1989). As in the case with receptor internalization, different βARs display various degrees of down-regulation: β2, β1 and β3 show marked, modest and little down-regulation, respectively (Lohse, 1993). The parallelism in receptor internalization and down-regulation suggests that the mechanisms of both processes act in a coordinated fashion.

Recently, as mentioned above, accumulating evidence indicates that GRK phosphorylation and arrestin binding constitute the crucial first steps for internalization of several GPCRs. It has been shown that a mutant β2AR (Y326A), which is a poor substrate for β-adrenergic receptor kinase (βARK), failed to internalize (Barak et al, 1994). However, its ability to internalize can be rescued by over-expression of βARK or arrestin (Lefkowitz, 1998). On the hand, dominant negative β-arrestins may strikingly impair receptor internalization of β2AR (Lefkowitz, 1998). Since β-arrestins have high affinity for clathrin, arrestin-bound phosphorylated β2ARs then bind to clathrin and are internalized through clathrin-coated pits. Not all GPCRs undergo endocytosis through clathrin-coated vesicle pathways, e.g. the angiotensin II 1A receptor (Lefkowitz, 1998).
and D2 dopamine receptor (Vickery and von Zastrow, 1999), which internalize through a pathway other than the clathrin-coated pits.

Another interesting point is that receptor endocytosis through clathrin-coated pits is found to be important for mitogenic signaling, e.g., activation of MAP kinase (Lefkowitz, 1998), a signaling element downstream of adenylyl cyclase and other effectors.

In summary, a great deal has been learned about the structure and function of GPCRs over the past decade. Yet, no definitive 3D structure is available due to the unsuccessful effort to obtain high resolution crystals of membrane proteins. Moreover, we do not fully understand the complexity and multiplicity of the structural changes that accompany agonist binding and lead to G protein coupling. This will not only require an understanding of the static 3D structure of the receptor, but also the ability to follow the dynamic conformational changes of the receptor in the presence of agonists, antagonists and G proteins.

1.2 Dopamine Receptors

Dopamine is a biogenic amine and has long been recognized as a major neurotransmitter in the brain. Dopamine action is involved in motor control, emotion, affect and neuroendocrine regulation in the central nervous system, and peripherally in functions such as regulation of sodium uptake in the kidney (Missale et al, 1998). There has been great interest in the sites of action of dopamine, namely the dopamine receptors. This interest was fueled by studies of such clinical diseases as Parkinsonism and
schizophrenia, for which dopamine was shown to play an important role either in the pathogenesis or in therapeutic treatment. Biochemical studies on dopamine receptors began in 1970s. In 1976 Cools and van Rossum (Cools and van Rossum, 1976) reviewed a large body of data on the actions of dopamine and concluded that there might be more than one receptor for dopamine in the brain. In 1979, two dopamine subtypes were proposed (Kebabian and Calne, 1979): D1 and D2. The D1 receptor was defined as the receptor linked to the stimulation of adenylyl cyclase with a lower affinity for butyrophenones, whereas D2 receptors were the receptors with a high affinity for butyrophenones such as haloperidol, but which either did not affect or inhibit adenylyl cyclase.

For a decade, the dual receptor concept served as the basis for studies of dopamine receptors. This relatively simple picture of dopamine receptor subtypes was swept aside in the late 1980s by the application of molecular cloning techniques. So far, the genes for five dopamine receptor subtypes have been cloned (Missale et al, 1998). All five dopamine receptors are members of the GPCR superfamily. Detailed structural, pharmacological and biochemical studies revealed that each of these receptor subtypes fall into one of the two initially recognized categories. D1 and D5 subtypes have properties resembling the original D1 receptor, whereas D2, D3 and D4 have features of the original D2 receptor. Thus the D1-like/D2-like classification concept developed in the late 1970s is still valid in a broad sense.

There are three major dopamine pathways in the central nervous system (Missale et al, 1998). The nigrostriatal pathway originates from the substantia nigra and is the primary source of dopaminergic innervation of the dorsal striatal neurons. It plays an
important role in regulating motor behavior and its deterioration is the major cause for the motor symptoms of Parkinson's disease. The mesolimbic pathway, which originates from neurons of the ventral tegmental area (VTA), innervates the ventral striatum, nucleus accumbens, olfactory tubercle, and parts of the limbic system, and is most probably involved in emotional and motivational aspects of behavior. It may well contribute to the etiology of schizophrenia and serve as the substrate for neuroleptic agents (along with mesocortical pathway from VTA to prefrontal cortex; this terminal area may be involved in learning and memory). The tuberoinfundibular pathway originates from hypothalamus to innervate the anterior pituitary and regulates the secretion of prolactin and α-MSH.

The distribution of dopamine receptors in the brain and peripheral tissues has been determined mainly through in situ hybridization (for mRNAs) (reviewed by Missale et al, 1998). D1 receptor is the most widely expressed among the five receptor subtypes. D1 mRNA was found in the striatum, nucleus accumbens and the olfactory tubercle; while D1 receptor proteins were detected in the limbic system, hypothalamus and thalamus in addition to the above sites. The discrepancy between the mRNA and protein distribution suggests that in some areas, the receptors are mainly present in axonal projections. D5 is poorly expressed in the brain and mainly restricted to the hippocampus, the lateral mamillary nucleus and the parafascicular nucleus of the thalamus. The difference in receptor distribution suggests that although D1 and D5 receptors exhibit similar pharmacology, they are not functionally redundant. D1 and D5 receptors were detected to have both pre- and postsynaptic localization, with the latter being predominant. D2 receptors have been found mainly in the striatum, the olfactory tubercle and the core of nucleus accumbens. D3 receptors are found in the ventromedial shell of
nucleus accumbens, and at low levels in the hippocampus, in the septal area and in various cortical layers of medial temporal lobe, but poorly expressed in the dorsal striatum. D4 receptor have been found at low levels in the basal ganglia, but appear to be highly expressed in the frontal cortex, amygdala, hippocampus, hypothalamus and mesencephalon.

The functional role of different dopamine subtypes in some behaviors has been investigated and reviewed (Missale et al, 1998). It is now clear that concomitant stimulation of D1 receptor is important for synergistic action of D2 agonists to promote maximal locomotor stimulation. In mesolimbocortical pathways, implicated in reward and reinforcement mechanisms, learning and memory, and drug-self administration, both D1 and D2 receptors are involved. D3 receptors, shown to be located mainly postsynaptically, seem to be inhibitory on locomotor function. The role of D4 receptors is still mostly unknown. The fact that they are expressed specifically in limbic and cortical regions involved in the control of cognition and emotion and, to a less extent in the dorsal striatum makes them attractive targets for new antipsychotic drugs with few extrapyramidal side effects.

In addition to the stimulation or inhibition of adenylyl cyclase in cells and brain tissues, dopamine receptors are also involved in modulation of immediate early gene (c-fos) and neuropeptide (substance P) expression in neurons (reviewed by Missale et al, 1998).

As to receptor distribution in the periphery, it has been shown that all the cloned receptors are present in the kidney and D4 is also expressed in the heart (Missale et al,
But the nature of dopamine receptors in blood vessels, postganglionic sympathetic nerve endings and adrenal cortex are largely studied pharmacologically.

Genes coding for D1-like receptors (D1 and D5) do not contain introns, whereas those for D2-like receptors (D2, D3 and D4) are interrupted by introns at similar locations (Neve and Neve, 1997; Missale et al, 1998). Splice variants have been found for D2, D3 and D4 receptors due to differential splicing of the introns.

Like all GPCRs, dopamine receptors possess two cysteine residues in the second and third extracellular loops, which have been considered to form an intramolecular disulfide bond and stabilize the receptor structure. The D1-like receptors have a short third intracellular loop and a long C-tail, as do many GPCRs coupled to Gs, whereas the D2-like receptors are characterized by a long third intracellular loop and short C-terminal, a common feature of receptors coupled to Gi. In fact, the carboxyl terminal is about seven times longer for the D1-like receptors than for the D2-like receptors (Missale et al, 1998). D1 and D5 receptors have two cysteines in their C-tail within a short distance of TM7. D2, D3 and D4 have one cysteine at the end of their short carboxyl terminal (Neve and Neve, 1997). These cysteines have been found in the majority of GPCRs and are considered to be putative sites for palmitoylation (Jin et al, 1999).

As mentioned earlier, mutagenesis studies and structural modeling with adrenergic receptors have localized ligand binding domains to the hydrophobic TM regions. In particular, the Asp in TM3 (for binding the amino group of catecholamines), the two Ser residues in TM5 (as hydrogen bond donors to bind the hydroxyl groups of the catechol ring), the Phe in TM6 (for stabilizing the orthogonal interaction with the aromatic moiety of adrenergic ligands), and the Asp in TM2 (for allosteric modulation by
Na\(^{+}\)) are also present in these receptors (Neve and Neve, 1997; Missale et al, 1998). The DRY (DRF in D4) motif of the second intracellular loop is also present in all the dopamine receptors. Mutagenesis studies of most of the above residues were performed mainly in D1 and D2 receptor subtypes, and the results were generally consistent with those of adrenergic receptors (Missale et al, 1998). Various studies using peptides, chimeric receptors, receptor antibodies and site-directed mutagenesis invariably point out that, like adrenergic receptors, multiple regions of the intracellular domains of dopamine receptors are involved in G protein-coupling (reviewed by Robinson and Caron, 1997).

In the past few years after the cloning of the five dopamine receptors, it has been found that activities of these receptors are more complicated than the original simple categorization of stimulation or inhibition of adenylyl cyclase as proposed by Kebabian and Calne (Kebabian and Calne, 1979). It has been shown that D1-like receptors may also stimulate PI hydrolysis, inhibit a Na\(^{+}/H^{+}\) exchanger and increase K\(^{+}\) outflow in certain cells. The D2-like receptors may also modulate intracellular Ca\(^{2+}\) levels, K\(^{+}\) currents, the arachidonic acid pathway, and even cell growth (Robinson and Caron, 1997).

The cloning and characterization of genes encoding the five dopamine receptor subtypes have led to a series of genetic linkage studies to search for possible correlations between the receptors and neuropsychiatric diseases (reviewed by Baldessarini, 1997; Missale et al, 1998). The lack of a clearly identifiable factor in most of the psychiatric disorders includes the entire subgenome of about 20,000 genes specific for human brain as the potential candidate genes. And since virtually all clinically effective antipsychotic agents are clearly syndrome-nonspecific, exerting beneficial effects also for mania,
psychotic depression and other disorders with severe agitation and psychotic features, it may not be surprising that currently no linkage has been found between any major psychiatric disorders and any of the five cloned dopamine receptors.

In summary, the diverse actions of dopamine are mediated by at least five GPCR subtypes. D1 and D5 mainly couple to Gs (D1-like), and D2, D3 and D4 couple to Gi (D2-like). The cloning, characterization, mutagenesis and other studies of these receptors have provided valuable information about the structure and function of the five dopamine receptor subtypes. However, a complete understanding of the structure-function relationship of dopamine receptors will have to await further mutagenesis studies, molecular modeling, and the eventual elucidation of the three dimensional structure in the future. Gene-targeting technology will also help to delineate the functional roles played by dopamine receptors in the brain and in the periphery.

1.3 Palmitoylation of GPCRs

Cellular proteins may be modified by a variety of biochemical processes including phosphorylation, glycosylation, acetylation, arginylation, ADP-ribosylation, methylation, and lipidation. Protein lipidations (Towler et al, 1988; Casey, 1995) are classified on the basis of the nature of the attached lipid moieties into myristoylation (acylation by myristic acid), prenylation (acylation by isoprenoids), palmitoylation (acylation by palmitic acid), arachidonoylation (acylation by arachidonic acid), and modification by glycosylphosphatidylinositol (GPI). Myristoylation (Resh, 1996; Johnson et al, 1994; Boutin, 1997), prenylation (Zhang and Casey, 1996; Resh, 1996;
Higgins and Casey, 1996) and GPI modification (Udenfriend and Kodukula, 1995; Stevens, 1995; England, 1993) have been extensively studied and reviewed, whereas arachidonoylation is a relatively recent finding (Hallak et al, 1994). These diverse lipid modifications serve to enhance the association of the modified proteins with the membrane and may also promote protein-protein interactions. Accumulating evidence demonstrates that palmitoylation of proteins may also function in a similar pattern (Munby, 1997; Bouvier et al, 1995; Bizzozero et al, 1994; Bizzozero, 1997).

A wide variety of proteins in eukaryotic cells have been found to be palmitoylated (Table 1.1). Among these are proteins of the Src-family, Ras-family, endothelial nitric oxide synthase (eNOS), G protein alpha subunits, and two G protein-coupled receptor kinases (GRKs) as well as numerous GPCRs listed separately in Table 1.2.

Palmitoylation is a reversible posttranslational modification (Bonatti et al, 1989) whereby a 16-carbon saturated fatty acyl chain is covalently attached to a protein through a thioester bond (Bizzozero et al. 1994). After the original report (Ovchinnikov et al. 1988) describing the palmitoylation of bovine rhodopsin on the two cysteines in its cytoplasmic tail, many GPCRs have been shown to be palmitoylated (Table 1.2). Since almost every member of the GPCR superfamily possesses cysteine(s) at the analogous position, it is believed that the majority of the GPCRs are palmitoylated in a comparable manner, although the function of palmitoylation still remains undetermined.

A complete investigation of a protein predicted to be palmitoylated should answer the following questions: (1) Is the protein acylated? (2) Is the fatty acid attached to the protein really palmitate? (3) What is the chemical nature of the bond? (4) Where is the palmitoylation site?
When a protein has been determined to be palmitoylated, the next step will be functional studies to investigate the role played by protein palmitoylation.

As summarized in several excellent reviews (Mumby, 1997; Casey, 1995; Bouvier et al, 1995; Bizzozero et al, 1994; Bizzozero, 1997), palmitoylation is a dynamic process and thus has the potential to be regulated, either enzymatically or non-enzymatically, for which there have been reports supporting both. As shown in Table 1.1, an extensive array of proteins has been proven to be palmitoylated, yet in only a few of them have studies been carried out to determine the functional role. In these investigations, these proteins share a common feature, namely, they are usually membrane-associated proteins and palmitoylation seems to increase the degree of membrane-association.

Palmitoylated proteins can roughly be classified into three categories (Bizzozero et al, 1994):
(a) Single transmembrane glycoproteins (e.g., transferrin receptor). After removal of both palmitoylation sites, the mutant receptor was internalized and mediated iron uptake as efficiently (Jing and Trowbridge 1990) as the wild type transferrin receptor or at an increased rate (Alvarez et al, 1990).
(b) Membrane-associated cytosolic proteins (e.g., Ras family, some members of Src family and G protein α subunits). As summarized (Resh, 1994; Casey, 1995), the Ras family proteins and members of Src family require dual lipidation (prenylation and palmitoylation or a stretch of basic residues near the carboxyl-terminal for Ras, and myristoylation and palmitoylation for Src near the amino-terminal) for membrane association, which may reflect the insufficiency of one lipid modification to anchor these
proteins stably to membranes. Interestingly, the α subunits of the G protein have a very similar pattern of myristoylation and palmitoylation (Resh, 1994).

(c) Multiple transmembrane proteins (e.g., GPCRs). The majority of GPCRs are believed to be palmitoylated at cysteine(s) in their C-terminal, although only about eighteen of them have been investigated experimentally (Table 1.2).

Two functional models of protein-palmitoylation can be hypothesized: (1) the palmitoyl chain promotes non-specific association with membrane; and (2) palmitoylation mediates protein localization to a specific subdomain/microdomain of cell membrane such as caveolae where caveolin and eNOS are aggregated (Mumby, 1997). Caveolae (Anderson, 1993) are specialized parts of plasma membrane formed as flask-shaped invaginations, and evidence is accumulating that a variety of molecules involved directly or indirectly in signal transduction are enriched therein. Whether GPCRs locate within, or are internalized through, caveolae during desensitization is still unresolved due to conflicting data (Mumby, 1997).

The palmitoylation of four proteins have been shown to be increased by agonist stimulation: β2AR by isoproterenol (Mouillac et al, 1992), D1DR by dopamine (Ng et al, 1994a), stimulatory GTP-binding protein (Gs) by β2AR (Mumby et al, 1994; Degtyarev et al, 1993), and eNOS by bradykinin (Robinson et al, 1995).

To determine the functional role of palmitoylation in GPCRs (see Refs in Table 1.2), several research groups have carried out a series of site-directed mutagenesis studies. The two main aspects of receptor function investigated were receptor-G protein coupling and receptor internalization/down regulation.
Elimination of the palmitoylation site (C341G) of β2-adrenergic receptors resulted in a partial loss of receptor-G protein coupling (O'Dowd et al, 1989; Moffett et al, 1993). Likewise, ET<sub>B</sub> receptor (Okamoto et al, 1997) was shown to have impaired receptor-G protein-coupling upon removal of the palmitoylation sites. In contrast, similar mutagenesis studies on other GPCRs have demonstrated that palmitoylation is not required for coupling. The elimination of the palmitoylation sites did not show any effect on rhodopsin-G<sub>t</sub> coupling (Karnik et al, 1993) and paradoxically, the chemical removal of palmitate from rhodopsin even increased the receptor-G<sub>t</sub> coupling (Morrison et al, 1991). A lack of effect on receptor-G protein coupling, assessed by the inhibition of adenylyl cyclase activity, has been demonstrated in both α2-adrenergic receptors (Kennedy and Limbird, 1993) and the M2 muscarinic receptors (van Koppen and Nathanson, 1991). Similarly, the substitution of glycine for Cys335 and Cys337 of the mouse TRHR did not affect receptor-mediated stimulation of IP<sub>3</sub> production (Nussenzveig et al, 1993). In LH/hCG receptors, the loss of palmitoylation did not affect the efficiency of coupling to Gs protein (Zhu et al, 1995; Kawate and Menon, 1994). In the single or double mutations in D1 dopamine receptors we have studied, which eliminated one or both of the palmitoylation sites, no difference was observed between the wild type and mutant D1 dopamine receptors (Jin et al, 1997). The lack of effect was also observed for ETA (Horstmeyer et al, 1996) and V2 receptors (Sadeghi et al, 1997) (Table 1.2). On the other hand, the effects of palmitoylation on receptor sequestration and down-regulation have been diverse (see Table 1.2), indicating that receptor palmitoylation may serve completely different roles among various members of this superfamily of GPCRs in receptor sequestration and receptor down regulation.
The relationship between palmitoylation and receptor-G protein coupling was shown to be unique for β2-adrenergic receptors. It is attributed to the unique feature of the C-terminal tail, in which a cAMP-dependent protein kinase (PKA) site is present in close proximity (4 amino acid residues apart) to the palmitoylation site. No such phosphorylation site is found in the C-tails of rhodopsin, α2A-adrenergic receptors, M2 muscarinic receptors, TRH receptors, ETA, V2 receptors and D1DR. A PKA site is found in LH/hCG receptors in its C-terminus, but it is 33 amino acid residues away from the sites that have been shown to be palmitoylated. The elimination of palmitoylation of the Gly341β2-adrenergic receptors results in constant exposure of the PKA site, allowing constitutive desensitization even in the absence of agonist treatment. In fact, it has been shown that the basal level of phosphorylation is about 4 times higher in the Gly341β2-adrenergic receptor mutant than in the wild type and that agonist treatment cannot promote any further phosphorylation or uncoupling of the mutant receptor (Moffett et al, 1993). Subsequently, it was also shown that elimination of the PKA site by substituting alanine residues for Ser345 and Ser346 of Gly341β2-adrenergic receptor restored both the phosphorylation and the effector activation to levels observed with wild type receptors (Moffett et al, 1996), lending further support that the unique presence of a PKA site in the immediate vicinity of the palmitoylation site of β2-adrenergic receptor is responsible for the discrepancy of the observations between the β2-adrenergic receptor and other GPCRs. Since there is no such PKA site in the other receptors, palmitoylation or non-palmitoylation therefore should make no difference in their phosphorylation state whether at basal level or in the presence of agonist treatment. However, ETB receptors
do not contain PKA sites despite the presence of several serines near the cysteines. Thus, the observation in ETB receptors can not be explained at present.

1.4 The Rationale of Ph.D. Projects

At present, the GPCR superfamily has over 300 members. Although all of these receptors have a common membrane topology, the conservation of specific amino acid sequence across the whole family is limited, and the variety of cellular responses mediated by these receptors is enormous. Much of the information available at the time when I initiated my Ph.D. projects concerning the structure and functions of GPCRs, was derived from studies of a single receptor, the β2-adrenergic receptor. The biggest challenge has been to determine how much of this information is applicable to other receptors, so that general principles can be formulated.

We chose the D1 dopamine receptor as the prime target, for the following reasons:
(1) D1DR resembles β2-AR both in structure and function (coupled to Gs); thus it is an ideal GPCR on which to test whether conclusions made from studies of β2-AR are generalizable to other GPCRs;
(2) D1DR has been shown to play an important role in many aspects of brain function and in peripheral organs as outlined above; and
(3) D1DR was cloned in our laboratory.

The objectives of the projects toward this thesis have been to clarify the structure-function relationship of D1 dopamine receptor. The role of specific amino acid residues
in the intracellular domains of D1DR was investigated. Specifically, for reasons outlined in the following paragraphs, we intended (1) to determine the actual sites and role of palmitoylation in G protein-coupling of D1DR, (2) to examine the role of a serine residue in the first intracellular loop that seemed to be conserved in several GPCRs, (3) to determine the significance of a cdc2 phosphorylation site in the second intracellular loop of D1DR in agonist-induced receptor internalization, and (4) to clarify the relationship between the receptor expression level and the effector response.

At the initiation of the project, only four GPCRs had been studied in terms of palmitoylation. In all these four receptors, palmitoylation occurred at the cysteine residues of carboxyl terminal in the vicinity of TM7. However, a controversy existed as to whether palmitoylation was required for receptor-G protein coupling. It was shown that palmitoylation was important for receptor-G protein coupling in β2AR (O'Dowd et al., 1989). But elimination of palmitoylation sites did not interfere with receptor-G protein coupling in the other three receptors, namely, rhodopsin (Karnik et al., 1993), β2-adrenoceptor (Kennedy and Limbird, 1993) and M2 muscarinic receptor (Van Koppen and Nathanson et al., 1991). It was hypothesized that the D1DR was palmitoylated at the Cys347 and Cys351. To address this controversy as to if palmitoylation was required for receptor-G protein coupling, we eliminated these two putative palmitoylation sites of D1DR both individually and together. Because of the similarity in both structure and function of D1DR and β2AR (both coupled to Gs, while rhodopsin to Gt and β2 and M2 to Gi), we predicted that the removal of the palmitoylation sites from D1DR would impair receptor-G protein coupling as in β2AR. However, our data revealed that elimination of the putative palmitoylation sites did not perturb receptor-G protein
coupling. Therefore, palmitoylation was not involved in G coupling in D1DR, challenging the hypothesis proposed earlier on the basis of β2AR (O'Dowd et al, 1989; Moffett et al, 1993). Our results were consistent with the majority of subsequent reports on GPCR palmitoylation studies (reviewed by Jin et al, 1999). These data are presented in Chapter 2.

Since there are five cysteines in the intracellular domains of D1DR, the question from the above study naturally became 'Are Cys347 and Cys351 rather than the other three, really the actual palmitoylation sites?' In further experiments, the actual sites for palmitoylation in D1DR were determined. The results confirmed that these two cysteines were indeed the palmitoylation sites. The next question then was what was the reason for the discrepancy between β2AR and D1DR (and others). Preliminary studies (Bouvier et al, 1995) suggested that the PKA site adjacent to the palmitoylation site in β2AR was responsible for the discrepancy between β2AR and other GPCRs concerning the relationship of palmitoylation and G protein-coupling. To test this hypothesis, we created a similar scenario by introducing a consensus PKA sequence into the C-tail of D1DR. The experimental data and analysis using the extended ternary complex model demonstrated that the PKA site close to the palmitoylation site did confer constitutive desensitization to D1DR, confirming the hypothesis mentioned above. These data are presented in Chapter 3.

A variety of naturally occurring mutations in GPCRs have been found to cause diseases, some by losing function and others by gaining function (reviewed by Raymond 1994). An interesting naturally occurring mutation was discovered in melanocyte-stimulating hormone receptor (MSH) (Robbins et al, 1993). A spontaneous mutation of
the serine to leucine in the first intracellular loop caused a constitutive activation of MSH receptor and enhanced receptor-G protein coupling. Since a serine residue seemed to be present in a number of GPCRs including D1DR, I decided to examine the significance of the serine residue of the first intracellular loop of D1DR in the activation of adenylyl cyclase. I expected that a similar mutation might also result in a constitutively active D1DR. The results are presented in Chapter 4.

Receptor internalization is a very important function of GPCRs. Being a component of receptor desensitization and signaling switch (Shwartz and IJzerman 1998), receptor internalization is controlled mainly by specific motifs, receptor phosphorylation, receptor ubiquitination, and the specific make-up of endocytotic machinery of different cell lines, in which endogenous or exogenous receptors are expressed (reviewed by Koenig and Edwardson 1997). Phosphorylation is regarded as a major factor in the process of receptor internalization. Traditionally, researchers focused on phosphorylation of GPCRs mediated by GRKs, PKA and PKC. However, other phosphorylation sites are also present in GPCRs. I have identified a cdc2 consensus sequence in the second intracellular loop of D1DR. A similar site is also present in the corresponding region of β2AR and D5 dopamine receptor. The significance of the cdc2 phosphorylation site in the second intracellular loop of D1DR was investigated. The results suggested that this phosphorylation site, in addition to PKA/PKC and GRK sites, might also be involved in agonist-induced receptor internalization. These results are presented in Chapter 5.

Ligand properties such as affinity, potency and efficacy have been the central concepts in the field of pharmacology. The concept of efficacy was first introduced and
defined by Stephenson in 1956. Stephenson stated that a maximum effect can be produced by an agonist when occupying only a small proportion of the receptors and that different drugs may have varying capacities to initiate a response and consequently occupy different proportions of the receptors when producing equal responses. This property was referred to as the efficacy of the drug (Stephenson 1956). In 1966, Furchgott revised the definition by introducing a concept of intrinsic efficacy and defining efficacy as the product of intrinsic efficacy and total number of receptors in the system \((\varepsilon = \varepsilon R_t)(Furchgott 1966)\). In the past decades, scientists have been trying to determine the chemical nature of efficacy. But even with abundance of information on the sequence and structure of GPCRs, the nature of efficacy still remains elusive (reviewed by Clarke and Bond 1998). In the past few years during which I studied D1 dopamine receptor using stable cell lines, I observed that the efficacy of dopamine in different cell lines as measured by the maximal stimulation of adenylyl cyclase varied with receptor expression levels. In order to determine if the maximal response \((Q)\) varied with total receptors available \((R_t)\) in a rectangular hyperbolic pattern \((Q = Q_{\text{max}} \frac{\varepsilon R_t}{1 + \varepsilon R_t})\) as predicted by Furchgott (Furchgott 1966), I decided to examine the relationship between receptor density and maximal cyclase response. The data confirmed that the maximal response \((Q\) or \(V_{\text{max}}\)) varied hyperbolically with receptor density \((R_t\) or \(B_{\text{max}}\)) in different clones. These results were consistent with studies carried out by Furchgott in 1966 and with more recent ones (see Chapter 6). These data also emphasized the importance of using cell lines expressing comparable receptor levels.

Figure 1.1 represents a topographic model of D1DR.
Figure 1.1 D1 Dopamine Receptor
### Table 1.1

**A List of Palmitoylated Proteins and Functions of Palmitoylation**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function of Palmitoylation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 55 kDa membrane protein of human erythrocytes, different from band 3</td>
<td>Anchoring to plasma membrane</td>
<td>[Das et al. 1992]</td>
</tr>
<tr>
<td>A 85-kDa protein in rat adipocytes similar to CD36 (platelet membrane glycoprotein IV)</td>
<td>Anchoring to plasma membrane</td>
<td>[Jochen and Hays 1993]</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>Chemical depalmitoylation decreased its activity</td>
<td>[Randall 1994]</td>
</tr>
<tr>
<td>Adenyl cyclase</td>
<td>Chemical depalmitoylation decreased its activity</td>
<td>[Mollner et al. 1995]</td>
</tr>
<tr>
<td>Asialoglycoprotein receptor (ASGP-R)</td>
<td>Not required for anion-exchange</td>
<td>[Zeng et al. 1995]</td>
</tr>
<tr>
<td>Cation-dependent mannose 6-phosphate receptor</td>
<td>Essential for the normal trafficking and lysosomal enzyme sorting</td>
<td>[Schweizer et al. 1996]</td>
</tr>
<tr>
<td>Caveolin</td>
<td>Not necessary for localization of caveolin to caveolae;</td>
<td>[Dietzen et al. 1995];</td>
</tr>
<tr>
<td></td>
<td>May stabilize the oligomer</td>
<td>[Monier et al. 1995]</td>
</tr>
<tr>
<td>CD4, the HIV Receptor</td>
<td>Not required for expression of CD4 on the cell surface or for binding of p56lck to its cytoplasmic domain</td>
<td>[Crise and Rose 1992]</td>
</tr>
<tr>
<td>CD44</td>
<td>May play an active role in receptor and receptor interactions and signal transduction in normal human T lymphocytes</td>
<td>[Guo et al. 1994]</td>
</tr>
<tr>
<td>Chloroplast 32-kDa herbicide-binding protein</td>
<td></td>
<td>[Mattoo and Edelman 1987]</td>
</tr>
<tr>
<td>Cyclic nucleotide phosphodiesterase</td>
<td></td>
<td>[Agrawal et al. 1990]</td>
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<tr>
<td>Protein/Molecule</td>
<td>Function/Characteristics</td>
<td>References</td>
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<tr>
<td>Endothelial Nitric Oxide Synthase (eNOS)</td>
<td>Required for targeting of NOS to caveolae</td>
<td>[Robinson et al. 1995; Garcia-Cardena et al. 1996]</td>
</tr>
<tr>
<td>Erythocyte ankyrin</td>
<td></td>
<td>[Staufenbiel 1987]</td>
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<td>GAD65 in pancreatic beta cells</td>
<td></td>
<td>[Solimena et al. 1994; Christgau et al. 1992]</td>
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<tr>
<td>GAIP (G Alpha Interacting Protein)</td>
<td>Membrane anchorage</td>
<td>[De Vries et al. 1996]</td>
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<tr>
<td>Glucose transporter GLUT</td>
<td></td>
<td>[Pouliot and Beliveau 1995]</td>
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<tr>
<td>Glutamate Kainate Receptor GluR6</td>
<td>Kainate-gated currents produced by the unpalmitoylated mutant receptor were indistinguishable from those of the wild-type</td>
<td>[Pickering et al. 1995]</td>
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<tr>
<td>Glycoprotein G of rabies virus</td>
<td></td>
<td>[Gaudin et al. 1991]</td>
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<tr>
<td>Glycoprotein Ib in megakaryocytes</td>
<td></td>
<td>[Schick and Walker 1996]</td>
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<tr>
<td>gp41 of HIV and SIV</td>
<td></td>
<td>[Yung et al. 1995]</td>
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<tr>
<td>Gramicidin transmembrane channel</td>
<td></td>
<td>[Koeppel et al. 1995]</td>
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<tr>
<td>GRK6 and GRK4</td>
<td>Essential for membrane association</td>
<td>[Stoffel et al. 1994; Premont et al. 1996]</td>
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<tr>
<td>Hemagglutinin of influenza virus</td>
<td>No effect on fusion activity.</td>
<td>[Steinhauer et al. 1991; Philipp et al. 1995];</td>
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<td></td>
<td>May regulate the maturation and budding of influenza virus</td>
<td>[Veit and Schmidt 1993; Portincasa et al. 1992]</td>
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<tr>
<td>Human CD36</td>
<td></td>
<td>[Tao et al. 1996]</td>
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<tr>
<td>Influenza A virus M2 protein</td>
<td></td>
<td>[Veit et al. 1991; Sugrue et al. 1990]</td>
</tr>
<tr>
<td>Binding partner</td>
<td>Description</td>
<td>Reference</td>
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<td>L-type calcium channel beta2a subunit</td>
<td>Palmitoylation-deficient beta2a mutant still localized to membrane particulate fractions and able to target functional channel complexes to the plasma membrane similar to wild-type; However, channels formed with a palmitoylation-deficient beta2a subunit exhibited a dramatic decrease in ionic current per channel</td>
<td>[Chien et al. 1996]</td>
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<td>Low affinity neurotrophin receptor (p75LNTR)</td>
<td></td>
<td>[Barker et al. 1994]</td>
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<td>Membrane glycoproteins of Semliki Forest virus</td>
<td></td>
<td>[Schmidt and Burns 1989; Scharer et al. 1993]</td>
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<td>Myelin proteolipid protein</td>
<td></td>
<td>[Bizzozero et al. 1990]</td>
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<td>Myelin-associated glycoproteins (MAG)</td>
<td></td>
<td>[Pedraza et al. 1990]</td>
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<tr>
<td>Na channel alpha subunit</td>
<td></td>
<td>[Schmidt and Catterall 1987]</td>
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<td>Neural cell adhesion molecules (NCAM)</td>
<td></td>
<td>[Murray et al. 1987]</td>
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<td>Neuromodulin (GAP-43)</td>
<td>Palmitoylation is important for membrane targeting, Golgi localization, and neurite transport of neuromodulin</td>
<td>[Sudo et al. 1992; Liu et al. 1993; Liu et al. 1994]</td>
</tr>
<tr>
<td>Nicotinic ACh-receptor</td>
<td></td>
<td>[Olson et al. 1984]</td>
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<tr>
<td>P-selectin</td>
<td></td>
<td>[Fujimoto et al. 1993]</td>
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<tr>
<td>p21H-ras, p21N-ras, and p21K-ras(A)</td>
<td>Combine with the CAAX motif to target specific plasma membrane localization; The posttranslational processing of ras p21 is critical for stimulation of yeast adenylate cyclase</td>
<td>[Jackson et al. 1990; Hancock et al. 1990]</td>
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<tr>
<td>p21rhoB</td>
<td></td>
<td>[Horiuchi et al. 1992]</td>
</tr>
<tr>
<td>p62</td>
<td>May be important in vesicular transport during mitosis</td>
<td>[Mundy and Warren 1992]</td>
</tr>
<tr>
<td>p63 of endoplastic reticulum</td>
<td></td>
<td>[Schweizer et al. 1995]</td>
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<tr>
<td>Pertussis toxin</td>
<td>Required for cellular toxicity</td>
<td>[Hackett et al. 1995]</td>
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<td><strong>Ras-related Rap2 protein</strong></td>
<td><strong>[Beranger et al. 1991]</strong></td>
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<td><strong>Ras-related YPT/rab proteins in S. pombe</strong></td>
<td><strong>[Newman et al. 1992]</strong></td>
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<td><strong>Rh polypeptides of RBC</strong></td>
<td><strong>[Hartel-Schenk and Agre 1992]</strong></td>
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<td><strong>SCG10 in the growth cones of developing neurons</strong></td>
<td><strong>[Di Paolo et al. 1997]</strong></td>
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<td><strong>Sindbis virus E2 glycoprotein</strong></td>
<td><strong>Important for budding</strong></td>
<td><strong>[Ivanova and Schlesinger 1993]</strong></td>
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<td><strong>SNAP-25</strong></td>
<td><strong>[Hess et al. 1992]</strong></td>
<td></td>
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<td><strong>Src-family tyrosine kinases (p56lck, p59fyn, p55fgr, and p59hck)</strong></td>
<td><strong>Confer localization to caveolae</strong></td>
<td><strong>[Wolven et al. 1997; Shenoy-Scaria et al. 1994; Rodgers et al. 1994; Robbins et al. 1995; Resh 1994; Paige et al. 1993; Koegl et al. 1994]</strong></td>
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<td><strong>Synaptotagmin</strong></td>
<td><strong>[Veit et al. 1996; Chapman et al. 1996]</strong></td>
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<td><strong>Torpedo Cysteine String Protein (T-Csp)</strong></td>
<td><strong>[Gundersen et al. 1994]</strong></td>
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<td><strong>Transferrin receptors</strong></td>
<td><strong>Associated with an inhibition of the rate of transferrin receptor endocytosis</strong></td>
<td><strong>[Alvarez et al. 1990]</strong></td>
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<td><strong>Transforming growth factor (TGF)-alpha</strong></td>
<td><strong>Critical in interaction with associated proteins</strong></td>
<td><strong>[Shum et al. 1996]</strong></td>
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<tr>
<td><strong>Transmembrane proteins in murine leukemia virus</strong></td>
<td><strong>Not affect its transport, processing, surface expression, or cell fusion activity</strong></td>
<td><strong>[Yang and Compans 1996; Hensel et al. 1995]</strong></td>
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<tr>
<td><strong>Tubulin</strong></td>
<td><strong>[Ozols and Caron 1997; Caron 1997]</strong></td>
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<td><strong>Vesicular Stomatitis Virus (VSV) G protein</strong></td>
<td><strong>[Rose et al. 1984]</strong></td>
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<td><strong>x1caax-1 of Xenopus</strong></td>
<td><strong>Mutation of the palmitoylation sites inhibited the association of x1caax-1 with the membrane</strong></td>
<td><strong>[Reddy et al. 1991; Kloc et al. 1991; Kloc et al. 1993]</strong></td>
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<td><strong>Yeast Ras2 proteins</strong></td>
<td><strong>Necessary for membrane association</strong></td>
<td><strong>[Kuroda et al. 1993]</strong></td>
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<td><strong>Yeast synaptobrevin homologue (Snc1)</strong></td>
<td><strong>Affects protein stability</strong></td>
<td><strong>[Couve et al. 1995]</strong></td>
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<tr>
<td>Receptor</td>
<td>Sites</td>
<td>Mutant</td>
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<tr>
<td>α2AR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C442</td>
<td>C442A/S</td>
</tr>
<tr>
<td>β2AR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C341</td>
<td>C341G</td>
</tr>
<tr>
<td>D1DR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C347, C351</td>
<td>C347/351G</td>
</tr>
<tr>
<td>ETA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C1215</td>
<td>C1215S, C1215A</td>
</tr>
<tr>
<td>ETB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C402, C403, C405</td>
<td>C402S; C403/405S; C402/403/405S</td>
</tr>
<tr>
<td>LH/CGR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C621, C622</td>
<td>C621/622A</td>
</tr>
<tr>
<td>Rhodopsin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C322, C323</td>
<td>C322S/C323S</td>
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<tr>
<td>TSHR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C699</td>
<td>C699A</td>
</tr>
<tr>
<td>V2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C341, C342</td>
<td>C341/342S</td>
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<tr>
<td>D2DR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>C442</td>
<td></td>
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<tr>
<td>5-HT1A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>C417, C420</td>
<td></td>
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<tr>
<td>5-HT1B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>C388</td>
<td></td>
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<tr>
<td>mGluR4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>C890</td>
<td></td>
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<tr>
<td>M2AChR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>C457</td>
<td>C457G</td>
</tr>
<tr>
<td>M3AChR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>C560, C562</td>
<td>C560/562S</td>
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<tr>
<td>SSTR5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>C320</td>
<td>C320A</td>
</tr>
<tr>
<td>TRHR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>C335, C337</td>
<td>C335/337S/G</td>
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<tr>
<td>MOR&lt;sup&gt;d&lt;/sup&gt;</td>
<td>C346/351A</td>
<td></td>
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</table>
a: Receptors have been shown to be palmitoylated, and the palmitoylation sites were clearly determined by site-directed mutagenesis.

b: Receptors have been shown to palmitoylated, but no mutagenesis study was carried out, hence sites still unknown.

c: The putative sites (cysteine residues) were mutated, but no palmitoylation data were available. Thus the palmitoylation site was not verified.

d: Receptors have been shown to be palmitoylated and mutagenesis of the putative sites (cysteines) was carried out. however, the elimination of cysteines in the C-tail did not eliminate palmitoylation signal. Therefore, the palmitoylation site remains undetermined.

**Abbreviations:** 5-HT1A=5-hydroxytryptamine1A receptor; 5-HT1B=5-hydroxytryptamine1B receptor; α2AR=α2-adrenergic receptor; β2AR=β2-adrenergic receptor; D1DR=D1 dopamine receptor; D2DR=D2 dopamine receptor; ETA=endothelin A receptor; ETB=endothelin B receptor; LH/CG=luteinizing hormone/chorionic gonadotropin receptor; M2AChR=m2 acetylcholine receptor; M3AChR=m3 acetylcholine receptor; mGluR4=metabotropic glutamate receptor subtype 4; MOR=µ opioid receptor; SSTR5=somatostatin receptor type 5; TRHR=thyrotropin releasing hormone receptor; TSHR=thyrotropin receptor; V2=V2 vasopressin receptor.
Chapter 2.

The Relationship between Palmitoylation and Receptor-G protein Coupling in D1 Dopamine Receptor

This chapter is a modified version of the following paper:

ABSTRACT

Using a site-directed mutagenesis approach, we have eliminated the putative palmitoylation sites in the C-terminal tail of the human D1 dopamine receptor by replacing the two cysteines with alanines either separately or together. The wild type and mutant D1 receptors were stably expressed in BHK cells and characterized to detect any differences in receptor-G protein interactions. Unlike the mutagenesis study of the β2-adrenergic receptor, mutant D1 receptors have the same proportion of high affinity state for agonists and no difference was observed in the stimulation of adenylyl cyclase activity. These results are consistent with studies of LH/hCG receptors, which are coupled to Gs, α2-adrenergic and m2 muscarinic receptors, which are coupled to Gi, and TRH receptors, which are coupled to the G proteins that mediate hydrolysis of phosphatidylinositol phosphate, and thus suggest that palmitoylation is not essential in G protein coupling for D1 and other receptors and that it may play a unique role in the β2-adrenergic receptors.
INTRODUCTION

Acylation of membrane-associated proteins by long-chain saturated fatty acids, such as myristic and palmitic acids, has been found to be a common phenomenon since it was first reported in 1951 (Towler et al., 1988). Myristoylation occurs co-translationally with little or no metabolic turnover (Towler et al., 1988). In contrast, palmitoylation is a posttranslational modification that takes place on a nascent protein in a post-endoplasmic reticulum or pre-Golgi compartment (Bonatti et al., 1989) and rapid turnover has been reported in several proteins including transferrin receptors (Alvarez et al., 1990), G proteins (Mumby et al., 1994) and β2-adrenergic receptors (Mouillac et al., 1992). Protein palmitoylation may play several important roles inside the cell, including anchorage of protein to cell membrane, protein-protein interaction and determination of intracellular transportation/localization (see Bizzozero et al., 1994 for review). Palmitoylated proteins include p21ras, GAP-43, some G protein α-subunits, several tyrosine kinases of the p60src family (reviewed by Bizzozero et al., 1994 and Bouvier et al., 1995), an integral membrane protein localized in the endoplasmic reticulum (p63) (Schweizer et al., 1995), the low affinity neurotrophin receptor p75 (p75LNTR) (Barker et al., 1994) and the GluR6 kainate receptor (Pickering et al., 1995). The alignment of the amino acid sequences (Fig. 2.1) shows that the majority of the members of the G protein-coupled receptor superfamily have one or more cysteines at their carboxyl tails. Several G protein-coupled receptors have been shown to be palmitoylated via thioester bonds at these cysteines (Bouvier et al., 1995), including rhodopsin (Karnik et al., 1993), β2-adrenergic receptors (O’Dowd et al., 1989), α2A-adrenergic receptors (Kennedy and Limbird 1993), LH/hCG receptors (Kawate and Menon 1994), D1 dopamine receptors
It has been shown that the palmitoylation at Cys341 in the C-terminal of β2-adrenergic receptors is important for receptor-G protein coupling, and a mutant β2-adrenergic receptor with its Cys341 replaced by glycine greatly reduced its ability to form the high affinity state for agonists and its ability to stimulate adenylyl cyclase activity (O'Dowd et al, 1989; Moffett et al, 1993). However, similar mutagenesis studies on other G protein-coupled receptors, including the rhodopsin (Karnik et al, 1993), α2-adrenergic receptors (Kennedy and Limbird, 1993), m2 muscarinic receptors (Van Koppen and Nathanson 1991), thyrotropin-releasing hormone receptors (TRHR) (Nussenzveig et al, 1993) and LH/hCG receptors (Kawate and Menon 1994), showed that elimination of the palmitoylation site(s) by substituting cysteine(s) with other amino acid residues did not perturb receptor-G protein coupling as was shown in the β2-adrenergic receptors. Similarly, a comparison of the electrophysiological properties of the wild-type GluR6 kainate receptor and the unpalmitoylated mutant receptor (C827A, C480A) showed that the kainate-gated currents produced by the unpalmitoylated mutant receptor were indistinguishable from those of the wild-type GluR6. Thus, those studies showed that prevention of palmitoylation could produce variable effects on different receptors. The human dopamine D1 receptor contains two putative palmitoylation sites (Cys347 and Cys351) in its carboxyl terminus (Fig. 2.2) (Sunahara et al, 1990). To clarify the role of palmitoylation in D1 dopamine receptors, we eliminated the two palmitoylation sites both separately and together using site-directed mutagenesis. We have found that introduction of the mutation(s) did not affect ligand binding, receptor-G protein coupling, agonist-induced stimulation of adenylyl cyclase activity, or receptor desensitization. These
results again contrast with those of the β2-adrenergic receptor (O'Dowd et al, 1989; Moffett et al, 1993) and are more consistent with other receptor studies such as α2A-adrenergic receptor (Kennedy and Limbird, 1993), m2 muscarinic receptor (Van Koppen and Nathanson 1991), TRHR (Nussenzveig et al, 1993) and LH/hCG receptor (Kawate and Menon 1994) mutagenesis studies. We believe that the unique role of the palmitoylation in the β2-adrenergic receptor is attributed to its unique feature of its C-terminal tail.
MATERIALS AND METHODS

Materials. [α-32P]ATP (800 Ci/mmol), [3H]SCH23390 and [3H]cAMP (30 Ci/mmol) were purchased from Du Pont/NEN. Lipofectin, geneticin (G418), penicillin and streptomycin were purchased from GIBCO/BRL. Dopamine, serotonin, leupeptin, benzamidine, soybean trypsin inhibitor, GTP, ATP, cAMP and forskolin were purchased from Sigma. SKF38393 and butaclamol were purchased from RBI (Natick, MA). Dowex Resin (100-200 mesh) was purchased from Bio-Rad.

Cell culture. BHK3 cells were maintained in Dulbecco's Modified Essential Medium (DMEM) (GIBCO/BRL) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Permanent cell lines expressing the wild type and the mutant receptors were maintained as monolayer culture in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml G418.

Site-directed mutagenesis of D1 dopamine receptor gene. Mutagenesis was carried out on the human D1 dopamine receptor gene using the PCR as described (Higuchi et al, 1988). The PCR products were subcloned as described (Jin et al, 1992) into expression vector pRC/CMV (Invitrogen), which was linearized by Xba I, blunt-ended with Klenow fragment and dephosphorylated by calf alkaline phosphatase (Pharmacia). The orientation of inserts was confirmed by restriction mapping, and the DNA sequences encoding the wild type and the mutant receptors were verified by double-stranded DNA sequencing using Sanger's dideoxynucleotide termination method with T7 DNA Sequencing Kit (Pharmacia).
Permanent transfection of BHK3 cells with wild-type and mutant D1 receptor genes.

Monolayers of cells on Petri dishes at about 50% confluence were transfected using Lipofectin according to the instructions of the manufacturer. Cells were trypsinized at 48 hours and diluted 1:30 in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml G418 and incubated at 37°C in 5% CO2. After two weeks, G418 resistant clones were isolated in small Petri dishes, expanded in 75 cm² flasks, and screened using D1 receptor antagonist, SCH23390, with saturation isotherm curves to determine the expression level of the receptors. One cell line was selected for each type of receptor with a similar expression level (approximately 5 pmoles/mg protein for the wild type, C347A and C347/351A) as assessed by Bmax of [³H]SCH23390 saturation curves (see below). However, the C351A mutant was expressed at 15 pmoles/mg protein because a cell line expressing 5 pmoles/mg protein could not be found.

Ligand binding assays. Radioligand binding was performed essentially as described (Sunahara et al, 1990). The final concentrations of binding buffer were 50 mM Tris-HCl, 5 mM EDTA, 1.5 mM CaCl₂, 5 mM MgCl₂ (and 120 mM NaCl for antagonists) with protease inhibitors (10 μg/ml benzamidine, 5 μg/ml leupeptin, and 5 μg/ml soybean trypsin inhibitor). Cells were collected in the binding buffer by scraping with a rubber policeman. Cell membrane suspensions were obtained by Polytron homogenization of the collected cells (6,500 rpm for 20 seconds). Saturation experiments were carried out in duplicates with [³H]SCH23390 in increasing concentrations (20-5000 pM) and non-specific binding was determined by binding that was not displaced by 1 μM (+)-butaclamol. The concentration of [³H]SCH23390 in competition assays was
approximately equal to 600 pM. In competition curves using dopamine, tubes contained a final concentration of 5 mM ascorbic acid to prevent dopamine oxidation.

**Desensitization study.** Prior to harvesting, cells were pretreated with 10 μM dopamine for 15 min (with 5 mM ascorbic acid), and competition assays of dopamine (10^{-10}-10^{-4}) against [3H]SCH23390 (600 pM) were performed.

**Adenylyl cyclase assay.** Cell membranes were prepared by Polytron homogenization (6,500 rpm for 20 seconds) in 5 mM Tris-HCl (pH 7.4) and 2 mM EDTA (pH 8.0), centrifugation at 800 X g for 10 minutes to remove unbroken cells, ultracentrifugation of the supernatant at 15,000 rpm for 30 minutes to collect membranes, and resuspension of the membrane pellet in the reaction buffer containing 75 mM Tris-HCl, 12.5 mM MgCl\_2 and 2 mM EDTA. Adenylyl cyclase assays were conducted in duplicates as described (Johnson and Salomon 1991) at 28°C for 20 min, in a total volume of 50 μl of the assay mix, containing 20 μg membrane protein, 12 μM ATP, 100 μM cAMP, 53 μM GTP, 2.7 mM phosphoenol-pyruvate, 0.2 units of pyruvate kinase, 1 unit of myokinase and 5 mM ascorbic acid, with 0.13 μCi of [α-32P]ATP, in the presence of indicated concentrations of dopamine or 100 μM forskolin. Reactions were stopped by the addition of 1 ml of an ice-cold solution containing 0.4 mM ATP, 0.3 mM cAMP and [3H]cAMP (approximately 25,000 cpm). The resulting solutions were decanted directly onto the Dowex columns. The elute was then subjected to either the purification on aluminum columns or ZnSO\_4/Ba(OH)\_2 precipitation. Both methods produced comparable, low blank values (24). Protein concentrations were measured using Bio-Rad reagents.

**Data analysis.** All data were analyzed by nonlinear least squares regression using the InStat and InPlot GraphPad computer programs.
RESULTS

Radioligand binding profiles for wild-type and mutants of D1 receptors. Alignment of the amino acid sequences of G protein-coupled receptors reveals the presence of one or two conserved cysteine residues in the carboxyl terminus of most members of this receptor superfamily, including the human D1 dopamine receptor (Figs. 2.1 and 2.2). The human D1 dopamine receptor has been shown to be palmitoylated via thioester bond (Ng et al, 1993a). There are two cysteines (Figs. 2.1 and 2.2) in the C-tail of D1 receptors corresponding to those that are shown to be palmitoylated in rhodopsin (Karnik et al. 1993), and β2-adrenergic (O'Dowd et al, 1989; Moffett et al, 1993), α2A-adrenergic (Kennedy and Limbird 1993), and LH/hCG receptors (Kawate and Menon 1994). To determine whether the introduction of single and double mutations at these cysteines affected the binding profiles for agonists and antagonists of D1 receptors, the dissociation constants (Kd values) for [3H]SCH23390 and the rank orders of potency for D1 agonists and antagonists were determined. The Kd values were nearly identical to that of D1 dopamine receptor in human caudate tissue (Table 2.1). Catecholamine agonists and some dopaminergic antagonists were selected to compete for [3H]SCH23390 binding. Agonist displacement was observed to be the same for all the four receptors, with the following rank order of potency: dopamine > SKF38393 > serotonin (Table 2.2). Similarly, antagonist competition exhibited the same rank order for both the wild type and mutant receptors: SCH23390 > (+)-butaclamol > haloperidol (Table 2.3). The parameters (Kd and Ki values) were almost identical to each other between the wild type and the mutant receptors, and were consistent with the published
literature (Seeman and Niznik 1988). These data indicated that the replacement of either one or both of the cysteines with alanine(s) in the C-terminal did not affect the proper ligand binding profile of D1 dopamine receptors. This is expected, because it has been shown that ligand binding mainly involves the transmembrane domains (Dohlman 1991).

Agonist binding studies using dopamine or SKF38393 revealed the presence of two affinity states of the receptor detected both D1 agonists in all the four cell lines (see Table 2.2). The proportion of the high affinity state was very similar for the wild type and mutant D1 receptors, indicating the same extent of receptor-G protein coupling (De Lean et al., 1980). Thus, Cys347 and Cys351 of the D1 dopamine receptor are not required for agonist and antagonist binding, or for ternary complex formation.

**Agonist-induced desensitizations were indistinguishable for wild-type and mutant D1 dopamine receptors.** To assess the ability of the wild-type and mutant D1 dopamine receptors to desensitize, cells expressing receptors were pretreated with 10 μM dopamine for 15 min. As shown in Figure 2.3, all four types of dopamine D1 receptors were detected by dopamine competition against [³H]SCH23390 to have two affinity sites, with similar receptor fractions in the high affinity state (approximately 20%) in the absence of agonist pretreatment. The high affinity state of the wild type and mutant receptors was converted almost completely to low affinity state after exposure to dopamine (10 μM, 15 min), suggesting a similar pattern of desensitization by agonist.

In addition, dopamine competition (without pretreating the cells with dopamine) in the presence of 100 μM Gpp(NH)p, a nonhydrolyzable GTP analogue, the high affinity state of both the wild type and mutant receptors were also decreased to undetectable
levels (data not shown), indicating that the high affinity state of these receptors were equally sensitive to guanine nucleotide (De Lean et al, 1980).

Therefore, unlike the β2-adrenergic receptor, for which high affinity sites were lost when cysteine was replaced by alanine, elimination of palmitoylation site(s) did not affect receptor-G protein coupling for D1 dopamine receptors or agonist-induced D1 receptor desensitization.

The effect of mutation(s) on adenylyl cyclase activity. To further characterize the effect on receptor-G protein coupling caused by elimination of one or both of the palmitoylation sites, we assessed stimulation of adenylyl cyclase activity mediated by the wild-type and mutant receptors (Fig. 2.4). The results demonstrated that the two single mutant (C347A and C351A) and the double mutant (C347/351A) receptors had the same stimulation pattern as the wild type, and the EC50 values for the adenylyl cyclase stimulation with dopamine were almost identical to each other (Table 4). In all cases, forskolin caused full stimulation of adenylyl cyclase activity (approximately 100 pmoles cAMP/min/mg protein). These effects were different to those of β2-adrenergic receptor, the elimination of palmitoylation of which caused partial loss of adenylyl cyclase stimulation (O'Dowd et al, 1989; Moffett et al, 1993), although both D1 dopamine receptors and β2-adrenergic receptors are coupled the stimulatory GTP-binding proteins (Gs).

The basal level of adenylyl cyclase activity in the cell line expressing the C351A mutant D1 receptors was relatively higher than the other three cell lines. It was probably because the level of receptor expression was three-fold higher (approximately 15 versus 5 pmoles/mg protein, see Materials and Methods) than the other cell lines. Therefore, more
copies of the active form receptors were available stochastically at basal state (Milligan et al, 1995; Kenakin 1995a; Kenakin 1995b). Similar phenomena have been observed in other receptors, including β2-adrenergic receptors, in transfected cell lines (Samana et al, 1993; Adie and Milligan 1994). On the other hand, it is noteworthy that although the expression level of C351A cells was about three times higher, the maximum activity of adenylyl cyclase stimulation is not further elevated, probably because adenylyl cyclase activity has already been stimulated to its maximum by high concentrations of dopamine, i.e., "saturated", in the presence of receptors at the level of 5 pmol/mg protein or lower. This is consistent with a quantitative evaluation between the variable β2-adrenergic receptor expression levels and the EC50 and Vmax for epinephrine stimulation of adenylyl cyclase activity, carried out by Whaley et al (Whaley et al, 1993), which showed that Vmax initially increased roughly proportionally to β2-adrenergic receptor levels until reaching a plateau at a certain high level of β2-adrenergic receptors.
Table 2.1

SCH23390 Affinity for Wild Type and Mutant D1 Receptors

[^3H]SCH23390 of six concentrations of $10^{-10}$ to $10^{-4}$ M was used in the saturation binding isotherm to determine the dissociation constants (Kd) of the wild type and mutant receptors (see Materials and Methods). Non-specific binding was the binding not displaced by the presence of 10 mM (+)-butaclamol. The data shown (mean ± SEM) were obtained from the indicated number of experiments performed in duplicates. All values are within normal range of Kd for SCH23390. In t test, compared with WT, a = $P$ value between 0.01 and 0.02 (single mutants), b = not significant, $P$ value between 0.1 and 0.2 (double mutant).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>WT</th>
<th>C347A</th>
<th>C351A</th>
<th>C347/351A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kd (pM)</td>
<td>284.2 ± 27.5</td>
<td>369.6 ± 38.6$^a$</td>
<td>363.0 ± 30.6$^a$</td>
<td>318.1 ± 32.2$^b$</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 2.2

Agonist Rank Order for Wild Type and Mutant D1 Receptors

The rank order of the agonists shown below were determined by competition curves against \([^3H]SCH23390\) (600 pM) with a series of concentrations \((10^{-10}-10^{-4} \text{ M})\) of the agonists listed in the table (see Materials and Methods). The values shown are averages of results obtained from at least two experiments in duplicates.

<table>
<thead>
<tr>
<th>Agonists</th>
<th>Ki high (nM)</th>
<th>Ki low (nM)</th>
<th>% High</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild Type</td>
<td>5.5</td>
<td>6410</td>
<td>11.1</td>
<td>2</td>
</tr>
<tr>
<td>Dopamine C347A</td>
<td>9.1</td>
<td>1165</td>
<td>33.6</td>
<td>2</td>
</tr>
<tr>
<td>Dopamine C351A</td>
<td>2.9</td>
<td>3081</td>
<td>15.3</td>
<td>2</td>
</tr>
<tr>
<td>Dopamine C347/351A</td>
<td>5.4</td>
<td>804.6</td>
<td>18.0</td>
<td>4</td>
</tr>
<tr>
<td>SKF38393 WILD TYPE</td>
<td>12.3</td>
<td>787.1</td>
<td>13.9</td>
<td>2</td>
</tr>
<tr>
<td>SKF38393 C347A</td>
<td>19.7</td>
<td>1784</td>
<td>17.0</td>
<td>3</td>
</tr>
<tr>
<td>SKF38393 C351A</td>
<td>25.6</td>
<td>641.7</td>
<td>17.9</td>
<td>3</td>
</tr>
<tr>
<td>SKF38393 C347/351A</td>
<td>19.4</td>
<td>1458</td>
<td>17.2</td>
<td>2</td>
</tr>
<tr>
<td>5-HT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild Type</td>
<td>28007</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>5-HT C347A</td>
<td>36037</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>5-HT C351A</td>
<td>53920</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>5-HT C347/351A</td>
<td>26240</td>
<td></td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>
Table 2.3

Antagonist Rank Order for D1 Receptors

Competition binding assays were performed to determine the rank order of potency for D1 antagonists (see Materials and Methods). The Ki values were averages of results determined by at least two experiments carried out in duplicates using InPlot and InStat computer programs.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Ki (nM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCH23390 Wild Type</td>
<td>3.9</td>
<td>2</td>
</tr>
<tr>
<td>SCH23390 C347A</td>
<td>3.1</td>
<td>2</td>
</tr>
<tr>
<td>SCH23390 C351A</td>
<td>5.9</td>
<td>2</td>
</tr>
<tr>
<td>SCH23390 C347/351A</td>
<td>3.4</td>
<td>2</td>
</tr>
<tr>
<td>Butaclamol Wild Type</td>
<td>31.8</td>
<td>2</td>
</tr>
<tr>
<td>Butaclamol C347A</td>
<td>26.3</td>
<td>2</td>
</tr>
<tr>
<td>Butaclamol C351A</td>
<td>42.5</td>
<td>2</td>
</tr>
<tr>
<td>Butaclamol C347/351A</td>
<td>19.1</td>
<td>2</td>
</tr>
<tr>
<td>Haloperidol Wild Type</td>
<td>177.4</td>
<td>2</td>
</tr>
<tr>
<td>Haloperidol C347A</td>
<td>159.9</td>
<td>2</td>
</tr>
<tr>
<td>Haloperidol C351A</td>
<td>223.8</td>
<td>2</td>
</tr>
<tr>
<td>Haloperidol C347/351A</td>
<td>183.0</td>
<td>2</td>
</tr>
</tbody>
</table>
**TM 7**

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hum D1R</td>
<td>... NP[IIYAF]-NADFRKAFSTLLG[CYRLCPATW ...</td>
</tr>
<tr>
<td>Hum Rhodopsin</td>
<td>... NPVIYIM</td>
</tr>
<tr>
<td>Hum □□AR</td>
<td>... NPLIYCR</td>
</tr>
<tr>
<td>Hum □□AR</td>
<td>... NPVIYTI</td>
</tr>
<tr>
<td>Rat D2R</td>
<td>... NOIIYTTFNIEFKAFMKILHC</td>
</tr>
<tr>
<td>Hum M2AChR</td>
<td>... NPACYAL</td>
</tr>
<tr>
<td>Hum 5-HT1AR</td>
<td>... NPVIYAY</td>
</tr>
</tbody>
</table>

...       ...     ...     ...     ...

**Figure 2.1. Alignment of Amino-Terminal Regions of the Carboxyl Tails of G Protein-Coupled Receptors.** The cysteine residues for palmitoylation are highlighted.
Figure 2.2. The TM7 to the N part of the C-terminal of the Human D1 Dopamine Receptor. The palmitoylation sites are indicated by zigzag lines. The two cysteines of the C-terminus were substituted by alanine either separately (C347A and C351A) or together (C347/351A) by site-directed mutagenesis (see Materials and Methods).
Figure 2.3. Desensitization of D1 Receptors Induced by Dopamine Pretreatment.

Cells expressing the wild type and mutant receptors were pretreated with 1 μM dopamine for 15 minutes. Dopamine (10^{-10} to 10^{-4} M) was used as the cold agonist to compete against 600 pM [^{3}H]SCH23390. The data shown are representatives of two to four independent experiments done in duplicates. Controls and pretreated samples are indicated by open and closed symbols, respectively.
Figure 2.4. Adenylyl Cyclase Activities Stimulated by Dopamine in Cells Expressing the Wild Type and Mutant D1 Receptors. Dopamine (10^-11 to 10^-4 M) was used to stimulate adenylyl cyclase in the four cell lines expressing the wild type and mutant D1 dopamine receptors. The results shown are representatives of two to four experiments done in duplicates. The parameter estimates (EC50) are shown in Table 2.4.
Table 2.4

EC50 Values for Dopamine Stimulation of Adenylyl Cyclase Activity by the Wild Type and Mutant D1 Receptors.

Adenylyl cyclase assays were carried out to determine the EC50s of the wild type and the mutant D1 receptors (see Materials and Methods). The values shown are means of two to four experiments in duplicates. All values are within normal range of EC50. No statistical analysis was applied.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>WT</th>
<th>C347A</th>
<th>C351A</th>
<th>C347/351A</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (nM)</td>
<td>28.7</td>
<td>68.0</td>
<td>72.6</td>
<td>35.2</td>
</tr>
<tr>
<td>n</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
Primary sequence comparison among G protein-coupled receptors reveals that at least one or two cysteine residues are conserved in the COOH-terminal of the majority of these receptors (Fig. 2.1). Palmitoylation has been demonstrated experimentally in a number of G protein-coupled receptors at these cysteines via thioester bonds, including rhodopsin (Karnik et al., 1993), β2-adrenergic receptors (O'Dowd et al., 1989; Moffett et al., 1993), α2A-adrenergic receptors (Kennedy and Limbird, 1993), and LH/hCG receptors (Kawate and Menon, 1994). D1 dopamine receptors (Ng et al., 1993a), 5-HT1B receptors (Ng et al., 1993b) and D2 receptors (Ng et al., 1994) have also been shown to be palmitoylated via thioester bonds. It was suggested that this high level of conservation of palmitoylation could be a general process with potentially important functions (Bouvier et al., 1995).

To determine the functional role of palmitoylation in G protein-coupled receptors, several research groups have carried out a series of site-directed mutagenesis studies on rhodopsin (Karnik et al., 1993), β2-adrenergic receptors (O'Dowd et al., 1989; Moffett et al., 1993), α2A-adrenergic receptors (Kennedy and Limbird, 1993), m2 muscarinic receptors (Van Koppen and Nathanson, 1991), thyrotropin releasing hormone receptors (Nussenzveig et al., 1993) and LH/hCG receptors (Kawate and Menon, 1994). The two main categories of receptor properties being investigated were receptor-G protein coupling and receptor internalization/down regulation.
Elimination of the palmitoylation site (C341G) of β2-adrenergic receptors resulted in a partial loss of receptor-G protein coupling, as demonstrated by the loss of agonist-detected high affinity state and decrease in the receptor mediated adenylyl cyclase stimulation (O’Dowd et al, 1989; Moffett et al, 1993). In contrast, similar mutagenesis studies on other G protein-coupled receptors including this study have demonstrated that palmitoylation at the cysteine(s) of their C tail is not required for receptor-G protein coupling. Although the chemical removal of palmitate from rhodopsin increased the receptor-Gt coupling (Morrison et al, 1991), the elimination of the palmitoylation sites (C322S and C323S) did not show any effect on rhodopsin-Gt coupling (Karnik et al, 1993). A lack of effect on receptor-G protein coupling, assessed by the inhibition of adenylyl cyclase activity, has been demonstrated in both α2A-adrenergic mutant receptors (C442A or C442S) (Kennedy and Limbird 1993) and the mutant M2 muscarinic receptors (C457G) (Van Koppen and Nathanson 1991), in which the palmitoylation site has been eliminated. Similarly, the substitution of glycine for Cys335 and Cys337 of the mouse thyrotropin-releasing hormone receptor did not affect the receptor-mediated stimulation of IP3 production (Nussenzveig et al, 1993). In addition, in LH/hCG receptors, which are coupled Gs like β2-adrenergic receptors, the EC50 and maximal production of cAMP among the wild type and non-palmitoylated mutants were comparable, suggesting that the loss of palmitoylation did not affect the efficiency of coupling to Gs protein (Kawate and Menon 1994). Thus, in the previous studies on receptor-G protein coupling, β2-adrenergic receptors were the only exception in which palmitoylation plays a critical role in its interaction with G proteins.
On the other hand, the effects of palmitoylation on receptor sequestration and down-regulation have been diverse. Although TRHR mutations (C335/337G) had no effect on its G protein coupling, the lack of palmitoylation significantly blocked rapid agonist-induced internalization (Nussenzveig et al, 1993). Interestingly, it has been shown (Eason et al, 1994) that in α2A-adrenergic receptor mutants (C442F or C442A), down-regulation after prolonged agonist exposure was completely abolished, although the mutation of Cys442 did not alter the extent or rate of agonist promoted sequestration or the recovery from sequestration. Finally, for LH/hCG receptors, abolition of palmitoylation by C621S and C622S mutations in the C-terminal tail increased agonist-induced internalization of the receptor (Kawate and Menon 1994). Thus, receptor palmitoylation may serve completely different roles among various members of this superfamily of G protein-coupled receptors in receptor sequestration and receptor down regulation.

D1 dopamine receptors, like β2-adrenergic receptors, are coupled to Gs. However, contrary to the results of the β2-adrenergic receptor mutagenesis study, the single or double mutations in D1 dopamine receptors, which eliminated one or both of the palmitoylation sites, did not perturb the ligand binding profile, receptor desensitization, agonist-receptor-G protein interaction, or the ability to mediate adenylyl cyclase stimulation, i.e., no difference was observed between the wild type and mutant D1 dopamine receptors. Nevertheless, this difference between the D1 dopamine and β2-adrenergic receptors in receptor-G protein coupling does not exclude the possibility that palmitoylation may be involved in other receptor functions such as receptor internalization/down-regulation in a pattern similar to other receptors as outlined above.
We believe that the relationship between palmitoylation and receptor-G protein coupling is unique for β2-adrenergic receptors and that it is attributed to the unique feature of its C-terminal tail. The β2-adrenergic receptor contains a cAMP-dependent protein kinase (PKA) site in its carboxyl tail in close proximity (4 amino acid residues apart) to the palmitoylation site, whereas no such a phosphorylation site is found in the C-tails of rhodopsin, α2A-adrenergic receptors, m2 muscarinic receptors, TRH receptors and D1 dopamine receptors. A PKA site is found in LH/hCG receptors in its C-terminus, but it is 33 amino acid residues away from the two cysteines that have been shown to be palmitoylated. We suggested before (O’Dowd et al, 1989) that palmitic acids on the β2-adrenergic receptors might serve to anchor its C-tail to the membrane, thereby, producing a fourth intracellular loop. This conformation of wild type β2-adrenergic receptors may prevent access to the PKA site in the vicinity by the cytosolic PKA. Therefore, palmitoylation/depalmitoylation can serve as a “switch” to cover or expose that particular PKA site in the wild type β2-adrenergic receptors, whereas the elimination of palmitoylation of the Gly341β2-adrenergic receptors can constantly expose the PKA site, thereby causing constitutive desensitization even in the absence of agonist treatment. In fact, Moffett et al. (Moffett et al, 1993) have shown that the basal level of phosphorylation is about 4 times higher in the Gly341β2-adrenergic receptor mutant than in the wild type and that agonist treatment cannot promote any further phosphorylation or uncoupling of the mutant receptor. In addition, Bouvier et al. reported (Bouvier et al, 1995) that elimination of the PKA site by substituting alanine residues for Ser345 and Ser346 of Gly341β2-adrenergic receptor restored both the phosphorylation and the effector activation to levels observed with wild type β2-adrenergic receptors, lending
further support to our conclusion that the unique presence of PKA site in the immediate vicinity of the palmitoylation site of β2-adrenergic receptor is responsible for the discrepancy on the observations between the β2-adrenergic receptor and other G protein-coupled receptors. Since there is no such a PKA site in the other receptors as aforementioned, palmitoylation or non-palmitoylation therefore should therefore make no difference to their phosphorylation state whether at basal conditions or in the presence of agonist treatment.

A less likely alternative explanation for the difference between D1 dopamine and β2-adrenergic receptors may lie in the different cell lines selected for receptor expression. In β2-adrenergic receptor studies (O’Dowd et al., 1989; Moffett et al., 1993), CHW1102 cells were used, whereas in our study we used BHK3 cells (for comparison, COS cells were used in rhodopsin study (Karnik et al., 1993), LLC-PK1 and CHO cells for α2A-adrenergic receptors (Kennedy and Limbird 1993; Eason et al., 1994), CHO cells for m2 muscarinic receptors (Van Koppen and Nathanson 1991), COS, Hela and CV cells for TRH receptors (Nussenzveig et al., 1993), and HEK-293 cells for LH/hCG receptors (Kawate and Menon 1994). It has been shown that signaling by G protein-coupled receptors may be dependent on the cell type (Milligan 1993). The different genetic background of the cells used for transfection may produce a different micro-environment in the vicinity of the receptor-G protein interaction. However, it does not seem very likely that cell lines were responsible for the different results of the two Gs-coupled receptors. In a subsequent study in Chapter 3, wild type and the double cysteine mutant D1 receptors were expressed in another mammalian cell line, Chinese Hamster Ovary (CHO) cells, and again the double mutant (designated as AA) exhibited normal receptor-
G protein coupling. The fact that elimination of palmitoylation sites from D1 receptor did not interfere with receptor-G protein coupling in both BHK and CHO cell lines argues against the possibility that different cell lines were the cause of the discrepancy between β2AR and D1DR.

During the conduction of this study, Jensen et al. (Jensen et al, 1995) reported a similar study on the carboxyl tail cysteine residues of D1 receptor and suggested that Cys347 was important for antagonist binding, activation of adenylyl cyclase and agonist-induced desensitization. We believe that their conclusions were based on technical differences. In their antagonist study, (+)-butaclamol (for both wild type and mutants) had a higher affinity for the D1 receptor than SCH23390, which was inconsistent with our study and the published literature (Seeman and Niznik, 1988), since the reported Ki values for (+)-butaclamol were two orders of magnitude smaller. In their adenylyl cyclase assays, their EC50 values (7.1-10.0 μM) for dopamine were several hundred times higher than our estimates (28.7-72.6 nM) and their SKF38393 EC50 (56-74 nM). The EC50 values and the reversed rank order of dopamine versus SKF38393 were also inconsistent with the published literature (Seeman and Niznik, 1988). Furthermore, in their desensitization study using adenylyl cyclase assays, EC50 values (5.8-9.7 μM) for preexposed samples were very similar to, actually smaller than, those of the controls (as listed above), suggesting that homologous desensitization for the wild type and the mutant receptors did not take place. Moreover, although they noticed that absence of Cys347 also caused a substantially diminished expression level in the membrane fraction of the cell, they failed to interpret their adenylyl cyclase data accordingly. Their Cys347 mutant receptors (0.4 pmol/mg protein for S347 and 0.5 pmol/mg protein for S347/S351)
were expressed only at about half to a third of the level of Cys351 mutants and wild type (1.2 pmol/mg protein for S351, 1.1 pmol/mg protein for T352 and 1.9 pmol/mg protein for WT). Their Vmax values (pmol of cAMP/(mg protein x min)) of adenylyl cyclase activity for C347 mutants (7.0 ± 0.5 for S347 and 6.4 ± 1.0 for S347/S351) were also about half of those for Cys351 mutants and wild type (13.2 ± 1.2 for S351, 13.9 ± 0.7 for T352 and 13.5 ± 1.2 for WT). Thus, we believe that the adenylyl cyclase activity for the wild type, Cys347 mutants and others would have been stimulated to a similar maximum level if the expression of those receptors had been at comparable levels in their study, and that proper interpretation of their data should actually confirm our conclusion indirectly.

Acknowledgements. This study has been funded by Ontario Mental Health Foundation Fellowship to HJ and grants from Medical Research Council of Canada and NIDA to SRG and BO'D.
Chapter 3

The Palmitoylation Sites and the Effect of an Engineered cAMP-Dependent Protein Kinase Consensus Site in the C Tail of D1 Dopamine Receptor

This chapter is based on the following manuscript:

ABSTRACT

To determine the palmitoylation sites in the human D1 dopamine receptor (D1DR), we expressed wild type and mutant receptors in which candidate cysteines in the carboxyl tail were substituted by alanines both individually (A347, A351) and together (AA). Our results showed that palmitoylation levels of A347 and A351 were reduced substantially and that AA had no detectable signal of palmitoylation. These data indicate that cysteines 347 and 351 were both palmitoylated and that they were the only sites of palmitoylation. We introduced a PKA site encompassing the position 351. We predicted that a functional PKA site might impair receptor-G protein coupling if it was not occluded by palmitoylation. Our results demonstrated that indeed the introduction of the PKA site reduced potency of dopamine stimulation of adenylyl cyclase and thus confirmed that when unoccluded, the PKA site introduced to position 351 of D1DR could confer partial constitutive desensitization.
INTRODUCTION

Many G protein-coupled receptors (GPCR) have been shown to be palmitoylated (recently reviewed by Jin, et al. 1999) at cysteine residues in the cytoplasmic tail and these include rhodopsin (Ovchinnikov, et al. 1988; Karnik, et al. 1993), β2-adrenoceptor (β2AR) (O'Dowd, et al. 1989), α2-adrenoceptor (α2AR) (Kennedy and Limbird 1993, Eason, et al. 1994), luteinizing hormone/chorionic gonadotropin (LH/CG) (Kawate and Menon 1994), endothelin A (ETA) (Horstmeyer, et al. 1996), endothelin B (ETB) (Okamoto, et al. 1997) and V2 vasopressin receptors (Sadeghi, et al. 1997). The serotonin 5-HT1A (Butkerait, et al. 1995) and 5-HT1B receptors (Ng, et al. 1993), dopamine D1 (D1DR) (Ng, et al. 1994a) and D2 receptors (Ng, et al. 1994b), and metabotropic glutamate receptor mGluR4 (Alaluf, et al. 1995) have also been reported to be palmitoylated, but the actual sites of palmitoylation for these receptors have not been demonstrated (Table 1.2), although it is assumed that palmitoylation occurs at the cysteine residues in their carboxyl tails. Recently, it was reported that a mutant μ-opioid receptor (MOR) with its two cysteines in the tail replaced by alanines was still palmitoylated as in wild type (Chen, et al. 1998), suggesting that palmitoylation of MOR occurred elsewhere.

Accumulating evidence reveals that palmitoylation serves to enhance the association of cytosolic proteins with the membrane (Mumby 1997, Bizzozero 1997). However, the function of GPCR palmitoylation remains unknown. The elimination of palmitoylation sites attenuated the receptor-G protein coupling in β2AR (O'Dowd, et al. 1989; Moffett, et al. 1993), ETB (Okamoto, et al. 1997) and SSTR5 receptors (Hukovic, et al. 1998), but not in other GPCRs studied including rhodopsin (Karnik, et al. 1993),
α2AR (Kennedy and Limbird 1993, Eason, et al. 1994), m2 muscarinic receptor (M2AChR) (van Koppen and Nathanson 1991), m3 muscarinic receptor (M3AchR) (Zeng, et al. 1999), thyrotropin releasing hormone receptor (TRHR) (Nussenzveig, et al. 1993), LH/CG (Kawate and Menon 1994), ETA (Horstmeyer, et al. 1996), V2 (Sadeghi, et al. 1997), D1DR (Jin, et al. 1997) and thyrotropin receptor (TSHR) (Tanaka, et al. 1998), indicating that palmitoylation is not essential for receptor-G protein coupling, although other functions such as receptor internalization and down-regulation may be affected by elimination of palmitoylation as summarized in Table 1.2.

The β2AR contains a cAMP-dependent protein kinase (PKA) consensus sequence in the close vicinity of the palmitoylation site, being separated by only one amino acid residue. It has been speculated that the elimination of palmitoylation of β2AR may expose the PKA site and cause constitutive desensitization of this receptor (Bouvier, et al. 1995). The fact that elimination of the PKA site from the depalmitoylated β2AR restored the normal receptor-G protein coupling relationship (Moffett, et al. 1996) lent further support to this hypothesis. No PKA consensus site at a similar position is found in the other receptors listed in Table 1.2.

The purpose of our study was two-fold. In the first part, we wished to elucidate the actual sites of palmitoylation of D1DR. We eliminated the putative palmitoylation sites by replacing Cys347 and Cys351 with alanine, both individually (designated as A347 and A351 hereafter) and together (designated as AA) (Jin, et al. 1997), and these receptors were expressed. In this report we showed that elimination of Cys347 or Cys351 substantially reduced the level of palmitoylation and no palmitoylation was shown in the double mutant (AA), indicating that these two cysteines are the only palmitoylation sites.
In the second part, we tested the hypothesis that the PKA site in β2AR is responsible for its difference from other GPCRs. We introduced a PKA site into D1DR at position 351. A functional PKA site would result in a similar constitutive desensitization. Indeed, we demonstrated that the addition of the PKA consensus site did cause a reduction in the potency of dopamine stimulation of adenylyl cyclase compared with the wild type. Thus, the introduced PKA site conferred a degree of constitutive desensitization when not interfered with by palmitoylation.
MATERIALS AND METHODS

Materials. Grace's supplemented medium, fetal bovine serum, gentamycin sulfate, fungizone, Goat serum, rabbit serum, lipofectin, geneticin (G418), penicillin and streptomycin were purchased from GIBCO/BRL (Toronto, ON). [9,10-^3^H]palmitic acid (70-80 Ci/mmol), [a-^32^P]ATP (800 Ci/mmol), [^3^H]SCH23390 and [^3^H]cAMP (30 Ci/mmol) were purchased from Du Pont/NEN (Boston, MA). Dopamine, serotonin, leupeptin, benzamidine, soybean trypsin inhibitor, GTP, ATP, cAMP and forskolin were purchased from Sigma (St. Louis, MO). Butaclamol were purchased from RBI (Natick, MA). Dowex Resin (100-200 mesh) was purchased from Bio-Rad (Richmond, CA).

Construction of D1DR recombinant baculoviruses. The wild type and mutant receptor genes (Jin, et al. 1997) were subcloned into Bacmid supplied with the Bac to Bac kit (Life Technologies) and subsequently expressed in Sf9 cells according to the instructions of the manufacturer.

Cell culture. Sf9 cells were grown in monolayer or suspension culture as described (Summers and Smith 1987). CHO cells were maintained in Dulbecco's Modified Essential Medium (DMEM) (GIBCO/BRL) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Permanent cell lines expressing the wild type and the mutant receptors were maintained as monolayer culture in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml G418.

Metabolic labeling with tritiated palmitate. At 24 hours post viral infection, Sf9 cells expressing D1DRs were cultured in serum-free medium for 18 hours prior to the labeling
experiment. Following this period, cells were resuspended in Grace's insect medium supplemented with 1% fetal bovine serum for 1 hour at 27°C. [9,10-3H]palmitate was dissolved in dimethyl sulfoxide and added to the suspension culture at the concentration of 0.2 mCi/ml for 4 hours. Palmitic acid labeling was terminated by centrifugation at 100 x g and cells washed twice with cold PBS.

**Solubilization and immunoprecipitation of D1DR and SDS-PAGE.** Cells were broken by sonication twice for 20 seconds at the setting of 3.5 in buffer A: 5 mM Tris-HCl and 2 mM EDTA with protein inhibitors (10 μg/ml benzamidine, 5 μg/ml leupeptin and 5 μg/ml soybean trypsin inhibitor). Unbroken cells were removed by centrifugation at 100 x g for 5 minutes. The supernatant was centrifuged at 27000 x g for 20 minutes and resuspended in solubilization buffer containing 100 mM NaCl, 20 mM Tris-HCl pH 7.4, 2% digitonin and 5 mM EDTA with protease inhibitors. The suspension was stirred at 4°C for 2 hours and centrifuged at 27000 x g for 20 minutes. The solubilized fraction was washed and concentrated in Centriprep 30 (Amicon) using buffer B: 100 mM NaCl and 10 mM Tris-HCl pH 7.4 with protease inhibitors. The resultant solution was precleared with 1/20 normal rabbit serum and protein A-Sepharose beads for 2 hours on ice. The solubilized receptors were immunoprecipitated with the rabbit antiserum for D1DR (kindly provided by Dr. Mark Brann, University of Vermont) at a 1/40 dilution in buffer B for 2 hours on ice, and agitated gently overnight at 4°C with 1/40 dilution of agarose fixed goat anti-rabbit IgG. The immunoprecipitate was washed several times with cold buffer B, solubilized in SDS sample buffer (50 mM Tris-HCl pH 6.5, 10% SDS, 10% glycerol and 0.003% bromophenol blue), sonicated and electrophoresed on SDS-PAGE.
Following electrophoresis, the gel was fixed and treated with Enlightning fluid (NEN) for 30 minutes, dried and exposed to Kodak X-AR film at -70°C for 6 weeks.

**Site-directed mutagenesis of D1 dopamine receptor gene.** Mutagenesis was carried out on the human D1 dopamine receptor gene using the PCR as described (Jin, et al. 1977). The PCR products were subcloned as described (Jin, et al. 1997) into expression vector pRC/CMV (Invitrogen), which was linearized by Xba I, blunt-ended with Klenow fragment and dephosphorylated by calf alkaline phosphatase (Pharmacia). The orientation of inserts was confirmed by restriction mapping, and the DNA sequences encoding the wild type and the mutant receptors were verified by double-stranded DNA sequencing using Sanger's dideoxynucleotide termination method with T7 DNA Sequencing Kit (Pharmacia).

**Permanent transfection of CHO cells with wild-type and mutant D1 receptor genes.** Monolayers of cells on Petri dishes at about 50% confluence were transfected using Lipofectin according to the instructions of the manufacturer. Cells were trypsinized at 48 hours and diluted 1:30 in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mg/ml G418 and incubated at 37°C in 5% CO₂. After two weeks, G418 resistant clones were isolated in small Petri dishes, expanded in 75 cm² flasks, and screened using D1 receptor antagonist, SCH23390, with saturation isotherm curves to determine the expression level of the receptors. One cell line was selected for each type of receptor with a similar expression level (approximately 200 fmoles/mg protein) as assessed by [³H]SCH23390 saturation.

**Ligand binding assay.** Ligand binding assays were carried out essentially as described (Jin, et al. 1997). The final concentrations in binding buffer were 75 mM Tris HCl, 5 mM
EDTA and 5 mM MgCl₂. Cells were collected in the binding buffer by scraping with a rubber policeman. Cell membrane suspensions were obtained by Polytron homogenization of the collected cells (6,500 rpm for 20 seconds). Saturation experiments were carried out in triplicates with [³H]SCH23390 in increasing concentrations (20-5000 pM) and non-specific binding was determined by binding that was not displaced by 1 µM (+)-butaclamol.

**Adenylyl cyclase assay.** Cell membranes were prepared by Polytron homogenization (6,500 rpm for 20 seconds) in binding buffer, centrifugation at 12000 rpm to collect membranes, and resuspension of the pellet in reaction buffer containing 75 mM Tris-HCl, 8 mM MgCl₂ and 5 mM EDTA. Adenylyl cyclase assays were conducted in duplicates as described (Johnson and Salomon 1991) at 28°C for 20 min, in a total volume of 50 µl of the assay mix, containing 20 µg membrane protein, 12 µM ATP, 100 µM cAMP, 53 µM GTP, 2.7 mM phosphoenol-pyruvate, 0.2 units of pyruvate kinase, 1 unit of myokinase and 5 mM ascorbic acid, with 0.13 µCi of [α-³²P]ATP, in the presence of indicated concentrations of dopamine. Reactions were stopped by the addition of 1 ml of an ice-cold solution containing 0.4 mM ATP, 0.3 mM cAMP and [³H]cAMP (approximately 25,000 cpm). The resulting solutions were decanted directly onto the Dowex columns. The elute was then subjected to purification on aluminum columns. Protein concentrations were measured using Bio-Rad reagents.

**Data analysis.** Signals of western blot and palmitoylation in SDS-PAGE were analyzed by densitometry using MCID-M4 (Imaging Research Inc., St. Catherine, Ontario). Ligand binding and adenylyl cyclase assay data were analyzed by nonlinear least squares regression using the InStat and InPlot GraphPad computer programs.
RESULTS

Palmitoylation occurs at both Cys347 and Cys351 of D1DR. To determine the sites of palmitoylation of D1DR, we replaced Cys347 and Cys351 with alanine both separately (A347 and A351) and together (AA), and expressed the wild type, A347, A351 and AA receptors in St9 cells. As shown in Fig. 3.1, WT, A347 and A351 (upper band in lanes 1, 2 and 3) were each palmitoylated; AA was not palmitoylated (lane 4). The levels of palmitoylation were found to be reduced in the single mutations as compared with the wild type. The level of receptor expression was corrected for by standardization with western blot signals. The A347 mutant was palmitoylated to 24.3% of the level of wild type, whereas the A351 was palmitoylated to 47.4% in comparison with the wild type (Table 3.1). No palmitoylation signal could be detected for the double mutant AA. The fact that the palmitoylation signal of wild type was at least two-fold greater than that of the single mutants and absent in the double mutant, indicated that these two cysteines were both palmitoylated, and that they were the only sites of palmitoylation for D1DR.

Cells expressing wild type and mutant receptors were treated with 10 μM dopamine for 20 minutes. We did not observe any increase in the level of palmitoylation in the wild type or single mutants (Table 3.1). Again, no signal was detected in the double cysteine mutant.

Introduction of PKA site in D1DR. In β2AR, elimination of palmitoylation site attenuated receptor-G protein coupling (O'Dowd, et al. 1989). It has been demonstrated (Moffet, et al. 1996) that PKA site in β2AR was responsible for the attenuated receptor-G protein coupling in the Gly341β2AR mutant. We introduced a PKA site into the wild
type D1DR and the AA double mutant. In order to create a consensus PKA sequence R-R-X-S, we replaced the wild type sequence R-L-C351-P with R-R-C351-S (designated as P) and replaced the R-L-A351-P in the double cysteine mutant with R-R-A351-S (designated as AAP) (Fig. 3.2). We subsequently expressed the WT, AA, P and AAP receptors in CHO cells. Stable cell lines expressing similar receptor densities were selected for further study. As shown in Table 3.2, the stable cell lines expressed WT, AA, P, and AAP at comparable levels. The dissociation constant Kd values for SCH23390 for the four receptors were also similar (Table 3.2), indicating that the introduction of the PKA site did not alter receptor affinity for the antagonist SCH23390.

The effect of the introduced PKA site on the receptor-G protein coupling. We had predicted that the PKA site introduced into the wild type receptor should not produce any constitutive desensitization because it might be occluded by palmitoylation. However, phosphorylation of the PKA site in the AAP was expected to confer some degree of constitutive desensitization, since the elimination of two palmitoylation sites might have promoted PKA-mediated phosphorylation. Therefore, we expected that WT, AA, and P should have the same level of basal activity and maximum dopamine stimulation of adenylyl cyclase, whereas the AAP should have lower potency and/or efficacy of agonist stimulation of adenylyl cyclase activity.

As shown in Figure 3.3 and summarized in Table 3.3, the basal adenylyl cyclase activities of WT, AA, P, and AAP were similar. However, the EC50 value for AAP was shifted to the right and was at least two-fold higher than the other three receptors (one-tailed t test, P<0.05). It was noted that the Vmax of P (P<0.01) and AAP (P<0.001) was higher than WT. Although the reason of the increased Vmax was not exactly clear, the
observation that forskolin also had increased stimulation in P and AAP cells might suggest that adenylyl cyclase was expressed higher in these two stable cell lines.
DISCUSSION

We have shown previously that D1DR is palmitoylated (Ng, et al. 1994); however, the palmitoylation site(s) was not determined. The two cysteines (Cys347 and Cys351) most proximal to the membrane were assumed to be the palmitoylation sites. Within the cytoplasmic domains, three other cysteine residues exist in D1DR, two in the third intracellular loop (Cys297 and Cys306) and one in the carboxyl tail (Cys385). In this report we have demonstrated the following: (1) wild type D1DRs were palmitoylated at both Cys347 and Cys351; (2) these two cysteines were palmitoylated independently, consistent with LH.CG (Kawate, et al. 1994) and V2 receptors (Sadeghi, et al. 1997) but in contrast to rhodopsin, in which the substitution of the first cysteine prevented the palmitoylation of the second cysteine (Karnik, et al. 1993); (3) palmitoylation occurred only at Cys347 and Cys351, consistent with most studies listed in Table 1 with the exception of MOR, where elimination of the two cysteines in the carboxyl tail did not affect palmitoylation of the mutant receptor; and (4) the other three cysteines in the cytoplasmic regions were not palmitoylated.

The study of receptors listed in Table 1.2 can roughly be classified into three categories:

(1) No palmitoylation data were available and only cysteine mutagenesis was carried out. These receptors include M2AchR (van Koppen and Nathanson, 1991), M3AchR (Zeng, et al. 1999), SSTR5 (Hukovic, et al. 1998) and TRHR (Nussenzveig, et al. 1993).
(2) Receptors were shown to be palmitoylated but no mutagenesis studies were carried out; therefore, the actual sites of palmitoylation were not determined. In these receptors, it has always been assumed that palmitoylation occurs at the cysteine residues of their carboxyl terminals close to the membrane. These receptors include 5-HT1A (Butkerait, et al. 1995), 5-HT1B (Ng, et al. 1993), D2DR (Ng, et al. 1994b) and mGluR4 receptors (Alaluf, et al. 1995).

(3) Receptors were shown to be palmitoylated and the actual palmitoylation sites were determined by peptide sequencing and mass spectrometry in rhodopsin (Ovchinnikov, et al. 1988) or by site-directed mutagenesis studies in the majority of other receptors as listed below. These receptors include the following: α2AR (Kennedy and Limbird 1993; Eason, et al. 1994), β2AR (O’Dowd, et al. 1989; Moffett, et al. 1993), ETA (Horstmeyer, et al. 1996), ETB (Okamoto, et al. 1997), LH/CG (Kawate and Menon 1994; Zhu, et al. 1995; Kawate, et al. 1997), rhodopsin (Karnik, et al. 1993), TSHR (Tanaka, et al. 1998) and V2 receptors (Sadeghi, et al. 1997). D1DR (Ng, et al. 1994a; Jin, et al. 1997; and this report) also falls into this category.

Of the receptors in which palmitoylation has been shown and the sites identified, ETA, ETB, LH/CG, rhodopsin and V2 receptors contain more than one cysteine residue in the carboxyl terminal. In ETB (Okamoto, et al. 1997), LH/CG (Kawate and Menon 1994), rhodopsin (Karnik, et al. 1993) and V2 (Sadeghi, et al. 1997), multiple cysteines of the palmitoylation sites were mutated both individually and together. In ETB, LH/CG and V2, these cysteines were palmitoylated individually and palmitoylation did not require the presence of the other cysteines. However, in rhodopsin, the mutation of the first of the adjacent cysteine residues prevented the palmitoylation of the next cysteine,
indicating that the first cysteine is the primary palmitoylation site (Karnik, et al. 1993). The five cysteines for palmitoylation in ETA (Horstmeyer, et al. 1996) were not mutated separately. Our results that in D1DR Cys347 and Cys351 were independent palmitoylation sites were consistent with the results of ETB, LH/CG and V2 receptors.

Recently, it was reported that palmitoylation of MOR occurred at sites other than the two cysteines of the carboxyl tail (Chen, et al. 1998). The reason for this discrepancy is not clear. Since there is another cysteine in the second intracellular loop of MOR, palmitoylation might occur at that cysteine. Our data in this report demonstrated that palmitoylation occurs only at Cys347 and Cys351 in the carboxyl tail of D1DR.

In the second part of this report, we introduced a PKA site in the vicinity of position 351 in the wild type and AA. Elimination of palmitoylation of β2AR has been suggested to constantly expose the adjacent PKA site to PKA leading to constitutive desensitization and impaired receptor-G protein coupling (Moffet, et al. 1996). A functional PKA site introduced in the AAP was expected to result in some constitutive desensitization. Indeed, EC50 of dopamine stimulation of adenylyl cyclase in AAP was shifted to the right (P<0.05). The decreased potency of dopamine stimulation suggested that the PKA site introduced into the carboxyl tail was functional, and that it conferred a certain degree of constitutive desensitization to this mutant receptor. The reason why this constitutive desensitization affected only potency but not efficacy is unknown. This phenomenon of desensitization affecting only potency (EC50) and not efficacy (Vmax) has been observed in other mutated receptors as well, such as a β2AR mutant with its βARK sites removed from the carboxyl tail (Hausdorff, et al. 1989), a chimeric β3AR
with a β2AR third intracellular loop (Jockers, et al. 1996), and a chimeric A1 adenosine receptor containing the A3 receptor tail (Palmer, et al. 1996).

The increased Vmax in P and AAP may be due to increased adenylyl cyclase levels in the two particular cell lines as indicated by the forskolin stimulation (Table 3.3). When standardized by forskolin stimulation, the ratio of mean Vmax over mean forskolin stimulation was close to each other (Table 3.3), suggesting that enhanced adenylyl cyclase activity rather than enhanced receptor-G protein coupling was responsible. The other possibility might be that the introduction of the PKA site somehow enhanced the receptor intrinsic efficacy (as measured by Vmax) while the rightward shift of EC50 was due to a concomitant reduction in receptor affinity for dopamine due to a conformational change due to the mutation. Although this possibility seems appealing, it is not likely to be the case according to the following analysis using the extended ternary complex model:

According to the extended ternary complex model (Figure 3.4) (Samama, et al. 1993), the apparent dissociation constant is given by

\[ K_{app} = \frac{1}{K} \cdot \frac{1 + J(1 + M[G])}{1 + \beta J(1 + \alpha M[G])} \]

Because dopamine is an agonist, \( \alpha \) and \( \beta \) are both > 1. Therefore any increase in \( \alpha, \beta, J \) and/or \( M \) would cause more increase in the denominator than in the numerator, which will cause \( K_{app} \) to decrease. That is, any increase in efficacy (increase in R*G and HR*G due to increase in \( \alpha, \beta, J, K \) and/or \( M \)) would inevitably lead to decrease in dissociation constant \( K_{app} \) or, in other words, increase of the agonist affinity.
Since $EC50 = \frac{Kd}{1+e}$ (Stephenson 1956), where $Kd$ is dissociation constant ($K_{app}$ in this case) and $e$ is efficacy, it is obvious that, if the phenomenon observed in AAP was due to increased efficacy, then the dissociation constant should decrease as mentioned above. The increase of $e$ and decrease of $Kd$ would cause $EC50$ to shift way to the left rather than significantly to the right as was observed in the experiments. Therefore, in AAP, efficacy was not likely to be increased. In fact, due to the partial constitutive desensitization induced by the PKA site, it was actually decreased. Therefore, the increase in $V_{max}$ of AAP could not be explained by increased efficacy and is more likely due to enhanced adenylyl cyclase activity as revealed by the enhanced forskolin response, possibly resulting from higher level of adenylyl cyclase in the particular stable cell line.

In summary, these results have shown for the first time that palmitoylation occurred exclusively at Cys347 and Cys351 in D1DR. Introduction of PKA in the carboxyl tail induced a constitutively desensitized state of the D1DR, which clarified the mechanism whereby palmitoylation of D1DR differed from β2AR.
Figure 3.1. Palmitoylation of D1 dopamine receptors. Metabolic labeling with tritiated palmitic acid was performed in Sf9 cells expressing the wild type D1DR and three mutant receptors as described in Materials and Methods. Cell membranes were immunoprecipitated with D1DR specific antibodies. The immunoprecipitated membrane proteins were resolved on SDS-PAGE and exposed to Kodak X-AR film at -70°C for 6 weeks. The upper bands (arrow) of the first three lanes from the left represent palmitoylated D1DRs: WT, A347 and A351. No palmitoylation was detected in the last lane representing the double cysteine mutant AA. The size of receptors was approximately 46 kDa. The lower bands represent a nonspecific protein immunoprecipitated with the anti-D1DR antibodies. This autoradiograph was a representative of two similar experiments.
Figure 3.2. A sequence alignment of the wild type and three mutant DIDRs. A partial sequence of wild type D1DR and three mutant receptors are aligned including a part of TM 7 and the adjacent carboxyl tail region. A consensus PKA sequence R-R-X-S that was introduced to the vicinity of position 351 in P and AAP is shown in bold. Cysteines and their alanine substitutions are underlined in WT, AA, P and AAP receptors.
Figure 3.3. Dopamine stimulation of adenylyl cyclase activity in D1D2R. Adenylyl cyclase assays were conducted on WT, AA, P, and AAP as described in the Materials and Methods. The curves are representatives of at least three independent experiments that were carried out in duplicate. EC50 and Vmax values are summarized in Table 3.3.
Figure 3.4 The Extended Ternary Complex Model.
Table 3.1

Palmitoylation Level of D1DR

Immunoprecipitated membrane proteins obtained from Sf9 cells expressing the WT, A347, A351 and AA receptors in the absence and presence of dopamine (DA) treatment (10 mM for 20 min) were electrophoresed on SDS-PAGE. Palmitoylation signal intensity was standardized by dividing the absorbence of palmitoylation signal read from the densitometer by the absorbence of Western blot signal. The percentage in the parentheses indicates the ratio of the mutant signal compared with the WT.

<table>
<thead>
<tr>
<th>D1DR</th>
<th>-DA</th>
<th>+DA</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.257  (100 %)</td>
<td>0.255</td>
</tr>
<tr>
<td>A347</td>
<td>0.063  (24.3 %)</td>
<td>0.039</td>
</tr>
<tr>
<td>A351</td>
<td>0.122  (47.4 %)</td>
<td>0.070</td>
</tr>
<tr>
<td>AA</td>
<td>0.010  (3.9 %)</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Table 3.2

The Binding Characteristics of Wild Type and Mutant D1 Dopamine Receptors

The Bmax and Kd values for the antagonist SCH23390 were measured in triplicates and shown as mean ± SEM. The numbers of independent experiments are indicated in the parentheses. All Kd values were within normal range of Kd for SCH23390. In t test for Kd values in comparison with wild type: a = P value between 0.8 and 0.9 (non-significant for AA), b = P value between 0.2 and 0.3 (non-significant for P), and c = P value between 0.02 and 0.05 (for AAP).

<table>
<thead>
<tr>
<th>D1DR</th>
<th>Bmax (fmol/mg)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>155.5 ± 15.1 (3)</td>
<td>311.6 ± 34.8 (3)</td>
</tr>
<tr>
<td>AA</td>
<td>151.4 ± 19.3 (3)</td>
<td>323.6 ± 51.7a (3)</td>
</tr>
<tr>
<td>P</td>
<td>236.7 ± 77.0 (4)</td>
<td>282.4 ± 28.4b (4)</td>
</tr>
<tr>
<td>AAP</td>
<td>218.8 ± 42.2 (3)</td>
<td>198.3 ± 53.1c (3)</td>
</tr>
</tbody>
</table>
Table 3.3

The Effect of PKA site in D1DR

The dopamine stimulation of adenylyl cyclase activity on the wild type and mutant receptors was carried out in duplicates as described in Materials and Methods. The concentration of forskolin was 10 μM. The values are shown as mean ± SEM. The number in parentheses indicates the number of replicated experiments. For comparison with wild type estimates using t test (one-tail t test for EC50 (a); two tail t test for Vmax (b, c and d) and Forskolin (e, f and g)): a, P<0.05; b, P>0.05; c, P<0.01; d, P<0.001, e, P>0.8; f, P<0.01; g, 0.05<P<0.1.

<table>
<thead>
<tr>
<th>D1DR</th>
<th>Basal (pmol cAMP/min/mg)</th>
<th>EC50 (μM)</th>
<th>Vmax (pmol cAMP/min/mg)</th>
<th>Forskolin (pmol cAMP/min/mg)</th>
<th>Ratio of Mean (Vmax/Forskolin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>42.2±4.3 (3)</td>
<td>0.56±0.15 (3)</td>
<td>90.3±9.1 (3)</td>
<td>300.5±54.5 (5)</td>
<td>0.30</td>
</tr>
<tr>
<td>AA</td>
<td>47.2±8.4 (4)</td>
<td>0.21±0.03 (4)</td>
<td>116.7±16.7b (4)</td>
<td>294.5±49.0e (5)</td>
<td>0.40</td>
</tr>
<tr>
<td>P</td>
<td>37.2±3.9 (5)</td>
<td>0.41±0.06 (5)</td>
<td>127.7±12.1f (5)</td>
<td>443.9±23.4f (5)</td>
<td>0.29</td>
</tr>
<tr>
<td>AAP</td>
<td>37.6±4.9 (3)</td>
<td>1.02±0.32a (3)</td>
<td>152.6±15.3d (3)</td>
<td>387.6±56.7# (3)</td>
<td>0.39</td>
</tr>
</tbody>
</table>
Acknowledgements:

This work was supported by Ontario Graduate Scholarship to HJ, and grants from Medical Research Council of Canada, National Institute on Drug Abuse and the Smokeless Tobacco Research Council, Inc. to SRG and BOD.
Chapter 4

A Mutagenesis Study of the First Intracellular Loop of D1 Dopamine Receptor

This chapter is a slightly modified version of the following paper:
ABSTRACT

The first intracellular loop of the G protein-coupled receptors (GPCRs) is probably the domain that has been studied least. According to the limited data available, mutations of this region can increase, decrease or not affect receptor-G protein coupling, depending on the receptor. Melanocyte-stimulating hormone (MSH) receptors with a Ser69Leu mutation of the first intracellular loop phenotypically confer tobacco color to the coat of mice, and have constitutive activity and enhanced agonist stimulation of adenylyl cyclase. Since the human D1 dopamine receptor (D1DR) has a serine at the equivalent position, we were interested to see if this serine is involved in receptor-G protein coupling in a similar fashion. Our site-directed mutagenesis study showed that the replacement of this serine by leucine (Ser56Leu) in D1DR did not affect dramatically the ability of the receptors to bind ligand or couple to G protein.
INTRODUCTION

A variety of naturally occurring mutations of GPCRs have been found in several diseases. Some mutations cause reduction or loss of function, e.g., hormone resistance to vasopressin in nephrogenic diabetes insipidus (Rosenthal et al, 1993). Others cause disease states by gaining function, e.g., retinitis pigmentosa (Dryja et al, 1990; Sung et al, 1991) and Jansen-type metaphyseal chondrodysplasia (Schipani et al, 1999), due to constitutively active mutant receptors. Different functional roles have been determined by studying the structural variations, occurring in different regions of the receptor proteins. However, studies on the functional role of the first intracellular loop of GPCRs are lacking. Table 4.1 lists the mutations that have been found or made in the first intracellular loop of GPCRs (see Discussion for details). An interesting mutation that occurs naturally in the first intracellular loop of the melanocyte-stimulating hormone (MSH) receptor, changes the pigmentation phenotype in mice (Robbins et al, 1993). The Ser69Leu mutation in mice with tobacco coat color was shown not only to have higher basal level of activity but also a greater agonist-mediated ability to stimulate adenylyl cyclase compared to the wild type receptor. The D1DR has a similar serine residue at the equivalent position in the first intracellular loop (Figure 4.1). We were interested to see if the substitution of leucine for serine at position 56 of D1DR by site-directed mutagenesis would also produce constitutive activity and/or increased maximal stimulation of adenylyl cyclase.
MATERIALS AND METHODS

Materials: [α-^32P]ATP (800 Ci/mmol), [α-^33P]ATP (2000 Ci/mmol), [^3H]SCH23390 and [^3H]cAMP (30 Ci/mmol) were from Du Pont/NEN. Geneticin (G418), penicillin and streptomycin were from GIBCO/BRL. Dopamine, GTP, ATP, cAMP and forskolin were from Sigma. Butaclamol was purchased from RBI (Natick, MA). Dowex Resin (100-200 mesh) was from Bio-Rad.

Cell culture: Chinese Hamster Ovarian (CHO) cells were maintained in alpha-MEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Permanent cell lines expressing the wild type and the mutant receptors were maintained as monolayer culture in alpha-MEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml G418.

Site-directed mutagenesis of D1DR gene: Mutagenesis was carried out on the human D1 dopamine receptor gene using the PCR as described (Jin et al, 1997). The PCR products were subcloned into expression vector pRC/CMV (Invitrogen) and the DNA sequences encoding the wild type and the mutant receptors were verified by double-stranded DNA sequencing using Sanger's dideoxynucleotide termination method with T7 DNA Sequencing Kit (Pharmacia).

Permanent transfection of CHO cells with D1DR genes: Monolayers of cells on Petri dishes at about 50% confluence were transfected using Ca++ co-precipitation according to the instructions of the manufacturer. Stable cell lines expressing the receptors were obtained using selection media containing G418 and selected as described (Jin et al, 1997).
**Ligand binding assays:** Radioligand binding was performed essentially as described (Jin et al, 1997). The final concentrations in binding buffer were 75 mM Tris-HCl, 5 mM EDTA, and 5 mM MgCl₂. Cells were collected in the binding buffer by scraping with a rubber policeman. Cell membrane suspensions were obtained by Polytron homogenization of the collected cells (6,500 rpm for 20 seconds). Saturation experiments were carried out in triplicates with [³H]SCH23390 in increasing concentrations and non-specific binding was determined by binding that was not displaced by 1 µM (+)-butaclamol.

**Adenylyl cyclase assay:** Cell membranes were prepared by Polytron homogenization (6,500 rpm for 20 seconds) in binding buffer, ultracentrifugation of the supernatant at 12,000 rpm for 15 minutes to collect membranes, and resuspension of the membrane pellet in buffer containing 75 mM Tris-HCl, 8 mM MgCl₂ and 5 mM EDTA. Adenylyl cyclase assays were conducted in duplicate as described (Johnson and Salomon 1991) at 37°C for 20 min, in a total volume of 50 µl of the assay mix, containing 20 µg membrane protein, 12 µM ATP, 100 µM cAMP, 53 µM GTP, 2.7 mM phosphoenol-pyruvate, 0.2 units of pyruvate kinase, 1 unit of myokinase and 5 mM ascorbic acid, with 0.13 µCi of [α-³²P]ATP or 3-5 µl of [α-³³P]ATP, in the presence of indicated concentrations of dopamine. Reactions were stopped by the addition of 1 ml of an ice-cold solution containing 0.4 mM ATP, 0.3 mM cAMP and [³H]cAMP (approximately 75,000 dpm). The resulting solutions were decanted directly onto the Dowex columns. The eluate was then subjected to purification on alumina columns. Protein concentrations were measured using Bio-Rad reagents.

**Data analysis:** All data were analyzed by nonlinear least squares regression using the
InStat and InPlot GraphPad computer programs.
RESULTS

CHO cell lines stably expressing similar levels of wild type and mutant D1DR were developed and selected (Table 4.2) to determine whether the Ser56Leu substitution in the first intracellular loop of D1DR would elicit the same effect as the MSH receptors, namely an increase in both basal activity and maximal adenylyl cyclase activity upon agonist stimulation. As shown in Table 4.2, the cell lines expressed receptors at about 200 fmol/mg protein, which is comparable to the physiological receptor concentration. The mutant receptor demonstrated somewhat higher binding affinity for the D1 selective antagonist [³H]SCH23390, with smaller dissociation constant (Kd) value. However, although the small changes were statistically significant, the values are well within the normal variation range of Kd for D1 receptors and therefore may not be of pharmacological significance. The observation that binding of the receptor to ligand was barely affected by the mutation in the first intracellular loop of D1DR is consistent with previous studies that ligand binding domains reside on the transmembrane domains for small molecule ligands and on the extracellular regions of the receptors for large molecules such as peptides (Strader et al, 1994).

We studied the effect of the Ser56Leu mutation on the effect of basal activity of adenylyl cyclase in these cells. As shown in Table 4.3 and Figure 4.2, the basal activity of the cell line expressing the mutant receptors was slightly decreased (P<0.001) when compared with the wild type receptor cell line. Therefore, no constitutive activity could
be demonstrated in the mutant receptors as shown in the analogous MSH receptors. Again, such a small decrease in the basal state activity was well within the normal range and may not be biologically significant.

Adenylyl cyclase activity upon maximal stimulation by dopamine was almost comparable between the wild type D1DR. No dramatic increase was observed, unlike the observations with the MSH mutant receptors. The mean Vmax value of the mutant D1 receptors was only slightly higher, which was within normal range of variation (approximately 100 pmol cAMP/mg/min) (Table 4.3 and Figure 4.2). EC50 value of the mutant was slightly decreased compared with the wild type (Table 4.3).

In summary, we did not observe any constitutive activity of adenylyl cyclase of the mutant D1 receptor as in the case of MSH receptor.
### Table 4.1

**Summary of the Mutations in the First Intracellular Loop of GPCRs**

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Mutations</th>
<th>Effects on Mutant(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MSH</strong></td>
<td>WT: KNRNLHSPMYY Mut: KNRNLHLPMYY</td>
<td>↑ Basal AC activity ↑ AC Vmax</td>
<td>Robbins et al, 1993</td>
</tr>
<tr>
<td><strong>β2AR</strong></td>
<td>WT: ERLQTVT Mut: ERL(--6--)QT VT ERL(--8--)QT VT</td>
<td>↓ Binding to CYP ↓ AC Vmax</td>
<td>Kobilka et al, 1987</td>
</tr>
<tr>
<td><strong>β2AR</strong></td>
<td>WT: KFERLQTVTN Mut: KFERGQTVTN KFEGLTVTN KFEDLETVTN</td>
<td>↓ Receptor expression ↓ AC Vmax</td>
<td>O'Dowd et al, 1988</td>
</tr>
<tr>
<td><strong>TSHR</strong></td>
<td>WT: TSHYKLNVPR Mut: AGQAQLAVPQ</td>
<td>Binding unchanged ↓ AC Vmax</td>
<td>Chazenbalk et al, 1990</td>
</tr>
<tr>
<td><strong>TSHR</strong></td>
<td>Mut: (1) CNRLRTVPR (2) TSHYKLTPTN (3) KFERLQTVPR (4) TSHYKLTVTN (5) AGQAQLAVPQ (6) TSHYKLVQPR</td>
<td>(1), (2), (5) and (6): loss of IP3 production; (3) and (4): ↓ IP3 production. All: ↓ AC basal activity and AC Vmax except (5) (no change in Vmax)</td>
<td>Kosugi and Mori, 1994</td>
</tr>
<tr>
<td><strong>Rhodopsin</strong></td>
<td>WT: VQHKKLRTPL Mut: VQHKKEL VQHKKLRL VQHKKLRAAL</td>
<td>↓ Transducin activation</td>
<td>Min et al, 1993</td>
</tr>
<tr>
<td><strong>TxA2-R</strong></td>
<td>WT: QGGSHTRSSFLT Mut: QGGSHTLSSFLT</td>
<td>↓ IP3 production</td>
<td>Hirata et al. 1994</td>
</tr>
<tr>
<td><strong>hCRFR II</strong></td>
<td>29 a.a. insert in the first intracellular loop when compared to its splicing variant hCRFR I</td>
<td>↓ AC Vmax and ↓ IP3 production (compared to R I)</td>
<td>Nabhan et al, 1995</td>
</tr>
<tr>
<td><strong>CCKBR</strong></td>
<td>The first intracellular loop of CCKBR was replaced by the counterpart of CCKAR</td>
<td>Gained the ability to stimulate adenylyl cyclase</td>
<td>Wu et al, 1997</td>
</tr>
</tbody>
</table>
Table 4.2

SCH23390 Binding Parameters for Wild Type and Mutant D1DR in CHO Cells

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Bmax (fmol/mg)</th>
<th>Kd (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>159.8±21.7 (11)</td>
<td>489.9±75.8 (11)</td>
</tr>
<tr>
<td>Mutant</td>
<td>214.8±21.4 (3)</td>
<td>212.6±50.8 (3)</td>
</tr>
</tbody>
</table>

The values shown in the table are the means ± SEM of saturation binding. The numbers in parentheses indicate the number of independent experiments that were carried out, each in triplicate. The data of the wild type receptor was the summation of eleven experiments carried out in four cell lines expressing similar levels of wild type receptors. Statistical analysis of comparing Kd with the wild type receptor using t test (a): P<0.001.
<table>
<thead>
<tr>
<th></th>
<th>TM 1</th>
<th></th>
<th>TM 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1DR</td>
<td>LVC\text{AVIT}</td>
<td>RFRHL - R</td>
<td>SKVTN</td>
</tr>
<tr>
<td>MSHR</td>
<td>VL\text{VIAIT}</td>
<td>KNRLH</td>
<td>SPMY</td>
</tr>
<tr>
<td>D2DR</td>
<td>LVCMA\text{VSH}</td>
<td>REKALQ - T</td>
<td>-</td>
</tr>
<tr>
<td>D5DR</td>
<td>LC\text{AIVR}</td>
<td>S\text{HRHL} - R\text{ ANMTN}</td>
<td></td>
</tr>
<tr>
<td>5-HT1AR</td>
<td>CVA\text{IALERS}</td>
<td>LQ -</td>
<td>-</td>
</tr>
<tr>
<td>(\alpha)2AR</td>
<td>LVI\text{ALT}</td>
<td>S\text{RSL} - RAPQ - N</td>
<td>LFLVSL</td>
</tr>
<tr>
<td>M2AChR</td>
<td>LV\text{MISFS} - K</td>
<td>V\text{NS} -</td>
<td>QLT \text{TVNNY}</td>
</tr>
<tr>
<td>IL8R</td>
<td>LV\text{MLVLLY}</td>
<td>S</td>
<td>I-</td>
</tr>
</tbody>
</table>

**Figure 4.1. An Alignment of Selected GPCRs.** The transmembrane domains 1 (TM1) and 2 (TM2) are indicated in brackets. D1DR has a serine at a similar position as MSHR. D1DR=D1 dopamine receptor, MSH=melanocyte-stimulating hormone, D2DR=D2 dopamine receptor, D5DR=D5 dopamine receptor, 5-HT1AR=5-hydroxytryptamine 1A receptor, \(\alpha\)2AR=\(\alpha\)2 adrenergic receptor, M5AChR=M5 acetylcholine receptor, and IL8R=interleukin 8 receptor.
Figure 4.2. Adenylyl Cyclase Activation by Dopamine in the Wild Type and Mutant Receptors. The curves shown in this figure are representatives of 15 cyclase assays performed in four cell lines for wild type receptor, and of 5 assays in a cell line expressing mutant receptors at a similar level. The basal level, Vmax and EC50 values are listed in Table 4.3.
Table 4.3

Basal and Dopamine-Stimulated Adenylyl Cyclase Activities of Wild Type and Mutant D1DR

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Basal (pmol cAMP/mg/min)</th>
<th>Vmax (pmol cAMP/mg/min)</th>
<th>EC50 (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>32.3±3.6 (15)</td>
<td>88.0±4.9 (15)</td>
<td>434.5±80.7 (15)</td>
</tr>
<tr>
<td>Mutant</td>
<td>24.5±3.7^a (5)</td>
<td>104.3±15.8^a (5)</td>
<td>284.2±56.0^c (5)</td>
</tr>
</tbody>
</table>

Basal activities and maximal activation of adenylyl cyclase in the cell lines expressing similar receptor levels were determined as described in the Materials and Methods. The values listed in the table are mean ± SEM of the number of experiments indicated in parentheses carried out in duplicates. The data for the wild type receptor were the summation of fifteen adenylyl cyclase assays carried out in four cell lines with approximately the same receptor expression level.

For comparison with the wild type parameters using t test, a: P<0.001; b: P<0.001; c: P<0.01.
DISCUSSION

Characterizations of the functional role of the first intracellular loop of GPCRs are generally lacking. However, in a limited number of studies, outlined in Table 4.1, this domain has been shown to play a critical role in receptor expression and receptor-G protein coupling in at least some receptors. One of the mutant receptors that is of particular interest has been the MSH receptor. A naturally occurring mutant receptor (Ser69Leu in the first intracellular loop) found in tobacco coat mice showed constitutive activity and greater activation of its effector, adenylyl cyclase, than the wild type when expressed in human 293 cells (Robbins et al, 1993).

In β2-adrenergic receptors, Kobilka et al. (Kobilka et al, 1987) showed that insertion of 4-8 amino acid residues into the middle of first intracellular loop led to a marked reduction in binding of the antagonist cyanopindolol and in the maximal agonist activation of adenylyl cyclase, although activation occurred with the same EC50 as in the wild type receptor. It is probable that normal folding and conformation of the receptor protein was disturbed by these insertions. In another study of the first intracellular loop of β2-adrenergic receptors, O'Dowd et al. (O'Dowd et al, 1988) found that the single substitution of Leu64 by Gly resulted in a receptor with a 10-fold increased binding affinity for agonist isoproterenol, but normal binding for cyanopindolol. This mutant receptor had an appreciably lower level of expression, accounting for its reduced potency and efficacy of cyclase stimulation. In the same study, substitutions of the amino acids flanking position 64 had similar but more deleterious effects on receptor expression, thus
preventing meaningful adenyl cyclase activation parameters to be obtained.

In the thyrotropin (TSH) receptor, the results are controversial. Chazenbalk et al. (Chazenbaulk et al., 1990) reported that a non-homologous substitution mutant involving the first intracellular loop caused the total loss of the TSH-induced cAMP response. However, Kosugi and Mori (Kosugi and Mori, 1994) reported that the first cytoplasmic loop is important for phosphoinositide signaling but not for agonist-induced adenyl cyclase activation when replaced with the corresponding homologous amino acids from α1- and β2-ARs.

In the characterization of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa, Min et al. (Min et al., 1993) discovered that deletion and replacement of two amino acids in the first intracellular loop resulted in a complete loss and a reduction of light-dependent transducin activation, respectively.

A mutation in the first cytoplasmic loop of the thromboxane A2 (TXA2) receptor was demonstrated by Hirata et al. (Hirata et al., 1994) in affected members of two unrelated families with a mild bleeding disorder characterized by defective platelet response to TXA2. In this receptor the replacement of Arg60 by Leu, a highly conserved basic residue among GPCRs, caused impaired ability to induce agonist-mediated PI hydrolysis.

Nabhan et al. (Nabhan et al., 1995) reported that corticotropin-releasing factor (CRF) receptor has two alternatively spliced forms. The two splice variants are identical except for a 29 amino acid insert present in the first intracellular loop of the type II receptor. When expressed in COS cells, type II CRF receptor had low binding affinity, a weak cAMP response and low inositol phosphate generation, suggesting that this receptor
is weakly coupled to G protein, as a result of the change in the first intracellular loop.

In summary, mutations in the first intracellular loop, whether occurring naturally or engineered by in vitro mutagenesis, may have variable effects on the function of different receptors, causing increased (e.g., MSH and CCKBR), decreased (e.g., β2AR, some TSHR mutants, rhodopsin and hCRFRII) or no change (e.g., some TSHR mutants) in receptor-G protein coupling. Our study showed that Ser56 in the first intracellular loop of the D1DR did not dramatically influence receptor-effector coupling. The differences that these various receptors manifest may relate to the relative position of residues within the intracellular loop, determined by the overall three dimensional architecture of the receptor, and may not therefore be directly comparable with each other. These findings emphasize the importance of studying structure-function determinants of GPCRs individually rather than to generalize from one receptor to another based on the common topography of GPCRs. Our efforts to elucidate the final determinants of the relationship between the structure and function of G protein-coupled receptors are still at a preliminary stage, until the structure of these receptors are definitively revealed by crystallography.

ACKNOWLEDGMENTS:
This study has been supported by grants from Medical Research Council of Canada and Addiction Research foundation of Ontario.
Chapter 5

Mutagenesis of the cdc2 Site in the Second Intracellular Loop of D1 Dopamine

Receptor Abolishes Agonist-Induced Receptor Internalization

This chapter is a modified version of the following manuscript:

ABSTRACT

A cdc2 consensus sequence is present in the second intracellular loop of the D1DR. To investigate if this phosphorylation site is involved in receptor internalization, we eliminated the sequence from D1DR. We found that the removal of the site abolished D1DR internalization, indicating that the cdc2 site is important for D1 receptor sequestration. In addition, in order to examine if cdc2 could provide a strong enough signal to cause D2DR to internalize, we engineered a cdc2 sequence into the corresponding position at the second intracellular loop of D2DR. Our data revealed that cdc2 site could not confer the capability of receptor internalization to D2DR. Our results point out the importance of studying other phosphorylation sites in addition to the most commonly investigated PKA, PKC and GRK kinases sites in GPCRs in order to elucidate their functional roles.
INTRODUCTION

Receptor internalization (synonymous with sequestration or endocytosis) is a common phenomenon observed in some GPCRs as well as other non-GPCRs (LDL and transferrin receptors) (Koenig and Edwardson, 1997).

GPCR internalization is controlled by several determinants: the endocytosis motifs (e.g., the Tyr at the cytoplasmic end of TM 7 of many GPCRs), agonist-induced phosphorylation of the receptors, ubiquitination (e.g., the ubiquitination of Ste2p, a receptor for the hormone α-factor in yeast), and differences in the components of the endocytotic machinery of different cell lines (reviewed by Koenig and Edwardson, 1997). The role played by phosphorylation in receptor internalization has been most extensively investigated.

Recent studies have shown that phosphorylation of β2AR promotes binding of β-arrestin (Ferguson et al, 1996), which subsequently attaches to clathrin in clathrin-coated pits (Goodmann et al, 1996). Apparently, β2AR internalization requires phosphorylation and is mediated by clathrin-coated pits. A number of GTPases have been implicated as key regulators of various stages of receptor endocytosis, including the 100 kDa dynamin. Dynamin is an essential, early acting component of the endocytotic pathway that operates through clathrin-coated pits. Dynamin controls the formation of clathrin coated vesicles by regulating the constriction and budding of clathrin-coated pits from the plasma membrane (De Camilli et al, 1995). Functional dynamin is required for β2AR internalization via clathrin-coated pits (Zhang et al, 1996).

Despite the large amount of information concerning β2AR sequestration, much
less is known of sequestration of other types GPCRs. Much has been learned in recent years regarding phosphokinases such as GRKs, PKA and PKC, and their roles in receptor function.

In fact, apart from GRKs, PKA and PKC, other protein kinases exist in the cells (Kennelly and Krebs, 1991). The protein kinase p34^{cd2} is one example. In eukaryotic cells, mitotic and meiotic M phase is initiated by activation of this protein kinase (Moreno and Nurse, 1990). Activation of p34^{cd2} may be responsible for phosphorylation of nuclear lamin, which plays a role in nuclear envelope disassembly, for phosphorylation of H1 histone, which contributes to chromosome condensation at mitosis, and for phosphorylation of the pp60^{c-src}, which is associated with cytoskeletal reorganization and spindle formation (Moreno and Nurse, 1990). A cdc2-like kinase was recently cloned in neurons of the central nervous system (Tang et al, 1996; Lazzaro et al, 1997), indicating that cdc2 family kinases are not restricted to modulating cell cycle. Consistent with this notion, p34^{cd2} is shown to be distributed throughout the cell (Moreno and Nurse, 1990).

Interestingly, a cdc2 phosphorylation consensus sequence (ISSPFRY) (Figure 5.1) is present in the second intracellular loop of D1DR (ISSPFRY) (Sunahara et al, 1990), D5DR (ISRPFRY) (Sunahara et al, 1991) and β2AR (ITSPFKY) (Kobilka et al, 1987). However, the functional role of cdc2 in GPCR function has not yet been fully investigated. Therefore, we postulated that the cdc2 site in the second intracellular loop of D1DR may have a significant role in receptor activation and/or internalization.

The β3AR does not desensitize nor internalize in the presence of agonist, and there are no phosphorylation sites for GRKs, PKA or cdc2 kinase (Strosberg, 1997; Jockers et al, 1996). However, the β3AR containing the second intracellular loop of the
β2AR (with the cdc2 site) caused the chimeric receptor (β3-3233) to endocytose (Jockers et al, 1996). Because D2DR does not internalize with agonist treatment (Itokawa et al, 1996; Ng et al, 1997), we were interested to investigate if similar mutations (Figure 5.1) could confer on D2DR an ability to internalize. Our results demonstrated that the removal of the cdc2 phosphorylation site by site-directed mutagenesis reduced D1DR internalization, suggesting that cdc2 phosphorylation site plays an important role in D1DR internalization. However, unlike the β3/β2 chimera (β3-3233), the introduction of the cdc2 sequence into the corresponding region of the second intracellular loop of D2DR did not result in receptor internalization, indicating that cdc2 phosphorylation site alone was not sufficient to confer on D2DR the capability to internalize.
<table>
<thead>
<tr>
<th></th>
<th>T M 3</th>
<th>T M 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT D1</td>
<td>I S V D R Y W A I S S P F R Y E R K M T P K</td>
<td>A A F I</td>
</tr>
<tr>
<td>Mutant D1</td>
<td>I S V D R Y W A I A A P F R Y E R K M T P K</td>
<td>A A F I</td>
</tr>
<tr>
<td>WT D2</td>
<td>I S S D R Y T A V A M P M L Y N T R Y S S K R</td>
<td>R V T V</td>
</tr>
<tr>
<td>Mutant D2</td>
<td>I S S D R Y T A I S S P F R Y N T R Y S S K R</td>
<td>R V T V</td>
</tr>
</tbody>
</table>

Figure 5.1 Alignment of Partial Sequences of Wild Type and Mutant D1 and D2 DR.
MATERIALS AND METHODS

**Materials:** [H]SCH23390 was purchased from Du Pont/NEN. DMEM, fetal bovine serum, PBS, trypsin-EDTA, Geneticin (G418), penicillin, and streptomycin were from Life Technologies, Inc. Dopamine was from Sigma. Butaclamol was purchased from RBI (Natick, MA).

**Cell culture:** Chinese Hamster Ovarian (CHO) cells were maintained in alpha-MEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Permanent cell lines expressing the wild type and the mutant receptors were maintained as monolayer culture in alpha-MEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml G418.

**Site-directed mutagenesis of D1DR gene:** Mutagenesis was carried out on the human D1 dopamine receptor gene using the PCR as described (Jin, et al. 1997). The PCR products were subcloned into expression vector pRC/CMV (Invitrogen) and the DNA sequences encoding the wild type and the mutant receptors were verified by double-stranded DNA sequencing using Sanger's dideoxynucleotide termination method with T7 DNA Sequencing Kit (Pharmacia).

**Permanent transfection of CHO cells with D1DR genes:** Monolayers of cells on Petri dishes at about 50% confluence were transfected using Ca^{++} co-precipitation according to the instructions of the manufacturer. Stable cell lines expressing the receptors were obtained using selection media containing G418 and selected as described (Jin et al. 1997).

**Ligand binding assays:** Radioligand binding was performed essentially as described (Jin et al, 1997). The final concentrations in binding buffer were 75 mM Tris-HCl, 5 mM
EDTA, and 5 mM MgCl2. Cells were collected in the binding buffer by scraping with a rubber policeman. Cell membrane suspensions were obtained by Polytron homogenization of the collected cells (6,500 rpm for 20 seconds). Saturation experiments were carried out in triplicates with [3H]SCH23390 in increasing concentrations and non-specific binding was determined by binding that was not displaced by 1 μM (+)-butaclamol. Protein concentrations were measured using Bio-Rad reagents.

Receptor Internalization Assays: For all internalization experiments, monolayers of CHO stable cell lines were incubated with serum-free DMEM 16 hours before the experiment, and treated for the designated time with 10 μM dopamine with 5 mM ascorbic acid (controls were treated only with ascorbic acid) at 37°C. Cells were then collected with a rubber policeman and homogenized as above. Unbroken cells were removed by centrifugation at 900 x g. The whole preparations were then layered on sucrose step gradients: 15%, 30%, 33% and 60% (Parker et al, 1995) for percentage fraction data (cell surface and intracellular fractions), and 32% only (Jockers et al, 1996) for saturation binding of the cell membrane receptors (for Bmax). In the latter the precipitate was resuspended in the binding buffer and receptors were measured as described above.

Data analysis: All data were analyzed by nonlinear least squares regression using the GraphPad Prism computer programs.
RESULTS

To determine the time course of receptor internalization following agonist activation, the stable cell line expressing the wild type D1DR was treated with 10 µM dopamine (with 5 mM ascorbic acid) for 0, 5, 10, 20, 30 and 60 minutes. As shown in Figure 5.2 and Table 5.1, the receptors internalized to the maximum extent by 20-30 min. Therefore, this time point was chosen as the standard duration of agonist treatment.

To examine the effect on receptor internalization after the removal of the cdc2 site from the second intracellular loop of D1DR, two stable cell lines expressing the wild type and the mutant D1DR at approximately the same level (200 fmol/mg) were selected. Sucrose step gradient concentration (Parker et al, 1995) was used to obtain the cell surface and intracellular fraction of receptors. The relative proportions of receptors in both compartments were measured. As shown in Table 5.2 and Figure 5.3, for the wild type D1 receptor, the dopamine treatment (10 µM for 30 minutes) increased the intracellular receptors from 34.5 ± 4.1 % (control) to 50.1 ± 1.2 % (p=0.001), and receptors on the cell surface decreased from 65.5 ± 4.1 % to 49.9 ± 1.2 % (p=0.001), indicating that the wild type D1 receptors internalized normally in CHO cells. However, for the mutant D1 receptor without the cdc2 site, the same agonist treatment did not cause any increase in the intracellular fraction or decrease in the cell surface fraction of receptors, suggesting that the mutant D1DR lost its ability to internalize (Figure 5.3 and Table 5.2).
Figure 5.2 Temporal Relationship between the Duration of Agonist Treatment and Extent of Receptor Internalization.
### Table 5.1

**Temporal Relationship of Dopamine Treatment with D1DR Internalization**

<table>
<thead>
<tr>
<th>Minutes</th>
<th>Intracellular Fraction (%) (mean ± SEM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>32.1 ± 1.2</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>44.8 ± 0.7</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>48.5 ± 4.0</td>
<td>4</td>
</tr>
<tr>
<td>20</td>
<td>49.3 ± 3.0</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>50.9 ± 1.3</td>
<td>6</td>
</tr>
<tr>
<td>60</td>
<td>47.4 ± 2.5</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 5.3 The Effect of the Removal of cdc2 Site on D1DR internalization.
To evaluate if the addition of the cdc2 site to the corresponding second intracellular loop could cause D2DR to sequester as for the \( \beta_3 \)AR chimera (Jockers et al., 1996), we selected two CHO cell lines that stably express the wild type and the mutant receptors at approximately 200 fmol/mg. The results are shown in Table 5.2 and Figure 5.4. Our data demonstrated that in both the wild type and the mutant D2 receptors, no increase in the intracellular compartment nor any decrease from cell surface was observed. Therefore, wild type D2 receptors did not sequester in CHO cells following dopamine treatment, and in contrast to the mutant \( \beta_3 \)AR, the cdc2 site engineered into the second intracellular loop of D2DR was not sufficient to induce receptor internalization.

To determine if the observation that cdc2 site is implicated in D1DR internalization could hold true for receptors expressed at higher density, two additional stable CHO cell lines were chosen (designated as WT D1DR-B and Mut D1DR-B), which expressed the wild type and mutant D1DR at approximately 400 fmol/mg protein, for further investigation. As summarized in Table 5.3 and shown in Figure 5.5, dopamine treatment increased the intracellular fraction of receptors from 51.3 \( \pm \) 2.0 % to 60.7 \( \pm \) 5.3 % (\( p = 0.17 \)), and decreased receptors on cell surface from 48.7 \( \pm \) 2.0 % to 39.3 \( \pm \) 5.3 % (\( p = 0.17 \)). However, no increase in intracellular receptors or decrease from cell surface was observed. Therefore, the data we observed in cells with lower expression levels could be replicated in stable cell lines with higher receptor density. These results confirmed that cdc2 did play a role in receptor internalization for D1DR.

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Figure 5.4 Effect of Insertion of cdc2 Site on D2DR Internalization.
Table 5.2

Agonist-Induced Internalization of D1DR and D2DR

The values (%) represent mean ± SEM. The number in the parentheses indicates the number of separate experiments performed in triplicates. The control is indicated by -, and the dopamine treated group by +. Statistical significance is determined with t test by comparing the values from control and from dopamine treated samples for the same fraction.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>DA</th>
<th>WT D1DR</th>
<th>Mut D1DR</th>
<th>WT D2DR</th>
<th>Mut D2DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular</td>
<td>-</td>
<td>34.5 ± 4.1 (3)</td>
<td>43.8 ± 7.7 (4)</td>
<td>40.5 ± 1.1 (5)</td>
<td>44.3 ± 3.0 (2)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>50.1 ± 1.2 (7)</td>
<td>43.0 ± 5.0 (5)</td>
<td>38.8 ± 2.4 (5)</td>
<td>43.9 ± 0.2 (2)</td>
</tr>
<tr>
<td>Cell Surface</td>
<td>-</td>
<td>65.5 ± 4.1 (3)</td>
<td>56.2 ± 7.7 (4)</td>
<td>59.5 ± 1.1 (5)</td>
<td>55.8 ± 3.0 (2)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>49.9 ± 1.2 (7)</td>
<td>57.0 ± 5.0 (5)</td>
<td>61.2 ± 2.4 (5)</td>
<td>56.1 ± 0.2 (2)</td>
</tr>
</tbody>
</table>

P values for t test: a and b = 0.001, c and d = 0.93, e and f = 0.53, g and h = 0.92.
Figure 5.5. Role of cdc2 Site in D1DR (in Cells with High Receptor Expression).
Table 5.3

Agonist-Induced Internalization of D1DR

The values (%) represent mean ± SEM. The number in the parentheses indicates the number of separate experiments performed in triplicates. The control is indicated by -, and the dopamine treated group by +. Statistical analysis of t test is carried out by comparing the values obtained from control and from dopamine treated samples for the same fraction.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>DA</th>
<th>WT D1DR-B</th>
<th>Mut D1DR-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular</td>
<td>-</td>
<td>51.3 ± 2.0 (3)b</td>
<td>52.3 ± 4.0 (3)c</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>60.7 ± 5.3 (3)a</td>
<td>53.8 ± 7.1 (3)c</td>
</tr>
<tr>
<td>Cell Surface</td>
<td>-</td>
<td>48.7 ± 2.0 (3)b</td>
<td>47.7 ± 4.0 (3)d</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>39.3 ± 5.3 (3)b</td>
<td>46.2 ± 7.1 (3)d</td>
</tr>
</tbody>
</table>

The p values for t test: a and b = 0.17, c and d = 0.86.
Furthermore, we also looked at the change in absolute numbers of receptors on cell surface before and after dopamine treatment (10 mM for 30 minutes as above) in the WT D1DR-B and Mut D1DR-B stable cell lines. For the wild type D1 receptor, agonist treatment reduced the receptor density on cell membrane from $485.2 \pm 59.3$ fmol/mg protein to $389.9 \pm 49.2$ fmol/mg (p=0.009, paired t test, two-tailed, df=3), indicating receptor internalization occurred after agonist treatment (Figure 5.6 and Table 5.4). However, the density of the mutant receptor on cell surface remained unchanged at about 400 fmol/mg protein (Figure 5.7 and Table 5.4), which further confirmed that cdc2 was involved in D1DR internalization.
Figure 5.6 Saturation Binding for the WT D1DR-B.

Figure 5.7 Saturation Binding for Mutant D1DR-B.
Table 5.4

[^3H]SCH23390 Saturation Binding of Cell Surface D1DRs

<table>
<thead>
<tr>
<th>Receptors</th>
<th>WT D1DR-B</th>
<th>Mutant D1DR-B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bmax</strong></td>
<td>485.2 ± 59.3 (control)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>408.5 ± 49.1 (control)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(fmol/mg)</td>
<td>389.9 ± 49.2 (treated)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>415.7 ± 58.6 (treated)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Kd</strong></td>
<td>0.338 ± 0.151 (control)</td>
<td>0.316 ± 0.142 (control)</td>
</tr>
<tr>
<td>(nM)</td>
<td>0.371 ± 0.168&lt;sup&gt;c&lt;/sup&gt; (treated)</td>
<td>0.476 ± 0.228&lt;sup&gt;d&lt;/sup&gt; (treated)</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>P values for paired t test for receptor density change: a = 0.009; b = 0.036. Membrane preparations of cells with or without dopamine treatment were compared to determine the statistical significance of difference observed in receptor density. For Kd values, unpaired t test was used: c, 0.7<P<0.8; d: 0.2<P<0.3, suggesting no change in receptor affinity for SCH23390.
Discusson

GPCR have four routes of trafficking within cells. Receptors synthesized de novo are transported to the cell surface from the Golgi complex. At basal state, slow endocytosis of receptors from cell surface into endosomes occurs constantly. With agonist stimulation, the endocytosis is increased markedly. When receptors reach endosomes, most of the receptors are resensitized and recycled back to the cell membrane, and a minority of receptors are routed to lysosomes for degradation (Koenig and Edwardson, 1997).

The mechanism of receptor internalization (or sequestration or endocytosis) is not completely clear. It seems to depend on several determinants, including specific motifs of protein primary structure, activation/phosphorylation state of the receptor, other chemical modification such as ubiquitination, and the specific endocytosis machinery present in cells where receptor expression occurs (Koenig and Edwardson, 1997).

Early reports on mutant β2-adrenergic receptors (β2AR) seemed to indicate that receptor sequestration was relatively independent of phosphorylation by PKA or βARK and of coupling to Gs, although agonist occupancy was clearly required for receptor sequestration (Campbell et al, 1991). However, subsequent studies (see review by Ferguson et al, 1996 and by Koenig and Edwardson, 1997) using the endocytosis-deficient Y326A mutant β2AR revealed that co-transfection with GRKs and/or overexpression of β-arrestin could rescue its endocytosis and the receptors were found to be phosphorylated. Rescue of endocytosis was blocked by deletion of βARK phosphorylation sites. In addition, both phosphorylation and endocytosis of receptors
could be reduced by the co-overexpression of a negative mutant of βARK (Koenig and Edwardson, 1997). Therefore, phosphorylation of GPCR may in fact play a role in endocytosis of receptors.

Despite the large amount of information concerning β2AR sequestration, much less is known of sequestration of other types GPCRs. From the fragmentary information available, some receptors seem to follow the same route, e.g., the chemokine receptor CXCR2 (Yang et al, 1999) and bradykinin B2 receptor (Pizard et al, 1999), but other receptors seem to deviate from the prototypical β2AR in several aspects. For example, angiotensin II AT1A receptors sequester in a dynamin-independent manner (Zhang et al, 1996). While m1, m3 and m4 mAChRs internalization is regulated by dynamin, m2 mAChR sequester independently of dynamin (Vogler et al, 1998). In another report, it was shown that m1, m3 and m4 AchRs internalized in an arrestin-independent manner (Lee et al, 1998). In the platelet-activating factor receptor, removal of the phosphorylation sites of the C tail reduced the extent of receptor sequestration, suggesting that phosphorylation may facilitate but is not essential for the process (Ishii et al, 1998). Several signaling and phosphorylation-deficient mutant FSH receptors were shown to internalize through an arrestin-dependent pathway (Nakamura et al, 1998). A truncated opioid receptor without phosphorylation sites was observed to still undergo a dynamin-dependent endocytosis involving clathrin coated pits (Murray et al, 1998). Instead of going through clathrin-coated pits, some receptors internalize through caveolae (Roettger et al, 1995) or non-coated pits (Raposo et al, 1989).

In the past decade, studies on GPCR phosphorylation have focused on PKA, PKC, and GRKs. Much less attention has been given to the possible functional roles of
other protein kinases. Only recently has another phosphorylation site been studied (Jockers et al, 1996). That is the cdc2 site, which is present in the second intracellular loop of several GPCRs including D1DR, β2AR and D5DR. Mitotic and meiotic M phase of eukaryotic cells starts by activation of this protein kinase (Moreno and Nurse, 1990). Activation of p34<sup>cdc2</sup> causes phosphorylation of nuclear lamin (nuclear envelope disassembly), phosphorylation of H1 histone (chromosome condensation at mitosis), and phosphorylation of the pp60<sup>Src</sup> (cytoskeletal reorganization and spindle formation) (Moreno and Nurse, 1990). The recent cloning of a cdc2-like kinase in neurons of the central nervous system (Tang et al, 1996; Lazzaro et al, 1997) suggests that functions of the cdc2 family kinases are not restricted to regulating cell cycle. Instead of restricted to nucleus, p34<sup>cdc2</sup> is shown to be distributed throughout the cell (Moreno and Nurse, 1990).

Interestingly, β3AR does not desensitize nor internalize in the presence of agonist, because of absence of phosphorylation sites for GRKs, PKA or PKC (Strosberg, 1997; Jockers et al, 1996). However, a mutant β3AR constructed to contain the second intracellular loop of the β2AR (with the cdc2 site) caused the chimeric receptor (β3-3233), to endocytose (Jockers et al, 1996). That is to say the cdc2 site-containing loop from β2AR conferred ability to internalize to the β3AR. This was the first indication that cdc2 kinase may play a role in receptor endocytosis.

Because D2DR does not internalize with agonist treatment like β3AR (Itokawa et al, 1996; Ng et al, 1997), we decided to investigate if similar mutations (Figure 5.1) could also cause D2DR to undergo agonist-induced internalization. Our results demonstrated that removal of the cdc2 phosphorylation site reduced receptor internalization for D1DR, suggesting that cdc2 phosphorylation site plays an important role in D1DR.
internalization. However, in contrast to the β3/β2 chimera (β3-3233), the cdc2 sequence placed into the corresponding intracellular loop of D2DR did not result in receptor internalization, indicating that the signal carried by cdc2 phosphorylation site is not sufficient to cause D2DR to internalize and that it is not the absence of this motif in the D2DR that hinders D2DR internalization after agonist treatment. Our study represents the first report, to our best knowledge, that investigate explicitly the role other phosphorylation sites play in the dopamine D1 receptor, in addition to the GRK and PKA/PKC sites.
Chapter 6
The Relationship between the Receptor Density and the Maximal Stimulation of Adenylyl Cyclase

This chapter is based on the following manuscript:

ABSTRACT

In the past few decades, researchers have been intrigued by the concept of drug efficacy, which has been shown to be related to receptor numbers present in the system. To directly address the issue of the relationship between receptor expression level and the measurement of functional parameters, we expressed wild type D1DR in Chinese hamster ovary cells. Fifteen stable cell lines, expressing receptors from about 10 fmol/mg protein to 2000 fmol/mg, were chosen and subjected to adenylyl cyclase assay. Bmax of receptor expression and Vmax of enzyme activity were correlated and analyzed. Our data demonstrated that at low levels, Vmax increased almost proportionally to Bmax. However, Vmax reached was saturable with higher receptor density. This report confirms previous observations of other GPCRs in native tissues and in transfected cell lines. It also emphasizes the importance of comparing wild type and mutant receptors in cell lines with similar receptor expression levels.
INTRODUCTION

The earliest quantitative and systematic study on the relationship between receptor numbers with cellular response was carried out in muscarinic receptor in native tissues (Stephenson 1966). Recently, several other papers have been published on the correlations of agonist stimulation with GPCR membrane expression in transfected cell lines (Whaley et al, 1994; Vogel et al, 1995; MacEwan et al, 1995; Thibonnier et al, 1997; Befort et al, 1999).

To investigate if the agonist stimulated cyclase activity is dependent on the receptor expression level and to determine the nature of the relationship for D1 dopamine receptor (D1DR), we expressed the wild type D1DR in Chinese hamster ovary cells. From the fifteen stable cell lines we generated, with receptor density ranging from about 10 fmol/mg protein to 2000 fmol/mg, our data revealed that at low levels, maximum agonist activation of adenylyl cyclase did vary with receptor density, and it increased proportionally to receptor density at low levels, but reached a plateau with higher receptor expression. Our report emphasizes the importance of comparing wild type and mutant receptors using cell lines with similar receptor expression levels.
MATERIALS AND METHODS

Materials. Grace's supplemented medium, fetal bovine serum, gentamycin sulfate, fungizone, Goat serum, rabbit serum, lipofectin, geneticin (G418), penicillin and streptomycin were purchased from GIBCO/BRL (Toronto, ON). [9,10-3H]palmitic acid (70-80 Ci/mmol). [α-32P]ATP (800 Ci/mmol). [3H]SCH23390 and [3H]cAMP (30 Ci/mmol) were purchased from Du Pont/NEN (Boston, MA). Dopamine, leupeptin, benzamidine, soybean trypsin inhibitor, GTP, ATP, and cAMP were purchased from Sigma (St. Louis, MO). Butaclamol were purchased from RBI (Natick, MA). Dowex Resin (100-200 mesh) was purchased from Bio-Rad (Richmond, CA).

Cell culture. CHO cells were maintained in Dulbecco's Modified Essential Medium (DMEM) (GIBCO/BRL) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Permanent cell lines expressing the wild type receptors were maintained as monolayer culture in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml G418.

Permanent transfection of CHO cells with wild-type D1 receptor genes. Monolayers of cells on Petri dishes at about 50% confluence were transfected using Lipofectin according to the instructions of the manufacturer. Cells were trypsinized at 48 hours and diluted 1:30 in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml G418 and incubated at 37°C in 5% CO₂. After two weeks, G418 resistant clones were isolated in small Petri dishes, expanded in 75 cm² flasks, and screened using D1 receptor antagonist, SCH23390, with a saturation concentration (50 nM) to determine the expression level of the receptors.
Ligand binding assay. Ligand binding assays were carried out essentially as described (Jin, et al. 1997). The final concentrations in binding buffer were 75 mM Tris HCl, 5 mM EDTA and 5 mM MgCl₂. Cells were collected in the binding buffer by scraping with a rubber policeman. Cell membrane suspensions were obtained by Polytron homogenization of the collected cells (6,500 rpm for 20 seconds). Saturation experiments were carried out in triplicates with [³H]SCH23390 at saturating concentration (50 nM) and non-specific binding was determined by binding that was not displaced by 1 μM (+)-butaclamol.

Adenylyl cyclase assay. Cell membranes were prepared by Polytron homogenization (6,500 rpm for 20 seconds) in binding buffer, centrifugation at 12000 rpm to collect membranes, and resuspension of the pellet in reaction buffer containing 75 mM Tris-HCl, 8 mM MgCl₂ and 5 mM EDTA. Adenylyl cyclase assays were conducted in duplicates as described (Johnson and Salomon 1991) at 28°C for 20 min, in a total volume of 50 μl of the assay mix, containing 20 μg membrane protein, 12 μM ATP, 100 μM cAMP, 53 μM GTP, 2.7 mM phosphoenol-pyruvate, 0.2 units of pyruvate kinase, 1 unit of myokinase and 5 mM ascorbic acid, with 0.13 μCi of [α-³²P]ATP, in the presence of indicated 10 μM of dopamine. Reactions were stopped by the addition of 1 ml of an ice-cold solution containing 0.4 mM ATP, 0.3 mM cAMP and [³H]cAMP (approximately 25,000 cpm). The resulting solutions were decanted directly onto the Dowex columns. The elute was then subjected to purification on aluminum columns. Protein concentrations were measured using Bio-Rad reagents.

Data analysis. All data were analyzed by nonlinear least squares regression using the GraphPad Prism computer program.
RESULTS

To determine the nature of the correlation between agonist activation of adenylyl cyclase and receptor expression level, fifteen stable cell lines were selected, for which both the receptor density (Bmax) and maximum stimulation of cyclase activity (Vmax) were measured. The receptor density for each cell line was determined by a saturating concentration (50 nM) of [3H]SCH23390, a D1-specific antagonist. The maximum cyclase activity was measured in the presence of 10 μM dopamine. The results were listed in Table 6.1 and the mean values of Bmax and Vmax were plotted in Figure 6.1.

Apparently, at low receptor density, e.g., below 400 fmol/mg protein, the magnitude of cyclase response was proportional to the receptor density, whereas as higher receptor expression level, the maximum stimulation of adenylyl cyclase reached a plateau and did not increase significantly with further increase of receptor density. This seemingly biphasic relationship between concentration (receptor level) and response (maximum activation of adenylyl cyclase) suggests that the cyclase maximal activation is dependent on receptor expression in a rectangular hyperbolic nature.
Figure 6.1 The Relationship Between Bmax And Vmax
Table 6.1

Receptor Density and Maximal AC Activity

The values listed are mean ± SEM, obtained from at least three separate experiments carried out in triplicates. The fifteen stable cell lines were numbered arbitrarily from 1 to 15. The mean values of Bmax and Vmax were plotted in Figure 6.1.

<table>
<thead>
<tr>
<th>Cell Clone</th>
<th>Bmax (fmol/mg)</th>
<th>Vmax (pmol cAMP/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>679.0±309.1</td>
<td>166.7±28.0</td>
</tr>
<tr>
<td>2</td>
<td>886.2±278.7</td>
<td>155.5±37.8</td>
</tr>
<tr>
<td>3</td>
<td>544.1±54.7</td>
<td>180.9±30.6</td>
</tr>
<tr>
<td>4</td>
<td>595.8±58.7</td>
<td>197.5±26.0</td>
</tr>
<tr>
<td>5</td>
<td>131.3±24.1</td>
<td>50.6±7.8</td>
</tr>
<tr>
<td>6</td>
<td>237.7±63.5</td>
<td>62.8±18.0</td>
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<td>7</td>
<td>281.1±125.9</td>
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<td>8</td>
<td>162.5±27.0</td>
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<td>205.6±40.5</td>
<td>114.9±30.1</td>
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<td>258.2±30.1</td>
<td>120.4±25.4</td>
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<td>14</td>
<td>148.3±46.0</td>
<td>94.3±32.4</td>
</tr>
<tr>
<td>15</td>
<td>2155±506.8</td>
<td>112.0±30.6</td>
</tr>
</tbody>
</table>
Table 6.1

Receptor Density and Maximal AC Activity

The values listed are mean ± SEM, obtained from at least three separate experiments carried out in triplicates. The fifteen stable cell lines were numbered arbitrarily from 1 to 15. The mean values of Bmax and Vmax were plotted in Figure 6.1.

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DISCUSSION

Ligand affinity, potency and efficacy have been the central concepts in pharmacology. The concept of efficacy was first defined in 1956 (Stephenson 1956). In the past decades, researchers have been trying to decipher the chemical nature of efficacy in more concrete terms as represented by the introduction of intrinsic efficacy (Furchgott 1966) and recently, under the influence of advancement in molecular biology, by several other attempts to explain the concept in molecular terms (Kenakin 1995a; Kenakin 1995b; Schwartz and JZerman 1998; Clarke and Bond 1998). Even with all these efforts and proposals of numerous models (ternary complex model (De Lean et al, 1980), the extended ternary complex model (Samama et al 1993), two-state model (Leff 1995), three state model (Leff et al, 1997), etc.), the black box of efficacy still remains to be worked out. Despite the ambiguity of efficacy in its chemical and physical meaning, many conclusions derived from this concept continue to hold true even today. For instance, according to Furchgott’s report (Furchgott 1966), response in native receptor-expressing tissue is given by

\[ Q_{\text{max}} = Q_M \frac{\varepsilon R_t}{1 + \varepsilon R_t} \]

where \( Q_{\text{max}} \) is the maximal response the agonist is capable of, \( Q_M \) is the maximal activity the whole system is capable of, \( \varepsilon \) is the intrinsic efficacy and \( R_t \) is total receptor number in the system.

That is to say the maximal response varies with total receptors available in the system hyperbolically. This equation has been corroborated by tissue studies (Furchgott 1966) and, more recently, by studies in transfected cell lines as discussed below.
To determine the relationship between receptor expression level and effector response in D1DR, I permanently transfected CHO cells with wild type D1DR. Clones with different levels of receptor expression, ranging from less than 10 fmol/mg to over 2000 fmol/mg were obtained and examined. The data revealed a biphasic, most probably a rectangular hyperbola, relationship between these parameters; namely, at low receptor expression, comparable to levels commonly observed in some native tissues, the cyclase activity varied proportionally with the receptor level, while at high receptor expression levels, a situation most commonly encountered in transfected cells, the maximum stimulation of adenylyl cyclase reached a saturated state.

Our results are consistent with several other studies that noted a similar relationship (Whaley et al, 1994; MacEwan et al, 1995). For decades, the classical concept of spare receptors or receptor reserve has been used to describe the phenomenon that not all receptors need to be activated to obtain maximal stimulation of effectors (Stephenson 1956). Whaley et al (Whaley et al, 1994) derived an equation (Fig. 6.2) showing that theoretically, the maximal activation of adenylyl cyclase should vary with receptor density in a rectangular hyperbola relationship, and the theoretical premise was proven experimentally with stable Ltk' cell lines expressing different levels of A R. MacEwan et al (MacEwan et al, 1995) extended the conclusions made with a full agonist (Whaley et al, 1994) at A R to partial agonists as well, using a combined method of stable cells treated with an irreversible antagonist (to block varying proportions of the receptor population) to obtain different (virtual) receptor densities. Recently, Befort et al (Befort et al, 1999) transiently transfected COS cells with varying amount of DNA to achieve different expression levels of δ opioid receptor. They observed that the
magnitude of agonist-stimulated activation was dependent on receptor expression level because \(^{[35\text{S}]}\text{GTP}\gamma\text{S}\) binding increased with increasing receptor density. They also documented that \(^{[35\text{S}]}\text{GTP}\gamma\text{S}\) binding plateaus at high receptor densities.

However, as Whaley et al pointed out (Whaley et al, 1994 and references within), not all GPCRs fit the same receptor density-cyclase activity pattern, especially receptors coupled to G proteins other than Gs. In fact Vogel et al (Vogel et al, 1995) used a slowly dissociating antagonist (varying amounts of QNB) to vary the available number of M2 muscarinic receptors in CHO cells. In that report, M2 receptors were shown to inhibit adenylyl cyclase (at low, physiological receptor levels) independently of receptor density, and to stimulate activity of adenylyl cyclase (at high receptor levels) proportionally dependent on receptor density (no saturation observed). The coupling to Gq also varied proportionally with available receptor numbers (again no saturation observed). Similarly in vasopressin V3 receptor, AVP triggered stimulation of phospholipase C in CHO-V3 cells with a potency directly proportional to receptor density (Thibonnier et al, 1997).

In fact, Whaley’s equation was essentially the same as Furchgott’s, which also predicted that the relationship between receptor number \((R_t)\) and maximal response \((Q_{\text{max}})\) was a rectangular hyperbolic one.

Another interesting study was reported by Roth et al on the effect of desensitization of β2AR (Roth et al 1991). In that report, both βARK (by inhibiting the PKA pathway with anti-peptide and inhibiting receptor internalization with Con A) and PKA (by inhibiting βARK pathway with heparin and internalization with Con A) mediated receptor phosphorylations were studied for different periods of time. It was shown that the degree of phosphorylation increased with the duration of agonist treatment.
but the maximal activation (representing efficacy) decreased with treatment time, indicating that desensitization was caused by phosphorylation and, more importantly, that agonist treatment had decreased the intrinsic efficacy (ε) of β2AR, hence the attenuation of maximal response (Q_max), because Q_max = Q_m \frac{εR_t}{1 + εR_t} \ (Furchgott \ 1966). In this report and in Whaley's, only R_t was changed, whereas in Roth's study, the intrinsic efficacy (ε) was altered by desensitization.

Interestingly in the same report (Roth et al 1991), Roth et al derived the following equation:

\[ E = E_m \frac{\tau[A]}{(K_A + [A]) + \tau[A]} \]

with E denoting the effect, E_m the maximum possible effect, [A] the agonist concentration, and K_A the dissociation constant of the agonist-receptor complex; \( \tau \) is a parameter describing the signal-transduction capacity of the system.

According to this equation, when \( [A] >> K_A \), \( E_{\text{max}} = E_m \frac{\tau}{1 + \tau} \). Therefore, Roth's equation was also the same as Furchgott's. Therefore, even though the true nature of efficacy has not been elucidated, the conclusions obtained from the application of this concept are still true and sound in both native tissues and transfected stable cell lines.

In summary, our results showed that the maximal D1DR activation of adenylyl cyclase was related to receptor density in a pattern simulating a rectangular hyperbola, and that, with all studies taken together, the use of V_max in assessing receptor activities should be paralleled with a determination of receptor levels before any meaningful evaluation can be made.
\[ V_{\text{max}} = V_{100} \frac{k \cdot r}{k \cdot r + k - 1} \]

Where \( V_{\text{max}} \) is the maximal activity possible at saturating concentrations of agonist, \( V_{100} \) is the adenylyl cyclase activity with 100% of receptors fully activated, \( k_l \) represents the rate constant of G protein activation, \( k_l \) is the rate constant for G protein inactivation, and \( r \) designates receptor density on cell membrane.

Figure 6.2 The equation describing the relationship between the receptor expression level and maximal adenylyl cyclase activity (Whaley et al, 1994).
Many cellular functions are controlled by signal transduction through G protein coupled receptors. The G protein-coupled receptor (GPCR) superfamily has been estimated to consist of over a thousand members that cover a wide range of stimuli, including senses (vision (both light and color), taste and odor), hormones (adrenelin, chemokines, TSH, TRH, MSH, GHRH, etc.) and neurotransmitters (5-HT, dopamine, adenosine, enkephalin, etc.). The cloning and characterization of genes encoding many of the GPCRs has enabled the scientists to gain a great deal of information on the structure and functions for this class of receptors in the past decade. Different regions of GPCRs play different roles in receptor function (Dohlman et al, 1991; Strader et al, 1994).

The intracellular domains (namely, the carboxyl terminus and three intracellular loops) are the most important regions of GPCR function, in that they constitute the interface with cellular signaling components in the cytosolic microenvironment. G protein binding sites, sites for PKA, PKC, and GRKs, sites for palmitoylation and sites for receptor internalization have all been identified in the these intracellular domains in a wide variety of GPCRs. Because of the importance of the intracellular domains of GPCR, these intracellular regions have been the main focus of research investigations in the past decade. In the late 80’s and early 90’s, most information on the structure and function of GPCRs was obtained through studies of the prototype β2AR. However, in the past few years, countless mutagenesis studies on many other GPCRs, especially in the intracellular domains, have been carried out. In line with the global efforts to decipher the information
contained in the intracellular domains of GPCRs, my Ph.D. projects have also been focusing on the main theme of the structure-functional relationship of the intracellular domains of GPCR, specifically, the functional role played by specific amino acid residues in the intracellular domains of the D1 dopamine receptor.

The first study on the intracellular domains of D1DR was on the putative palmitoylation sites of the carboxyl tail, namely Cys347 and Cys351. Palmitoylation is a reversible posttranslational modification, whereby a 16-carbon saturated fatty acid chain is attached to the cysteine residue via a thioester bond (Bonatti et al, 1989). Many cellular proteins have been found to be palmitoylated, including 14 GPCRs and 59 other proteins (e.g., adenylyl cyclase, caveolin, G protein α subunits, GRK4 and GRK6) (reviewed by Jin et al, 1999), most of which are cytosol proteins. Palmitoylation seems to increase membrane association of cytosolic proteins. The majority of the members of the G protein-coupled receptor superfamily have one or more cysteines at their intracellular carboxyl tails in a short distance downstream of TM7. These intracellular cysteines are considered the palmitoylation sites, although no consensus sequence has been identified. Since these palmitoylation sites are conserved in most GPCRs, it is conceivable to speculate that palmitoylation may play an important role in receptor function.

At the inception of my investigation into the role of palmitoylation in D1 receptor in 1994, there was a controversy as to whether palmitoylation was involved in receptor-G protein coupling. The mutant β2-adrenergic receptor with its Cys341 replaced by glycine greatly reduced its ability to form the high affinity state for agonists and its ability to stimulate adenylyl cyclase activity (O'Dowd et al, 1989; Moffett et al, 1993). However, similar mutagenesis studies on other G protein-coupled receptors, including the
rhodopsin (Karnik et al, 1993), α2-adrenergic receptors (Kennedy and Limbird, 1993) and m2 muscarinic receptors (Van Koppen and Nathanson 1991) showed that elimination of the palmitoylation site(s) by substituting cysteine(s) with other amino acid residues did not perturb receptor-G protein coupling. To clarify the role of palmitoylation in D1 dopamine receptors, I eliminated the two palmitoylation sites both separately and together using site-directed mutagenesis and found (in Chapter 2) that introduction of the mutation(s) did not affect ligand binding, receptor-G protein coupling, agonist-induced stimulation of adenylyl cyclase activity, or receptor desensitization. These data were consistent with most subsequent GPCR palmitoylation studies (Table 1.2).

For the characterizations of binding properties of the wild type and three cysteine mutant D1 receptors, some of the parameters showed small differences that were statistically significant. However, these small changes lie within the variation range reported by other groups in published literatures and therefore were not necessarily biologically relevant.

Since there are 3 other cysteines in the intracellular domains of D1DR (2 in the intracellular loop and another in the C tail), the next question naturally was ‘Are these two cysteines really the palmitoylation sites?’ The data presented in Chapter 3 have demonstrated clearly that Cys347 and Cys351 (not the other 3) are indeed the actual and only palmitoylation sites.

The other category of receptor properties being investigated was receptor internalization/down-regulation.

It seems that palmitoylation may affect receptor internalization/down-regulation. However, the effects of palmitoylation on receptor sequestration and down-regulation
may be diverse in different receptors. In mutant TRHR (C335/337G), the lack of palmitoylation significantly blocked rapid agonist-induced internalization (Nussenzveig et al, 1993). In α2A-adrenergic receptor mutants (C442F or C442A), down-regulation was completely abolished, although the mutation did not alter the extent or rate of agonist promoted sequestration or the recovery from sequestration (Eason et al, 1994). For LH/hCG receptors, abolition of palmitoylation increased agonist-induced internalization of the receptor (Kawate and Menon 1994).

Then what was the reason for the apparent discrepancy between β2AR and others in G protein-coupling?

I found that the β2AR contains a PKA site in its carboxyl tail in close proximity (4 amino acid residues apart) to the palmitoylation site, whereas no such a phosphorylation site is found in the C-tails of rhodopsin, α2A-adrenergic receptors, m2 muscarinic receptors, TRH receptors and D1 dopamine receptors. A PKA site is found in LH/hCG receptors in its C-terminus, but it is 33 amino acid residues away from the two cysteines that have been shown to be palmitoylated. Palmitoylation of wild type β2-adrenergic receptors may prevent access to the PKA site by PKA in the cytosol. Therefore, palmitoylation/depalmitoylation can serve to cover or expose that particular PKA site in the wild type β2-adrenergic receptors, whereas the elimination of palmitoylation of the Gly341β2-adrenergic receptors can constantly expose the PKA site, thereby causing constitutive desensitization. In fact, it was reported (Bouvier et al, 1995) that elimination of the PKA site from Gly341β2-adrenergic receptor restored both the phosphorylation and the effector activation to wild type levels.
In Chapter 3, I tested the hypothesis that the PKA site in β2AR was responsible for its difference from other GPCRs. I introduced a PKA site into D1DR at position 351. A functional PKA site would be expected to result in a similar constitutive desensitization. The data showed that the addition of the PKA consensus site did cause a reduction in the potency of dopamine stimulation of adenylyl cyclase compared with the wild type. Analysis with the extended ternary complex model confirmed that the introduced PKA site conferred a degree of constitutive desensitization when not interfered with by palmitoylation (in AAP).

The changes in the receptor activation capability of adenylyl cyclase were suggestive of a functional PKA site, but a conformational change (although less likely) induced by the mutation cannot be ruled out definitely unless the basal phosphorylation status was determined.

In summary, the results presented in Chapters 2 and 3 have shown clearly that palmitoylation occurred exclusively at Cys347 and Cys351 in D1DR. Unlike β2AR, palmitoylation was not required for receptor-G protein coupling as measured by adenylyl cyclase activities. Introduction of PKA in the carboxyl tail elicited a constitutively desensitized state of the D1DR. These results may help to clarify the mechanism whereby palmitoylation of D1DR differs from β2AR.

With all the palmitoylation studies of the 18 GPCRs taken together (Table 1.2), it is now clear that palmitoylation occurs in the cysteines of the intracellular carboxyl terminal tail of GPCRs close to TM7, and is not required for G protein interaction for most GPCRs. Although the observation that removal of palmitoylation may affect receptor internalization and down-regulation in certain receptors may suggest that
Palmitoylation may be involved in receptor intracellular trafficking, the clear functional role of palmitoylation, which is conserved in the majority of GPCRs, is still unknown.

Some evidence may suggest that the palmitate attached to receptor tails may serve to anchor this intracellular portion of GPCR to the cell membrane. In fact, it was shown directly in rhodopsin (Milligan et al, 1995): the two covalently attached fluorescent analogues of palmitate chains were shown to lie in the cell membrane, instead of embedding themselves within the protein itself. However, how the anchorage of this portion of C tail affects receptor function and what receptor functions are being affected are not yet clear.

Although palmitoylation of many G protein coupled receptors has been recorded at the intracellular cysteine residues on C tail, it is not clear if this acylation is specific for palmitoylation. A recent study on prostacyclin receptor revealed that cysteine residues in the C tail could also be modified by another form of lipidation, isoprenylation (Hayes et al, 1999). In that report, prostacyclin receptor was shown to be attached by the 15-carbon farnesyl isoprenoid also through a thioester bond with the cysteine residue. Disruption of the isoprenylation motif by site-directed mutagenesis yielded a mutant prostacyclin receptor that exhibited normal binding properties, but displayed diminished coupling to adenylyl cyclase and to phospholipase C. Therefore, in contrast to palmitoylation, receptor prenylation was required for G protein coupling in prostacyclin receptor. Whether other GPCRs are modified by isoprenylation at the cysteines of the intracellular carboxyl tail will have to await further investigations.

Palmitoylation levels of receptors may be modified by agonist (reviewed by Jin et al, 1999) or other treatment (e.g., nitric oxide) (Adam et al, 1999). Nitric oxide was
recently found to decrease cAMP production and palmitoylation level in β2AR. The basal palmitoylation status level of the β2AR was also found to be affected by nitric oxide, which suggested that nitric oxide acted directly on the palmitoylation status of the receptor (Adam et al, 1999). It seems that such regulation of palmitoylation by nitric oxide is more widely spread, and was also observed previously in the interacting proteins of growth-cone-associated protein 43 (GAP43) and Goα. Nitric oxide-induced alteration in palmitoylation of GAP43 and/or Goα may cause growth cone collapse (Milligan et al, 1995).

Interestingly, Loisel et al recently demonstrated in β2AR-Gαs fusion protein, which undergoes activation but not desensitization, internalization or down-regulation, that agonist isoproterenol treatment led to a rapid depalmitoylation of both the receptor and Gαs (Loisel et al, 1999). The extent of depalmitoylation was correlated with the intrinsic efficacy of the agonists to activate adenylyl cyclase. Given that the fusion protein represent the early activation steps, the results suggest that activation of receptor-G protein complex may cause depalmitoylation, while, to reconcile the previous observations that agonist treatment caused increased incorporation of tritiated palmitate (Mouillac et al, 1992; Ng et al, 1994a), Loisel et al suggested that later steps after activation of receptor-G protein (e.g., desensitization) was responsible for receptor repalmitoylation (Loisel et al, 1999).

Therefore, many studies suggest that receptor acylation (palmitoylation and isoprenylation) are reversible and regulated. Given that palmitoylation is highly conserved among GPCRs, it is conceivable that palmitoylation may play an important role in receptor function. However, many questions remain to be solved in the future.
First, is palmitoylation/depalmitoylation that is concurrently found with activation/deactivation the cause of change or an effect of the change or just an accompanying co-incidence? Second, if palmitoylation is not involved in receptor-G protein coupling, then what is its general function? Third, does palmitoylation play a common role in receptor function or different roles in different receptors? Considering our current knowledge (or lack of it) and sometimes conflicting and perplexing observations, the complete elucidation of this phenomenon will not be an easy task and will need much more effort in the future.

The second intracellular domain that I examined was the first intracellular loop of D1 receptor. Different functional roles of different regions of the receptor proteins have been determined. However, studies on the functional role of the first intracellular loop of GPCRs are generally lacking. However, in the limited number of studies, listed in Table 4.1, this domain has been shown to play a critical role in receptor expression and receptor-G protein coupling in some receptors, causing increased (e.g., MSH and CCKBR), decreased (e.g., β2AR, some TSHR mutants, rhodopsin and hCRFRII) or no change (e.g., some TSHR mutants) in receptor-G protein coupling.

One mutant receptor that is of particular interest has been the MSH receptor. A naturally occurring mutant receptor (Ser69Leu in the first intracellular loop) was identified in tobacco coat mice, and showed constitutive activity and enhanced activation of adenylyl cyclase when expressed in human 293 cells (Robbins et al, 1993). Since a similar serine residue was also found in several GPCRs including D1 receptor, I decided to investigate if a similar mutation (Ser56Leu) in D1DR would also elicit increased basal activity and enhanced stimulation of adenylyl cyclase. Our study showed that in contrast
to MSH receptor, Ser56 in the first intracellular loop of the D1DR did not dramatically influence receptor-effector coupling. These findings emphasize the importance of studying structure-function determinants of GPCRs individually rather than to generalize from one receptor to another based on the common topography of GPCRs. These results also point out that our efforts to elucidate the final determinants of the relationship between the structure and function of G protein-coupled receptors are still at a preliminary stage without definitive knowledge of receptor structures by crystallography.

The third intracellular domain of D1DR that I studied was the second intracellular loop. Receptor internalization (synonymous with sequestration or endocytosis) is a common phenomenon. GPCR internalization seems to be determined by several factors: the endocytosis motifs, phosphorylation of the receptors, ubiquitination and the components of the endocytotic machinery of different cell lines (reviewed by Koenig and Edwardson, 1997). Among these, phosphorylation is considered a major determinant and has been most extensively investigated.

In β2AR, phosphorylation promotes binding of β-arrestin (Ferguson et al, 1996), followed by attachment to clathrin in clathrin-coated pits (Goodmann et al, 1996) and dynamin-controlled budding of clathrin-coated pits from the plasma membrane (De Camilli et al, 1995).

Despite the opulence of information on β2AR sequestration, much less is known of sequestration of other types GPCRs. Traditionally, researchers focused on phosphorylation mediated by GRKs, PKA and PKC. In fact, apart from GRKs, PKA and PKC sites, other protein kinase consensus sequences also exist in GPCRs.

For instance, a cdc2 phosphorylation consensus sequence (ISSPFRY) (Figure 5.1)
is present in the second intracellular loop of D1DR (ISSPFRY), D5DR (ISRPFRY) (Sunahara et al, 1991) and β2AR (ITSPFKY) (Kobilka et al, 1987). However, the functional role of cdc2 in GPCR function has not yet been investigated. To clarify the functional role played by this intracellular sequence, I decided to study the cdc2 site in D1DR. The results demonstrated that the removal of the cdc2 phosphorylation site by site-directed mutagenesis reduced D1DR internalization, suggesting that cdc2 phosphorylation site plays an important role in D1DR internalization. However, the introduction of the cdc2 sequence into the corresponding region of the second intracellular loop of D2DR did not result in receptor internalization, indicating that cdc2 phosphorylation site alone was not sufficient to confer onto D2DR the ability to internalize. The results of Chapter 5 indicate that phosphorylation mediated by other protein kinases (than GRK, PKA and PKC) may also play important roles in receptor function.

These findings regarding the cdc2 site mutation may be consistent with a phosphorylated cdc2 site mediating receptor internalization. However, the phosphorylation state of receptor mutants was not experimentally determined and therefore the possibility that the mutation caused a conformational change (rather than change in phosphorylation status of the mutant receptor) could not be ruled out in the absence of phosphorylation data.

Despite the large amount of information concerning β2AR sequestration, much less is known of sequestration of other types GPCRs. From the fragmentary information available, some receptors seem to follow the same route, e.g., the chemokine receptor CXCR2 (Yang et al, 1999) and bradykinin B2 receptor (Pizard et al, 1999), but other
receptors seem to deviate from the prototypical β2AR in several aspects. For example, angiotensin II AT1A receptors sequester in a dynamin-independent manner (Zhang et al, 1996). While M1, M3 and M4 AChRs internalization is regulated by dynamin, M2 AchR sequester independently of dynamin (Vogler et al, 1998). In another report, it was shown that M1, M3 and M4 AchRs internalized in an arrestin-independent manner (Lee et al, 1998). In the platelet-activating factor receptor, removal of the phosphorylation sites of the C tail reduced the extent of receptor sequestration, suggesting that phosphorylation may facilitate but is not essential for the process (Ishii et al, 1998). Several signaling and phosphorylation-deficient mutant FSH receptors were shown to internalize through an arrestin-dependent pathway (Nakamura et al, 1998). A truncated opioid receptor without phosphorylation sites was observed to still undergo a dynamin-dependent endocytosis involving clathrin coated pits (Murray et al, 1998). Instead of going through clathrin-coated pits, some receptors internalize through caveolae (Roettger et al, 1995) or non-coated pits (Raposo et al, 1989). Therefore, deviations exist in almost every step from the ‘typical’ receptor internalization pathway of β2AR, which again emphasizes the importance of studying GPCRs individually rather than to generalize conclusions only on the basis of homologous structure or common topography.

The study presented in Chapter 5 represents the first report, to our best knowledge, that investigate explicitly the role other phosphorylation sites play in the dopamine D1 receptor, in addition to the GRK and PKA/PKC sites. In the future, further investigations into the roles played by phosphorylation of other protein kinases and into internalization patterns of more GPCRs may eventually clarify the mechanisms underlying the intriguing phenomenon of receptor internalization.
The study presented in Chapter 6 addressed a very important issue in pharmacology. The theme underpins all investigations of wild type and mutant receptors in pharmacology (Chapters 2, 3, 4 and 5). This issue was on the relationship between receptor numbers with cellular response. The studies on this topic were first carried out in the 60's in muscarinic receptor in native tissues (Stephenson 1966) and rekindled more recently, in transfected cell lines (Whaley et al, 1994; Vogel et al, 1995; MacEwan et al, 1995; Thibonnier et al, 1997; Befort et al, 1999).

To determine the nature of the relationship for D1 dopamine receptor (D1DR), I expressed the wild type D1DR in Chinese hamster ovary cells. From the fifteen stable cell lines with receptor density ranging from about 10 fmol/mg protein to 2000 fmol/mg, the data in Chapter 6 revealed that at low levels, maximum agonist activation of adenylyl cyclase did vary with D1 receptor density, and it increased proportionally to receptor density at low levels, but reached a plateau with higher D1 receptor expression. That is, the maximal D1DR activation of adenylyl cyclase was related to receptor density in a pattern simulating a rectangular hyperbola. This study emphasizes the importance of comparing wild type and mutant receptors using cell lines with similar receptor expression levels and that the use of Vmax in assessing receptor activities should be paralleled with a determination of receptor levels before any meaningful evaluation can be made. This point was obviously missed by some researchers (e.g., Jensen et al, 1995), who studied receptors using cell lines with different receptor expression levels. This kind of mistake was intentionally avoided in all my studies in Chapters 2, 3, 4 and 5 on wild type and mutant receptors by using stable cell lines with comparable levels of receptor expression.
The study in Chapter 6 also touched upon a central concept of pharmacology, efficacy. The concept of efficacy was first defined in 1956 (Stephenson 1956). In the past decades, researchers have been trying to decipher the chemical nature of efficacy in more concrete terms (intrinsic efficacy by Furchgott 1966) and recently, under the influence of advancement in molecular biology, by several other attempts to explain the concept in molecular terms (Kenakin 1995a; Kenakin 1995b; Schwartz and IJzerman 1998; Clarke and Bond 1998). Even with all these efforts and proposals of numerous models (ternary complex model (De Lean et al., 1980), the extended ternary complex model (Samama et al. 1993), two-state model (Leff 1995), three state model (Leff et al., 1997), etc.), the black box of efficacy still remains to be opened. According to Furchgott’s concept of intrinsic efficacy (Furchgott 1966), response in native receptor-express tissue is given by

\[ Q_{\text{max}} = Q_M \frac{\varepsilon R_t}{1 + \varepsilon R_t} \]

where \( Q_{\text{max}} \) is the maximal response the agonist is capable of. \( Q_M \) is the maximal activity the whole system is capable of, \( \varepsilon \) is the intrinsic efficacy and \( R_t \) is total receptor number in the system.

That is to say the maximal response varies with total receptors available in the system hyperbolically, which was exactly what I observed in stable D1 receptor cell lines.

The results are consistent with several other studies (Whaley et al., 1994; MacEwan et al., 1995; Befort et al., 1999). Whaley et al. (Whaley et al., 1994) derived an equation (Fig. 6.2) showing that the maximal activation of adenylyl cyclase should vary with receptor density in a rectangular hyperbola relationship. In fact, Whaley’s equation was essentially the same as Furchgott’s, which also predicted that the relationship
between receptor number \((R_i)\) and maximal response \((Q_{max})\) was a rectangular hyperbolic one.

Another interesting equation was derived by Roth et al (Roth et al 1991):

\[
E = E_m \frac{\tau[A]}{(K_A + [A]) + \tau[A]},
\]

with \(E\) denoting the effect, \(E_m\) the maximum possible effect, \([A]\) the agonist concentration, and \(K_A\) the dissociation constant of the agonist-receptor complex; \(\tau\) is a parameter describing the signal-transduction capacity of the system.

According to this equation, at maximal concentration of agonist (when \([A]\gg K_A\), \(E_{\text{max}} = E_m \frac{\tau}{1 + \tau}\). Therefore, Roth’s equation was also the same as Furchgott’s.

On the other hand, not all studies of GPCRs on this issue seem to fit the same receptor density-cyclase activity pattern (Vogel et al, 1995; Thibonnier et al, 1997). In those studies, the maximal response seemed to increase proportionally with receptor density and no saturation could be reached even at very high receptor expression levels. I believe the reason was because the intrinsic efficacy \((\varepsilon)\) of the agonist/receptor complex was so small that even at high receptor density, the maximal response was still at the early phase of the rectangular hyperbola, and eventually (provided that even higher expression levels were achievable) would reach a saturated status, as predicted by the above three equations.

Obviously, even though the true nature of efficacy has not been elucidated yet, the ambiguity does not prevent conclusions from being made by the application of this concept in both native tissues and transfected stable cell lines. It is possible that the apparent, expressed properties of agonists (affinity and efficacy) should be explained
with detailed analysis of their component parts and with less intuitive parameters such as the constants underlying the two-state model (Leff, 1995). To understand the real nature of efficacy, even the three-state model may not be sufficient, because in reality, there may be multiple active states (Leff et al, 1997). To formulate such a complex model seems to be a formidable task. Sometimes, one pharmacological phenomenon can be explained by several different ways. For instance, the ternary complex model (and the extended version) could be used to explain the two affinity states of agonists, two-state model and three-state model could also explain the observation. In fact, a cooperativity theory could also be used to explain the same phenomenon (Wreggett and Wells, 1995). What is the real situation that occurs in the microenvironment obviously still need further investigations to resolve.
Chapter 8

Conclusions

In conclusion, research conducted toward this Ph.D. thesis has clarified and resolved several interesting and intriguing, sometimes very perplexing problems in the field of GPCRs.

This thesis has clarified the following points:

1. Palmitoylation is not required in receptor-G protein coupling for D1DR as for the majority of GPCRs.

2. The D1 dopamine receptor is palmitoylated at Cys347 and Cys351 of the carboxyl tail, and palmitoylated exclusively at these two cysteine residues. In contrast to rhodopsin, palmitoylation of Cys351 does not require Cys347 to be palmitoylated. The PKA site in the β2AR C-tail encompassing the palmitoylated site appears responsible for the discrepancy observed between D1DR (and other GPCRs) and β2AR.

3. The Ser56Leu point mutation in the first intracellular loop does not render the mutant D1DR constitutively active as in MSHR.

4. The cdc2 consensus sequence in the second intracellular loop of D1DR is involved in D1 receptor internalization, but is not sufficient to cause D2DR to internalize.

5. The maximal activation of adenylyl cyclase varies with receptor expression level in a pattern that resembles a rectangular hyperbola, which emphasizes the importance of having comparable receptor density in order to compare effector responses.
It should be pointed out that through all these years of research in the field of GPCRs, it has become increasingly clear that each receptor is unique and deserves to be studied carefully and individually, rather than to generalize from a prototype (AR) only on the basis that they share the same topography.

The intensive investigations into the structure and functions of GPCRs and their role in pathogenesis of diseases will undoubtedly lead to a full understanding of how defective receptors could cause diseases. On the other hand, the opulence of information on GPCRs offers a great opportunity for development of therapeutic agents for many diseases.
REFERENCES


