# Comprehensive Model of G Protein-coupled Receptor Regulation by Protein Kinase C: Insight from Dopamine D1 and D5 Receptor Studies.

By

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## Abstract

Dopamine receptors belong to the G protein-coupled receptor (GPCR) superfamily and are classified into two families: D1-like (D1R and D5R) and D2-like (D2R, D3R and D4R), based on their ability to stimulate or inhibit adenylyl cyclase (AC). Classically, GPCRs (including D2R and D3R) are desensitized by the activation of the serine/threonine protein kinase C (PKC) upon phorbol-12-myristate-13-acetate (PMA) treatment. Previous studies demonstrate that while human D5R (hD5R) is also strongly desensitized upon PMA treatment, the human D1R (hD1R) undergo a robust PMA-induced sensitization. The aim of this PhD thesis was to explore how the canonical PKC- or phorbol ester-linked pathway can control the responsiveness of two similar GPCRs like hD1R and hD5R in an opposite fashion. Our data indicate that hD1R sensitization and hD5R desensitization are not mediated by a direct modulation of AC activity by PKC. Using a chimeric approach, we identified the third intracellular loop (IL3) as the key structural determinant controlling in an opposite manner the PMA-mediated regulation of hD1R and hD5R. To delineate the potential PKC phosphorylation sites, a series of mutation of serine (Ser) and threonine (Thr) located into IL3 of hD1R and hD5R were used. No hD1R mutation decreased the PMA-mediated sensitization. This suggests that hD1R phosphorylation is not required for PMA-induced sensitization. In contrast, our results indicate that PMA-mediated hD5R desensitization occurs through a hierarchical phosphorylation of Ser260, Ser261, Ser271 and Ser274. Notably, these hD5R mutants exhibited a PMA-induced sensitization, reminiscent of the PMA-induced hD1R sensitization. Additionally, using short hairpin RNAs (shRNAs), we showed that PKCε is

the potentiating PKC while the desensitizing isoform is δ. Overall, our work suggests the presence or absence of specific Ser residues on IL3 of hD1-like receptors dictate if phosphorylation-dependent desensitization (through PKCδ) or phosphorylation-independent potentiation (via PKCε) will occur.

# Table of Contents

# List of Tables

**CHAPTER 2**



**CHAPTER 3**

**CHAPTER 4**

**CHAPTER 5**

# List of Figures

**CHAPTER 1**



**CHAPTER 2**

**CHAPTER 4**



**CHAPTER 5**

## List of Abbreviations

**AA:** Arachidonic acid

**ABP-280:** Actin-binding protein 280

**AC:** Adenylyl cyclase

**ACh:** Acetylcholine

**AD:** Alzheimer's disease

**AGS:** Activator of G protein signaling

**Ala:** Alanine

**AMPA:** α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

**AP-2:** Adaptor protein-2

**aPKC:** Atypical protein kinase C

**ATP:** Adenosine triphosphate

**β'COP:** Beta cotamer protein

**$Ca^{2+}$:** Calcium

**cAMP:** Cyclic adenosine monophosphate

**CHO:** Chinese hamster ovary

**COMT:** Cathechol-O-methyltransferase

**cPKC:** Conventional protein kinase C

**CREB:** cAMP response element-binding protein

**CT:** Cytoplasmic tail

**D1R:** D1 dopaminergic receptor

**D2R:** D2 dopaminergic receptor

**D3R:** D3 dopaminergic receptor

**D4R:** D4 dopaminergic receptor

**D5R:** D5 dopaminergic receptor

**DA:** Dopamine

**DAG:** Diacylglycerol

**DARPP-32:** Dopamine and cyclic AMP-regulated phosphoprotein 32 kDa

**DAT:** Dopamine transporter

**DDC:** Dopa-decarboxylase

**EGFR:** Epidermal growth factor receptor

**EL:** Extracellular loop

**EL1:** First extracellular loop

**EL2:** Second extracellular loop

**EL3:** Third extracellular loop

**EPAC:** Exchange protein directly activated by cAMP

**ERK:** Extracellular signal-regulated kinase

**GABA:** Gamma-amino butyric acid

**GAP:** GTPase-activating protein

**GDP:** Guanosine diphosphate

**GEF:** Guanine nucleotide exchange factor

**GIRK:** G protein-regulated inwardly rectifying potassium channel

**GPCR:** G protein-coupled receptor

**GPe:** Globus pallidus externa

**GPi:** Globus pallidus interna

**GPR motif:** G protein regulatory motif

**G protein:** Guanine nucleotide-binding protein

**GPS:** G protein-coupled receptor proteolytic site

**GRK:** G protein-coupled receptor kinase

**GTP:** Guanosine triphosphate

**H8:** Helix 8

**hD1R:** Human D1 dopaminergic receptor

**hD5R:** Human D5 dopaminergic receptor

**HEK:** Human embryonic kidney cells

**HSP90:** Heat shock protein-90

**IL:** Intracellular loop

**IL1:** First intracellular loop

**IL2:** Second intracellular loop

**IL3:** Third intracellular loop

**IL4:** Fourth intracellular loop

**$IP_3$:** Inositol-1,4,5-triphosphate

**JNK:** c-Jun amino-terminal kinase

**kDa:** Kilodalton

**L-Dopa:** Levo-dihydroxyphenylalanine

**LTP:** Long-term potentiation

**M1R:** M1 muscarinic acetylcholine receptor

**M2R:** M2 muscarinic acetylcholine receptor

**MAO:** Monoamine oxidase

**MAPK:** Mitogen-activated protein kinase

**MEK5:** Mitogen-activated protein kinase kinase 5

**mGluR:** Metabotropic glutamate receptor

**MSN:** Medium spiny neurons

**mTORC2:** Mammalian target of rapamycin complex 2

**NAc:** Nucleus accumbens

**NADPH:** Nicotinamide adenine dinucleotide phosphate

**NF-κB:** Nuclear factor kappa-light-chain-enhancer of activated B cells

**NMDA:** N-methyl-D-aspartate

**nPKC:** Novel protein kinase C

**NT:** Amino-terminal

**NTR1:** Neurotensin type 1 receptor

**PAR-6:** Partitioning defective-6

**PB1:** Phox and Bem 1

**PDE:** cAMP phosphodiesterase

**PDGFR:** Platelet-derived growth factor receptor

**PDK-1:** Phosphoinositide-dependent kinase-1

**PFN:** Parafascicular nucleus

**PH:** Pleckstrin homology

**PI3K:** Phosphoinositide 3-kinase

**$PIP_2$:** Phosphatidylinositol-4,5-biphosphate

**$PIP_3$:** Phosphatidylinositol-triphosphate

**$PLA_2$:** Phospholipase $A_2$

**PLCβ:** Phospholipase Cβ

**PKA:** cAMP-dependent protein kinase

**PKB:** Protein kinase B

**PKC:** Protein kinase C

**PMA:** Phorbol 12-myristate 13-acetate

**PP1:** Protein phosphatase 1

**PP2A:** PKA-stimulated protein phosphatase-2A

**PP2B:** Protein phosphatase-2B

**RACK:** Receptor for activated c-kinase

**RasGRP:** Ras guanyl-releasing protein

**RGS:** Regulator of G protein signaling

**RKIP:** Raf kinase inhibitor protein

**RTK:** Regulator tyrosine kinase

**Ser:** Serine

**SNpc:** Substantia nigra pars compacta

**SNpr:** Substantia nigra pars reticulata

**STN:** Subthalamic nucleus

**Thr:** Threonine

**TM:** Transmembrane

**TPA:** 12-O-tetra-decanoyl 4β-phorbol 13-acetate

**Unc13:** Uncoordinated mutant #13

**VMT:** Vesicular monoamine transporter

**VTA:** Ventral tegmental area

## Acknowledgments

This present thesis represents the final result of five years of my life. Since September 2006, I learned so much about neuroscience and GPCRs but most importantly about myself. I discovered my own limits but also to not give up easily at the first problem. Paradoxically, I learned to trust more my intuition and to realize that we often know much more than what we think we know. This would not have been possible without a lot of people for which I am extremely grateful.

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## Thesis Format

This thesis is written in the collection of manuscripts format. Chapter 1 is a general introduction describing the theoretical background of the work presented herein. Chapter 2 is a manuscript titled "*The third intracellular loop of D1 and D5 dopaminergic receptors dictates their subtype-specific PKC-induced sensitization and desensitization in a receptor conformation-dependent manner*". Chapter 3 consists of a second manuscript titled "*Multiple serine and threonine clusters in the third intracellular loop of human D1 and D5 dopaminergic receptors modulate agonist binding and activation properties*". Chapter 4 deals with a manuscript titled "*Molecular mechanisms underlying the opposite PKC-induced regulation of dopamine D1 and D5 receptor coupling to cAMP pathway*". Chapter 5 includes a fourth manuscript titled "*Role of novel PKC isoforms in dopamine D1 and D5 receptor signaling*". Finally, chapter 6 is the overall discussion of this PhD thesis.

# Chapter 1

# General Introduction

## 1. G protein-coupled receptors

Receptors represent an extraordinary way to relay external information (stimuli) from the outside to the inside of cells. G protein-coupled receptors (GPCRs) represent the largest family of cell-surface receptors with more than one thousand identified since the 1980's. This diversity allows a huge number of intracellular responses. Our daily perception of life would not be the same without GPCRs. In fact, vision, olfaction and taste rely on activation of these membrane receptors.

GPCRs are seven transmembrane-spanning proteins and signal via heterotrimeric G proteins coupled on the cytoplasmic side (**Fig. 1**). Transmembrane (TM) domains are predominantly composed of hydrophobic residues. This contributes to the TM insertion into membrane. Each TM domain (individually termed as TM1 to TM7) is linked to the next one by an intracellular loop (IL) or an extracellular loop (EL). There are three IL (termed as IL1, IL2 or IL3) and three EL (termed as EL1, EL2 or EL3). The region located upstream to TM1 is extracellular and called the amino-terminal or N-terminal (NT) region. Finally, the region located after TM7 is intracellular and is known as the cytoplasmic tail (CT).

Membrane spanning domains are the main ligand binding structures (Gether 2000). Amino acids in contact with the ligand form the binding pocket of the GPCR. Additionally, in some GPCRs, the NT region is also involved in ligand binding. IL3 (the longer IL and the more divergent among GPCRs) is the main domain where G protein coupling occurs (Gether 2000). However, many studies suggest IL2 and IL4 are also involved in GPCR coupling with G proteins (Kobilka 1992, Savarese & Fraser 1992, Wess 1997, Dohlman *et al.* 1991, Strader *et al.* 1994, Wess 1998).

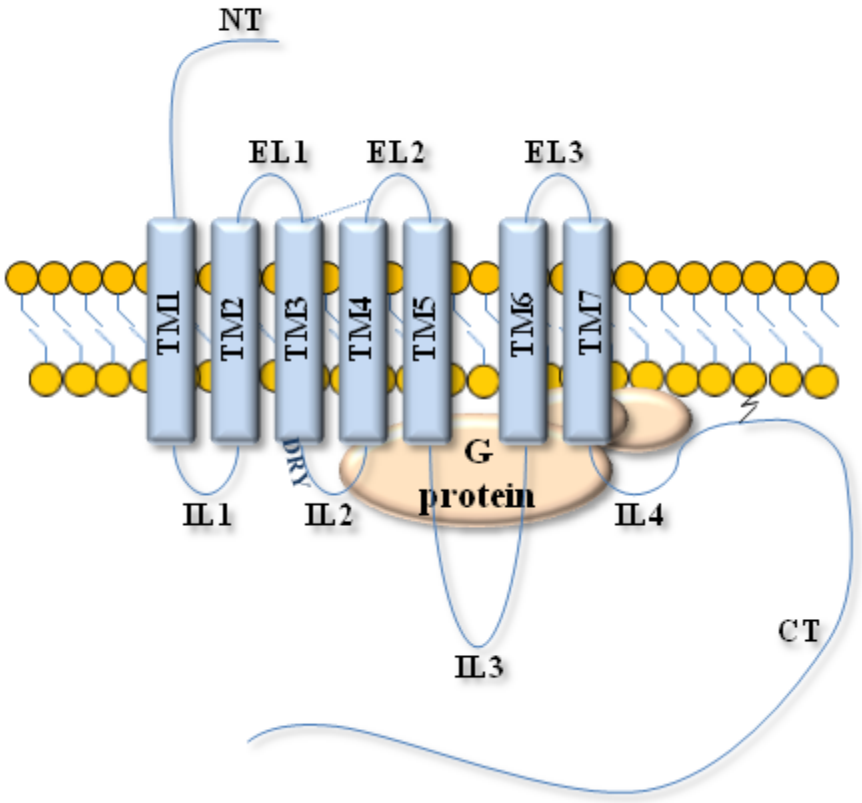


**Figure 1. General structure of a G protein-coupled receptor (GPCR).**
Different intracellular domains (IL: intracellular loop and CT: cytoplasmic tail) are shown. Extracellular domains (EL: extracellular loop and NT: amino-terminal) are also represented. Transmembrane (TM) domains 1 to 7 cross the plasma membrane. The transmembrane-spanning receptor forms a functional complex with their heterotrimeric G proteins. Presence of a disulfide bridge connecting EL2 and EL3 is a also a common feature for almost all GPCRs. Rhodopsin receptors generally have a palmitoylated cysteine in CT creating a putative IL4.

## 1.1. GPCR classification

GPCRs are generally classified on the basis of their structure and sequence homology using the GRAFS classification system. According to this system, there are five main families: glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin (Fredriksson *et al.* 2003). The rhodopsin-like family is the largest one. It comprises biogenic amine receptors (dopamine, adrenergic, serotonin, histamine and muscarinic), adenosine, cannabinoid, opioid, melanocortin, olfactory, opsins, vasopressin and others. Members of rhodopsin-like family have a relatively short NT and share a conserved DRY (aspartate-arginine-tyrosine) motif located in IL2 region at the cytoplasmic side of TM3 (Fig. 1). This motif is important in the functional and structural integrity of rhodopsin family receptors and can vary among receptors. However, the arginine is always present (Probst *et al.* 1992). Additionally, most of the rhodopsin receptors have a palmitoylated cysteine in CT. This creates a putative IL4 (**Fig. 1**). This region oscillates between a helical conformation (termed as H8) and a looplike structure (Krishna *et al.* 2002). The presence of a disulfide bridge connecting EL2 and EL3 is characteristic of almost all GPCRs families. This bridge has a role to play in the ligand binding and/or proper receptor folding (Savarese & Fraser 1992). Secretin-like receptors bind large peptides and are characterized by a long NT (60-80 amino acids) having several cysteine residues probably forming stabilizing disulfide bridges and serving as an extracellular hormone-binding domain. This family comprises glucagon, calcitonin, secretin receptors and others. The glutamate family members have a short IL3 but a very long NT (around 600 amino acids). The NT of glutamate family receptors is thought to serve as a ligand-binding site (Fredriksson et al. 2003). This family comprises metabotropic glutamate

(mGluR) and gamma-amino butyric acid (GABA) receptors, calcium, taste receptors and others. The adhesion family GPCRs have long, diverse and highly glycosylated NT regions (Baud *et al.* 1995). Their NT contains adhesion motifs mediating cell to cell adhesion (Stacey *et al.* 2000). The NT also contains an intracellular autocatalytic processing site also called GPS domain (G protein-coupled receptor proteolytic site) (Krasnoperov *et al.* 1997). This cleavage site is important for a proper cell-surface expression of the receptor (Krasnoperov *et al.* 2002). The frizzled receptors are involved in proliferation and cell polarity during development through mediation of signals from Wnt (secreted glycoproteins). The NT region (200 amino acids) as frizzled receptors participates in Wnt binding. Taste2 receptors are included in the same family than frizzled receptors because they share many consensus sequences that are not found in other families. Taste2 receptors have very short NT and are present in the tongue and palate epithelium. These receptors are responsible for the bitter taste.

## 1.2. Heterotrimeric G proteins

Heterotrimeric guanine nucleotide-binding proteins (G proteins) represent the link between the ligand-binding receptors and intracellular responses. These proteins are composed of three subunits: α, β and γ. In humans, there are 21 different Gα subunits, 6 Gβ and 12 Gγ (Downes & Gautam 1999). This variety of heterotrimeric G proteins allows specifity in the wide spectrum of possible intracellular events upon ligand binding to GPCRs. Based on the primary sequence, members of Gα subunits family (39-52 kDa) are divided into four classes: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ according to which effector they stimulate or inhibit (Simon *et al.* 1991). The Gα subunit contains a guanine nucleotide

binding domain, a GTPase domain (for guanosine triphosphate (GTP) hydrolysis) and binding sites for seven transmembrane-spanning proteins, Gβγ dimers and downstream effectors. The Gβ subunits (~36 kDa) and the Gγ subunits (7-8 kDa) form a functional unit together. There are 60 possible combinations of Gβγ dimers (Clapham & Neer 1997). G proteins can be regulated by regulators of G protein signaling (RGS) and by activators of G protein signaling (AGS). RGS proteins enhance Gα GTPase activity while AGS proteins activate G proteins in a GPCR-independent manner (Cismowski *et al.* 2001, Hollinger & Hepler 2002). By regulating G proteins, AGS and RGS can also modulate the activity of GPCRs.

## 1.3. GPCR activation

There are three main components of GPCR signaling: the seven transmembrane-spanning receptor, the heterotrimeric G protein and the enzymatic effector. The signal transmission from the outside to the inside of the cell is mediated through the heterotrimeric G protein cycle (**Fig. 2**).

### 1.3.1. Heterotrimeric G protein cycle

Binding of the agonist (first messenger) to GPCR triggers a conformational change of TM domains (Luttrell 2006). This TM movement promotes coupling between heterotrimeric G protein and the receptor leading to activation of Gα subunit. Activated Gα subunit adopts a conformational state catalyzing the exchange of guanosine diphosphate (GDP) for GTP, but also dissociation from the Gβγ dimer (**step 1 on Fig. 2**). Once dissociated, GTP-bound Gα subunit and Gβγ dimer regulates the activity of

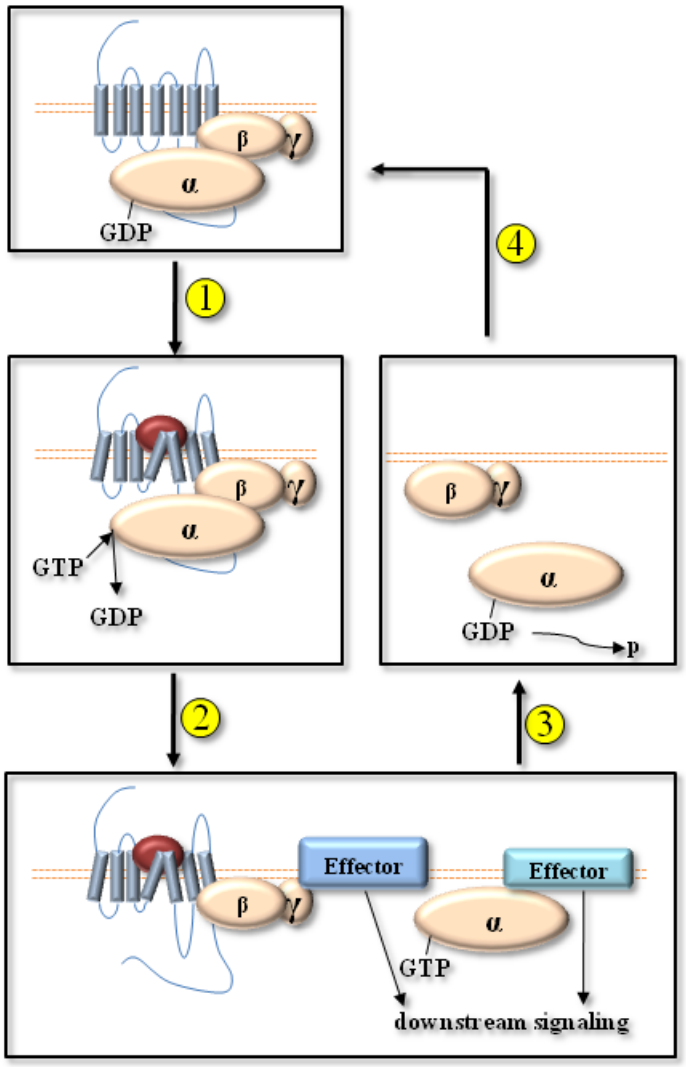


**Figure 2. Heterotrimeric G protein cycle.**
In its inactive form, the Gα subunit binds GDP and is associated with the βγ dimer. Agonist binding to the receptor induces a change of GPCR conformation leading also to a conformational change of Gα subunit. This causes the exchange of GDP for GTP on Gα subunit (**1**). Binding of GTP permits Gα dissociation from the receptor and βγ dimer. This dissociation allows GTP-bound Gα and βγ dimer to interact with and activate their respective effectors leading to downstream signaling events (**2**). The intrinsic GTPase activity of the Gα subunit then catalyzes GTP hydrolysis into GDP (**3**). GDP-bound Gα subunit reassociates with βγ dimer and a receptor to repeat another cycle (**4**).

enzymatic effectors (**step 2**). Activation of the effectors leads to production of second messengers. These second messengers activate protein kinases regulating several intracellular signaling pathways. The signal ends when the Gα subunit catalyzes GTP hydrolysis into GDP (through Gα intrinsic GTPase activity) (**step 3**) and then reassociates with βγ dimer to the inactive heterotrimeric state (**step 4**). The heterotrimeric G protein can then repeat another cycle.

### 1.3.2. Extended ternary complex model

The extended ternary complex model is the most commonly used to explain the link between ligand binding and the corresponding activity inside the cell. The ternary complex consists of the agonist (A), the receptor (R) and the G protein (G). In this model, the receptor oscillates between two states: an inactive state (R) and an active state (R*) (Park *et al.* 2008b). In the inactive state, the receptor adopts a conformation that does not allow G protein binding to the receptor and for which agonists have a low affinity. In contrast, in the active state, the receptor conformation allows G protein binding. Agonist has a high affinity for R*.

This model also provides an explanation for the differences observed between agonists, inverse agonists and antagonists. Agonists lead to activation of the receptor signaling pathway because they bind preferentially to the active conformation of the receptor (R*). Binding of agonists to R* stabilizes this conformation by shifting the equilibrium in favor of the active state. Conversely, the inverse agonist binds preferentially to and stabilizes the inactive conformation of the receptor (R) shifting the equilibrium in favor of the inactive state. This shifted equilibrium explains why inverse

agonists reduce the receptor activity at a level below the receptor constitutive activity (i.e. the receptor activity in absence of ligand). Lastly, antagonists (neutral ligands) do not alter the receptor constitutive activity because they bind with the same affinity to R and R*. Therefore, in the presence of antagonist, no agonist or inverse agonist can bind to the receptor and the thermodynamic equilibrium is unaffected.

In reality, the extended ternary model is incomplete because the existence of only two states (R and R*) cannot accommodate partial agonists. Studies with adrenergic receptors and rhodopsin support the idea of the existence of multiple states (Vilardaga *et al.* 2003, Vilardaga *et al.* 2005, Swaminath *et al.* 2004, Swaminath *et al.* 2005, Shichida & Imai 1998, Kandori *et al.* 2001). In this multistate model, different agonists promote different receptor conformations. Full agonists bind to the more active state, while partial agonists preferentially bind to less active states.

### 1.3.3. Main GPCR-mediated pathways

Once the seven transmembrane-spanning receptor is activated (by agonist binding), the signaling pathways are determined by the nature of Gα subunit coupled to the receptor itself (**Fig. 3**). The activation of Gα subunits that belong to the $G\alpha_s$ class (composed of $G\alpha_s$ and $G\alpha_{olf}$) leads to activation of adenylyl cyclase (AC) while the activation of Gα subunits that belong to the $G\alpha_i$ class (composed of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_o$) inhibits this enzyme. AC is a twelve membrane-spanning enzyme catalyzing the conversion of adenosine triphosphate (ATP) into the second messenger cyclic adenosine monophosphate (cAMP). cAMP is an activator of the serine (Ser)/threonine (Thr) cAMP-dependent protein kinase (PKA). In addition, cAMP can also activate the exchange

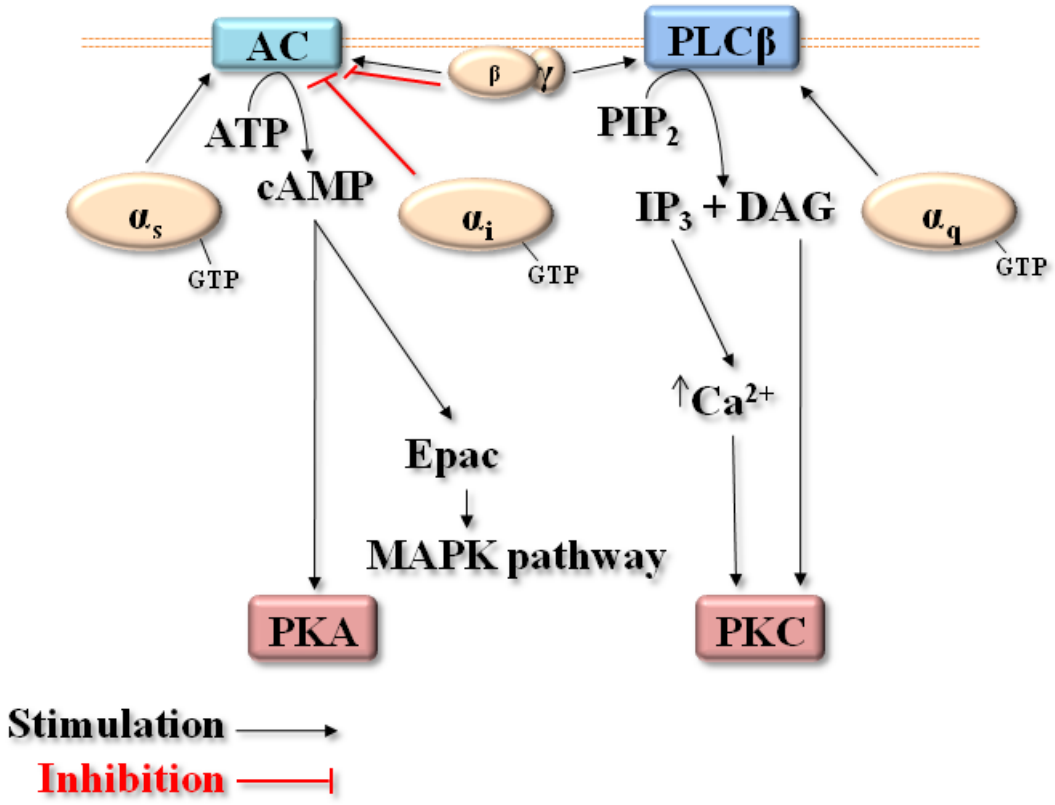


**Figure 3. Main GPCR-mediated pathways.**
Upon agonist exposure, $G\alpha_s$-coupled receptors activate adenylyl cyclase (AC) leading to an increase of intracellular cyclic adenosine monophosphate (cAMP). This triggers the activation of cAMP-dependent protein kinase (PKA) but also mitogen-activated protein kinase (MAPK) activation through stimulation of the exchange protein directly activated by cAMP (Epac). $G\alpha_i$-coupled receptors inhibit AC and the corresponding downstream pathways. GTP-bound Gαq subunit or free Gβγ dimers (from Gαq- or Gαi- coupled receptors) activate phospholipase Cβ (PLCβ). Free Gβγ dimers also stimulate or inhibit AC activity in an AC isoform-dependent manner. Stimulation of PLCβ activity leads to production of diacylglycerol (DAG) and inositol-1,4,5-triphosphate ($IP_3$) through phosphatidylinositol-4,5-biphosphate ($PIP_2$) hydrolysis. DAG activates protein kinase C (PKC) while the $IP_3$-mediated elevation of intracellular calcium concentration also activates some PKC isoforms.

protein directly activated by cAMP (Epac). Epac activation results in mitogen-activated protein kinases (MAPKs) stimulation. The MAPK pathway is common to another family of membrane receptors called tyrosine kinase receptors. MAPK activation induces several responses such as proliferation, apoptosis, survival, migration, transcription and metabolism (Dhanasekaran & Johnson 2007). In contrast to $G\alpha_s$ subunits, activation of Gα subunits that belong to the $G\alpha_q$ class (composed of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{15}$) and the resulting free βγ dimer both stimulate phospholipase Cβ (PLCβ) activity. This enzyme catalyzes the hydrolysis of phosphatidylinositol-4,5-biphosphate ($PIP_2$) present in cellular membrane into diacylglycerol (DAG) and inositol-1,4,5-triphosphate ($IP_3$). DAG is an activator of the Ser/Thr protein kinase C (PKC) while $IP_3$ stimulates calcium release from endoplasmic reticulum. However, this $IP_3$-mediated increase in cytosolic calcium also activates some PKC isoforms. The $G\alpha_i$-coupled receptors can also activate PLCβ through their free βγ dimer upon agonist exposure. In addition to PLCβ, free βγ dimer generated by $G\alpha_i$-coupled receptors can mediate a conditional increase of $G\alpha_s$-stimulated AC2, AC4, AC5, AC6, AC7 activity while inhibiting AC1, AC3 and AC8 (Sadana & Dessauer 2009). GPCR can also regulate ion channels through GTP-bound Gα subunit or βγ dimer. (Wickman & Clapham 1995, Albert & Robillard 2002).

## 2. Regulation of GPCR responsiveness

The GPCR responsiveness is tightly controlled by cells using several mechanisms. As previously stated, G protein activity can be regulated by AGS and RGS (Ross 1995, Ross & Wilkie 2000, Berman & Gilman 1998). The effector activity is dependent of the amount of free G protein subunits available but can also be regulated by

second messenger-dependent PKA and PKC. The effector products (second messengers) can also be enzymatically degraded by cAMP phosphodiesterases (PDE), phosphatidylinositol phosphatases and diacylglycerol kinases. These enzymes can be recruited to GPCRs after receptor activation promoting termination of the signal (Perry *et al.* 2002, Baillie *et al.* 2003). The GPCR itself can also be negatively regulated. It can occur through homologous and heterologous regulation, which requires a receptor phosphorylation step.

## 2.1. Homologous regulation

Homologous regulation leads to phosphorylation, desensitization and internalization or receptors after their activation (**Fig. 4**). Agonist-bound or activated receptors recruit the Ser/Thr GPCR kinases (GRKs). This targeting mechanism is different among GRKs isoforms (GRK 1 to 7) and is conferred by their C-terminal domain. GRKs phosphorylate CT or IL3 of agonist-occupied GPCRs (**step 1 on Fig. 4**). Once the GPCR is phosphorylated, the affinity of the receptor for β-arrestin is increased resulting in binding of β-arrestin to GPCRs (**step 2**). Because β-arrestin binds the receptor on regions involved in G protein coupling, binding of β-arrestin blocks the receptor-G protein interaction. At that step, the receptor is uncoupled. The most common arrestins are arrestin 2 (β-arrestin 1) and arrestin 3 (β-arrestin2) (Ferguson 2001, Luttrell & Lefkowitz 2002). Arrestin 1 and C-arrestin are only expressed in retina to regulate visual function. In addition to their function in GPCR uncoupling, non visual arrestins act as adapter proteins. The β-arrestin C-terminal domain contains binding motifs for clathrin and the adaptor protein-2 (AP-2) complex (Goodman *et al.* 1996, Laporte *et al.* 1999).

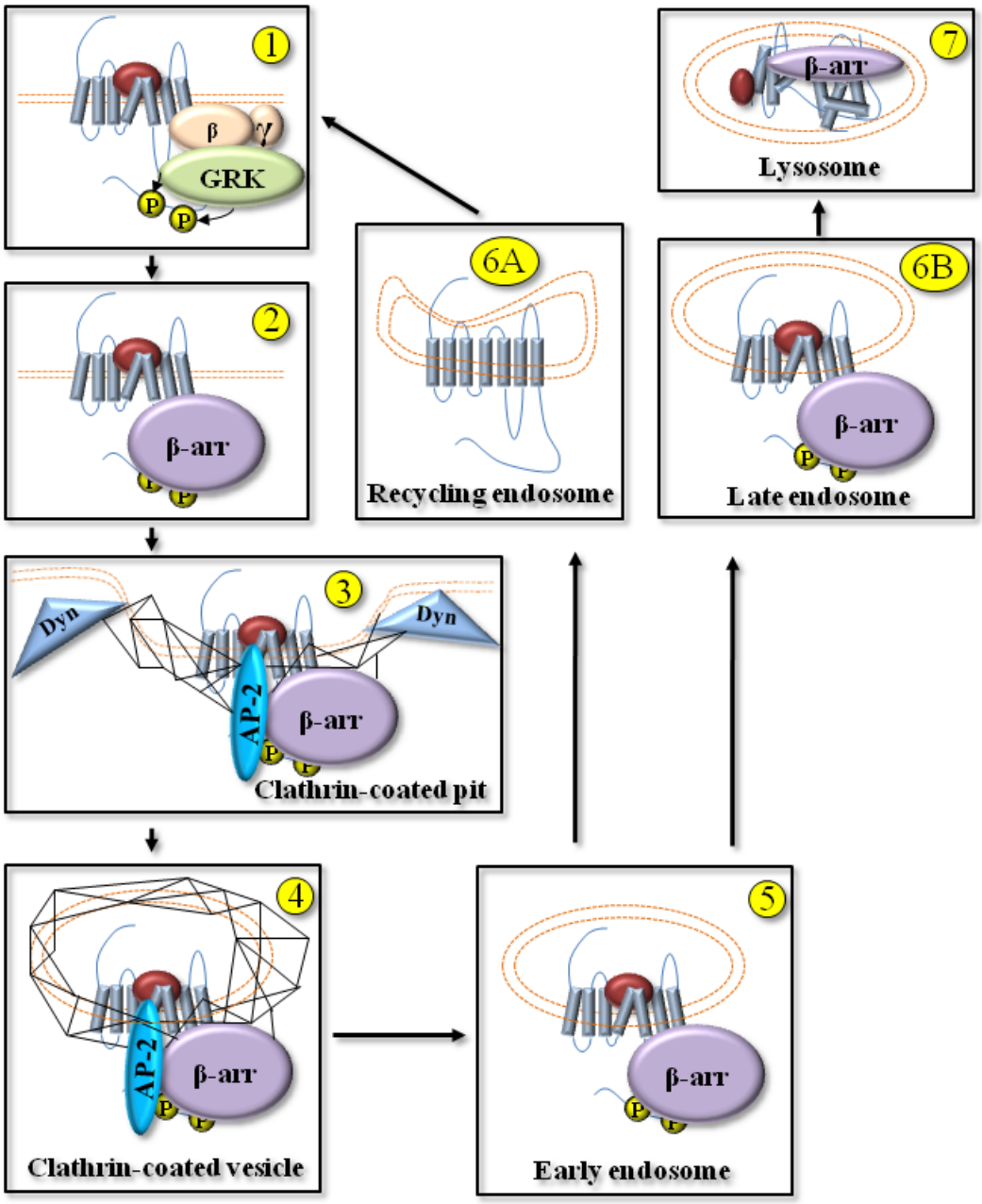


**Figure 4. Homologous regulation of GPCRs.**
Upon agonist binding to GPCRs, GRK is rapidly targeted to activated GPCR (**1**). GRK then phosphorylates the receptor. This phosphorylation triggers β-arrestin (β-arr) recruitment (**2**). β-arr binding to GPCR enables G protein coupling with the receptor and promotes recruitment of clathrin and adaptor protein-2 (AP-2) (**3**). Once the clathrin-coated pit is completed, dynamin (Dyn) pinches the clathrin-coated vesicle off the cell membrane (**4**). In the early endosomes (**5**), a sorting occurs where class A receptors bind transiently to β-arr and are targeted into recycling endosomes to be recycled to cell surface (**6A**), while class B receptors are tightly bound to β-arr in late endosomes (**6B**) and are mostly targeted toward lysosomes to be degraded (**7**).

This allows β-arrestin to target GPCR to clathrin-coated pits for endocytosis (also termed internalization or receptor sequestration) (**step 3**). Fission of the clathrin-coated pit from the plasma membrane by dynamin (**step 4**) allows the receptor complex to become part of early endosomes in the cytoplasm (**step 5**). At that point, the stability of GPCR-β-arrestin complex is the major determinant of sorting fate of the sequestered receptor. Class A receptors bind to β-arrestin 2 with higher affinity than β-arrestin 1 (Barak *et al.* 1997, Oakley *et al.* 2001, Oakley *et al.* 2000). Interaction between class A GPCRs and β-arrestin is transient and the GPCR-β-arrestin complex dissociates upon receptor internalization (**step 6A**). β-arrestin is recycled to cytoplasmic membrane and the receptor is dephosphorylated, the ligand dissociates from GPCR and the receptor returns to the cell membrane through recycling endosomes. In contrast, class B receptors bind β-arrestin 1 and β-arrestin 2 with equal affinity. The interaction between class B GPCRs and β-arrestin is strong and long-lasting. The GPCR-β-arrestin complex is targeted to late endosomes (**step 6B**). Phosphorylation of β-arrestin and ubiquitination of both β-arrestin and receptor then occurs. The receptor recycles slowly and tends to be degraded in lysosomes (**step 7**). In addition, many other proteins like Rab GTPases are involved in the transport, budding, docking and vesicle fusion (Dale *et al.* 2004).

## 2.2. Heterologous regulation

In contrast to homologous regulation where receptor desensitization occurs on the ligand-bound receptor, agonist occupancy of the target receptor is not required for heterologous desensitization. An activated receptor, through stimulation of second messenger-dependent kinases (PKA or PKC) can modulate the activity of another

receptor. In contrast to GRKs, PKA or PKC-mediated phosphorylation of GPCR directly impairs G protein binding to the receptor (Pitcher *et al.* 1992a). Second messenger-dependent kinase-mediated phosphorylation produces changes in GPCR charge distribution leading to alterations in the conformation of the phosphorylated region. This change in conformation, generally targeted to key regions on the GPCR where the G protein binds, is responsible for the decreased ability of the receptor to couple to G proteins. In the homologous regulation mechanism, the reduction in G protein-GPCR coupling is mediated by β-arrestin recruitment and not by GRK-mediated phosphorylation of GPCR by itself. In general, GRKs and second messengers-dependent kinases phosphorylate GPCRs on different sites (Lefkowitz *et al.* 1990). Classically, homologous and heterologous desensitizations are two independent processes. However, many studies show that PKA and PKC do not only phosphorylate GPCRs. In fact, these Ser/Thr kinases can also phosphorylate GRKs and so regulate homologous regulation (Chuang *et al.* 1996). This regulation can be positive or negative according to which GRK isoform is regulated. Additionally, numerous studies show that PKA and PKC can also regulate GPCR activity through effector phosphorylation. It was shown that PKA inhibits the activity of AC5 and AC6. Additionally, it was reported that phosphorylation of AC1, AC2, AC3, AC5 and AC7 by PKCα stimulates activity of these AC isoforms but inhibits AC4 activity. Moreover, PKCδ and PKCε were reported to inhibit AC6 activity and PKCζ to stimulate AC5 (Sadana & Dessauer 2009).

## 3. Protein kinase C

PKC, like PKA and PKB (also termed Akt), are members of the large AGC protein kinase family. AGC proteins are characterized by the presence of a conserved C-terminal Ser/Thr kinase domain having a binding site for ATP (the phosphate donor) and for their substrates. AGC proteins also have N-terminal regulatory domain that maintains the kinase inactive in absence of the appropriate ligand. PKC is a transducer of several events promoting lipid hydrolysis in the cell. Moreover, any signaling pathway leading to an increase of intracellular calcium is susceptible to activate specific PKCs isoforms.

### 3.1. PKC classification

There are 10 PKC isoforms. These isoforms are divided into 3 groups (conventional, novel and atypical) according to their cofactor/activator requirement (**Fig. 5**). Conventional PKC (cPKC) isoforms (composed of α, γ and the alternatively spliced βI and βII) need the presence of anionic lipids (such as phosphatidylserine), phosphatidylinositol-4,5-biphosphate ($PIP_2$), DAG (or phorbol esters) and calcium ($Ca^{2+}$) to be activated, while novel PKC (nPKC) (composed of δ, ε, η and θ) need anionic lipids and DAG/phorbol esters only. Atypical PKC (aPKC) (composed of ζ and τ) only need phosphatidylserine.

PKC primary structure also discriminates PKC groups. The regulatory domain of all PKC isoforms have an autoinhibitory sequence (termed as pseudosubstrate) and one or two membrane targeting modules (called C1 and C2). However, cPKC isoforms are characterized by the presence of a C2 domain binding anionic lipids in a $Ca^{2+}$-dependent manner. In contrast to cPKC, nPKC and aPKC do not bind calcium. The presence of

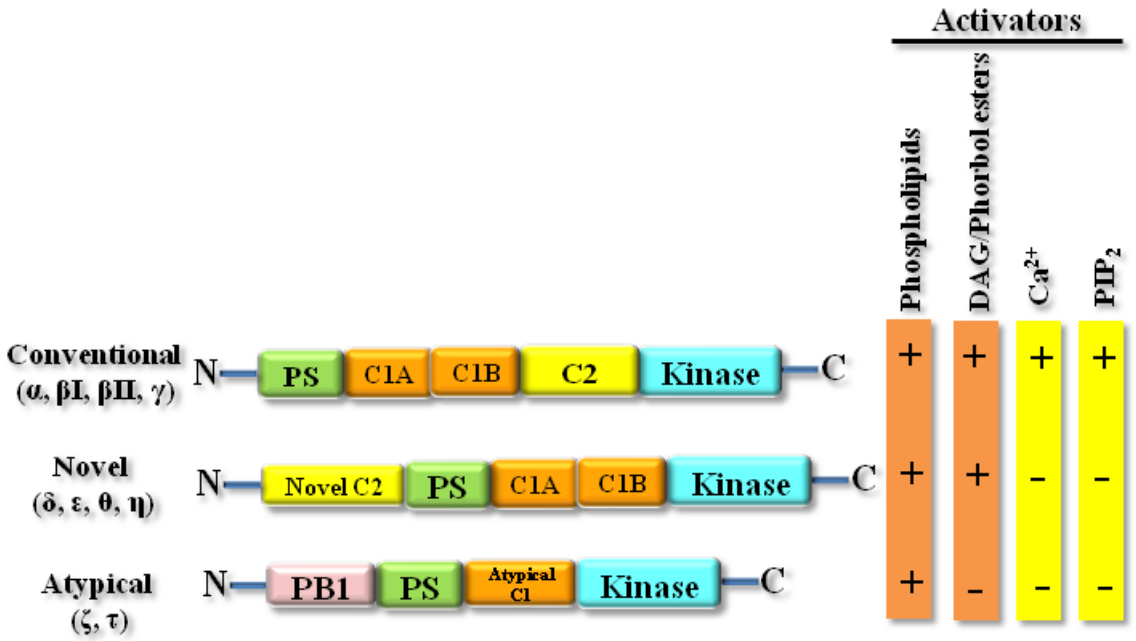


**Figure 5. Classification of PKC isoforms.**
PKC isoforms are classified according to their structure and their activator requirements. All PKC isoforms have a catalytic domain (kinase) and an autoinhibitory pseudosubstrate (PS) site in their regulatory region. Conventional (composed of α, βI, βII and γ isoforms) and novel (composed of δ, ε, θ and η isoforms) PKCs both have a cystein-rich C1 domain in tandem (C1A and C1B). This membrane-targeting domain binds diacylglycerol (DAG), phorbol esters and anionic phospholipids such as phosphatidylserine. Atypical PKCs (composed of ζ and τ isoforms) have only one copy of the cystein-rich region, called atypical C1 domain, binding phosphatidylinositol-triphosphate and ceramide, but not DAG/phorbolesters. Atypical PKCs also have a Phox and Bem 1 (PB1) domain promoting protein-protein interaction with other proteins having the same domain. The C2 domain of conventional PKCs has a calcium-dependent phosphatidylserine binding site while the novel C2 domain of novel PKC isoforms does not bind calcium. Atypical PKCs do not have C2 or novel C2 domains.

several negatively charged amino acids (aspartic acid residues) in the C2 domain of cPKCs allows $Ca^{2+}$ binding (Sutton & Sprang 1998). Binding of $Ca^{2+}$ on C2 domain of cPKC increases the kinase affinity for anionic membranes (containing phosphatidylserine). The C2 domain of nPKCs (called novel C2 domain) does not have these aspartic acid residues to bind $Ca^{2+}$. However, the affinity of nPKC C1 domain for DAG is higher than the one from cPKC C1 domain (Giorgione *et al.* 2006). This increased affinity compensate for the lack of involvement of novel C2 domain in membrane recruitment, while aPKC simply does not have a C2 domain (Sossin & Schwartz 1993). When PKC is in its active conformation, the C2 domain is also exposed and can interact with scaffolding or anchoring proteins called receptors for activated c-kinase (RACKs). Hence, C2 domain plays a critical role in PKC subcellular location and translocation (Kheifets & Mochly-Rosen 2007). The other membrane targeting module, the cysteine-rich C1 domain, is present as a tandem repeat (C1A and C1B) for cPKC and nPKC but as a single copy for aPKC (Hurley *et al.* 1997). The tandem C1A/B domain binds to DAG and phorbol esters while the aPKC C1 domain (also called atypical C1 domain) does not. However, the atypical C1 domain can bind phosphatidylinositol-triphosphate ($PIP_3$) or ceramide. For cPKC and nPKC isoforms, binding of phorbol esters or DAG on C1A/B domain increases PKC affinity for anionic lipids by creating a hydrophobic surface favorable for membrane interaction and PKC insertion into the lipid bilayer (Zhang *et al.* 1995). Alternatively, phosphatidylserine breaks the electrostatic interaction between C1 and C2 domains resulting in a "free" C1 domain that it can penetrate the lipid bilayer and bind DAG easily (Stahelin *et al.* 2005). The uniqueness of aPKC is the presence of a protein-protein interaction Phox and Bem 1 (PB1) domain.

This domain mediates interactions with other proteins in which a PB1 domain is present, like p62, partitioning defective 6 (PAR-6) and mitogen activated protein kinase kinase 5 (MEK5) (Moscat *et al.* 2006a, Moscat *et al.* 2006b, Moscat & Diaz-Meco 2000). Thus, aPKC activity in mainly controlled by protein-protein interactions.

## 3.2. PKC maturation

The catalytic competence and stability of PKC isozymes requires a maturation step, i.e. phosphorylation of three sites on the kinase domain: the activation loop, the turn motif and the hydrophobic motif (Keranen *et al.* 1995). These phosphorylation sites are conserved among AGC kinases family members. Only phosphorylated PKCs can be signal transducers. However, prior to phosphorylation, a conserved PXXP motif on PKC kinase domain must bind the heat chock protein-90 (HSP90). Following HSP90 binding, the first phosphorylation occurs on the PKC activation loop and is mediated by phosphoinositide-dependent kinase (PDK-1), a close cousin of PKB (Le Good *et al.* 1998). Phosphorylation of the activation loop correctly positions the kinase domain for catalysis. Substitution of phosphorylated threonine by valine (a neutral and non phosphorylable residue) leads to an inactive enzyme. In contrast, phosphomimetic mutation of this threonine into glutamic acid (a negatively charged amino acid) results in a fully catalytically active PKC (Orr & Newton 1994). This indicates that the correct positioning of the kinase domain is dependent of the presence of a negative charge on the activation loop (added by threonine phosphorylation by PDK-1 or by substitution of threonine by glutamic acid). Interestingly, the PKCδ isoform has the particularity to have a glutamic acid close to this phosphorylable threonine (Stempka *et al.* 1999). This

accounts for this PKC isoform activity (and not other PKC isoforms) when expressed in bacteria (which do not express PDK-1) (Stempka *et al.* 1997). Following PDK-1-mediated phosphorylation of the activation loop, the mammalian target of rapamycin complex 2 (mTORC2) mediates phosphorylation of another important region in the kinase domain: the turn motif (Ikenoue *et al.* 2008). This step of maturation locks PKC in a phosphatase and oxidation-resistant, thermally stable and catalytically competent conformation (Bornancin & Parker 1996). Interestingly, the turn motif can also serve as a protein-protein interaction docking site (Yaffe *et al.* 1997). mTORC2 is also responsible of phosphorylation of another region in the kinase domain: the hydrophobic motif. Lack of phosphorylation of this region increases phosphatase sensitivity and increases thermal stability of the enzyme (Edwards & Newton 1997, Bornancin & Parker 1997, Gysin & Imber 1997).

## 3.3. PKC activation

Once PKC is properly phosphorylated, this enzyme is mature and responsive (ready to phosphorylate a substrate). However, PKCs need the presence of an activator, i.e. lipid hydrolysis and/or intracellular calcium to respond. Until this event occurs, the kinase domain is kept silent by binding its own pseudosubstrate site (**Fig. 6A**). The pseudosubstrate sequence resembles that of a PKC substrate phosphorylation site except an alanine residue occupies the phosphorylable residue (Thr or Ser) (House & Kemp 1987). The pseudosubstrate, by occupying the substrate-binding pocket of PKC keeps the enzyme inactive (but responsive). An event leading to generation of DAG and/or increase of intracellular calcium (like activation of PLCβ by a $G\alpha_q$-coupled receptor) targets PKC

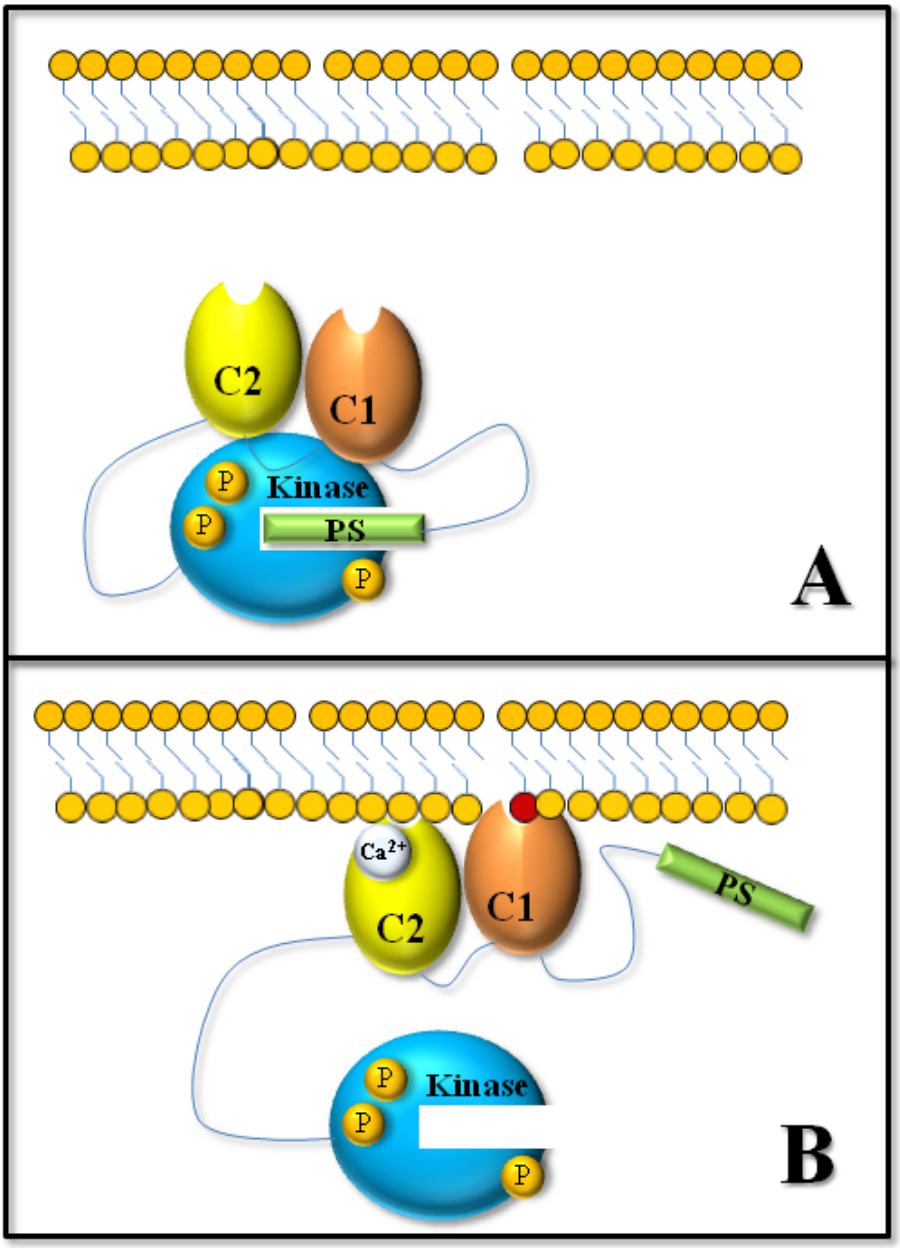


**Figure 6. PKC activation.**
After the maturation step, kinase domain is phosphorylated and its substrate-binding pocket is occupied by its pseudosubstrate (PS) (**A**). When a cellular event generates lipid hydrolysis, the C1 domain is recruited to the membrane to bind firmly to DAG (in red) (**B**). In addition, for conventional PKC isoforms, binding of $Ca^{2+}$ to C2 domain targets PKC to the plasma membrane, where C2 domain then binds $PIP_2$. This step is required for cPKC C1 domain binding to DAG. Lipid binding to the regulatory C1 and C2 domains provides enough energy to release the pseudosubstrate from the substrate-binding pocket. This allows PKC to phosphorylate its substrates.

to the membrane (**Fig. 6B**). In fact, binding of $Ca^{2+}$ to the C2 domain of cPKC, targets the kinase to plasma membrane, where it bind $PIP_2$. Then, C1 PKC domain binds its membrane-embedded DAG. Binding of C1 to phosphatidylserine increases interaction strength between DAG and C1 domain. Lipid binding to regulatory domains C1 and C2 provide enough energy to release the PKC pseudosubstrate from the kinase substrate-binding pocket (Johnson *et al.* 2000). Once the kinase domain is free from the pseudosubstrate domain, PKC can phosphorylate its substrates.

Cleavage of the region located just between the regulatory domains and the catalytic domain (called the hinge region) leads to the release of the catalytic domain fragment, which is freed from the autoinhibitory action of pseudosubtrate site (Frutos *et al.* 1999). This cleavage leads to constitutively activated fragments. It was observed that caspase-mediated cleavage of some PKC isoforms naturally occurs in response to an apoptotic stimulus (Frutos et al. 1999). Interstingly, in the brain, the atypical PKCζ is transcripted as a catalytic domain without its regulatory domain and is called PKMζ (Hernandez *et al.* 2003). This persistently active kinase has an important role to play in maintaining long-term memory (Sacktor 2011).

In addition to lipids, protein-protein interactions between PKC and other proteins also contribute to the enzyme location. Several proteins are known to interact with the PKC regulatory domains, pseudosubstrate site and regions where the sequence is variable among PKC isoforms (Ron & Kazanietz 1999).

## 3.4. PKC inhibition

PKC-mediated effects can be inhibited by disruption of any step of the PKC activation process. PKC inhibitors are classified according to which step they interfere. The main PKC inhibitor classes and their mechanism of action are explained here.

The first target to block PKC activity is the catalytic function. As previously described, the kinase domain of PKC contains an ATP binding site. This site is located in a narrow hydrophobic pocket. Inhibitors of PKC catalytic function compete with ATP for binding to that site. Without ATP binding, the kinase domain cannot phosphorylate substrates. However, PKC inhibitors belonging to this class generally lack specificity since the hydrophobic pocket is conserved throughout PKC isoforms. Staurosporin and midostaurin are two potent but non-selective PKC inhibitors from this class (Takahashi *et al.* 1987, Meyer *et al.* 1989). Chemical modification of these inhibitors has lead to increased specificity among PKC isoforms. Ruboxistaurin and enzastaurin are two examples of inhibitors exhibiting increased specificity (in this case for the ATP-binding site of PKCβ) (Jirousek *et al.* 1996, Green *et al.* 2006). High throughput screening was also used to identify arylamino-3-pyrimidine carbonitriles, 2-alkenylthieno[2,3-b]pyridines and diamino-pyrimidines as selective PKCθ inhibitors (Cole *et al.* 2008, Nathan Tumey *et al.* 2008, Cywin *et al.* 2007).

A second target is the substrate-binding site. PKC inhibitors in this class are generally oligopeptides. Their sequence is similar to the natural substrate but with a non-phosphorylable amino acid at the target phosphorylation site. These substrates compete with the real PKC substrates in a similar manner to the pseudosubstrate site on PKC. A myristoyl group is added to the oligopeptidic inhibitor to promote cell penetration.

Chelerythrine is a plant-derived small molecule inhibitor (non peptidic) that also competes for the protein substrate binding pocket (Herbert *et al.* 1990). Another interesting PKC inhibitor is sphingosine. However, instead of binding on the substrate-binding site on PKC, sphingosine blocks the association of substrates with membranes (Bazzi & Nelsestuen 1987). Sphingosine is positively charged and neutralizes the negatively charged lipids of membranes and consequently decreases the affinity between membranes and substrates. Generally, substrates located close to membranes, where PKC is activated, are more easily phosphorylated than those in solution. Consequently, sphingosine interferes with phosphorylation of substrates by PKC.

Another way to inhibit PKC activity is to interfere with the C1 regulatory domain. As an example, bryostatin 1 competes with DAG/phorbol ester binding on PKC C1 domain (Smith *et al.* 1985, Szallasi *et al.* 1994a, Szallasi *et al.* 1994b). This inhibitor activates cPKC and nPKC and mediates differential downregulation of these isoforms. This results in suppression of selective responses. Calphostin C is another inhibitor from this class. However, in contrast to bryostatin 1, calphostin C inactivates PKC through light-dependent production of free radical (Kobayashi *et al.* 1989, Bruns *et al.* 1991). However, it is reported that this inhibitor also binds to and inhibit other proteins having a C1 domain like uncoordinated mutant #13 (Unc-13) from *C. elegans* (Kazanietz *et al.* 1995).

Interfering with the C2 regulatory domain is another mechanism used by PKC inhibitors. These inhibitors have a similar sequence to RACKs and consequently interfere with binding of PKC C2 domain to RACKs. Thus, these inhibitors prevent translocation

of activated PKC. Dequalinium is an example of this class of inhibitor and is UV light dependent (Rotenberg *et al.* 1998, Rotenberg & Sun 1998).

Atypical PKCs have a PB1 domain involved in protein-protein interactions. This interaction can be disrupted by aurothioglucose or aurothiomalate (Stallings-Mann *et al.* 2006, Regala *et al.* 2008).

## 3.5. Non PKC phorbol ester/DAG receptors

PKC is not the only kinase to respond to phorbol esters or DAG. Originally considered as a PKC member, PKCμ (or called PKD in mice) is also a Ser/Thr kinase activated by DAG/phorbol esters (Valverde *et al.* 1994). In contrast to PKC, PKCμ has no pseudosubstrate domain, has a pleckstrin homology (PH) domain and its catalytic domain does not phosphorylate PKC substrates. In fact, the PKCμ kinase domain has more homology with calcium-calmodulin-dependent kinase II. Moreover, PKCμ is localized at the Golgi compartment (Prestle *et al.* 1996). It is activated by phosphorylation and is a PKC substrate (Zugaza *et al.* 1996). This kinase acts as a scaffold protein for certain enzymes promoting phosphoinositide synthesis (Nishikawa *et al.* 1998).

Many non-kinase proteins also bind to phorbol esters/DAG and mediate non-PKC phorbol ester responses. Mammalian α- and β-chimaerins, Ras guanyl-releasing protein (RasGRP) and Unc-13 possess a single copy of the cysteine-rich domain (named C1 in PKC). Deletion of this cysteine-rich domain in α1-chimaerin has been shown to completely abolish phorbol ester binding (Ahmed *et al.* 1990). It has been shown that similar to PKC, β2-chimaerin translocates to the membrane upon phorbol ester treatment

(Caloca *et al.* 1997, Caloca *et al.* 1999). Chimaerins have a GTPase-activating protein (GAP) domain and catalyze hydrolysis of GTP from Rac (a member of the small GTP-binding Rho protein family) and consequently inhibits Rac activity (Hall *et al.* 1993, Ahmed *et al.* 1993, Diekmann *et al.* 1991). Rac mediates several responses like activation of c-Jun aminoterminal kinase (JNK), cytoskeleton organization, cell cycle and cell growth regulation, and control of the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxydase in neutrophils (Bokoch 1994, Coso *et al.* 1995, Khosravi-Far *et al.* 1995, Mackay & Hall 1998). Additionally, presence of an SH2 domain in α2- and β2-chimaerins suggests these proteins can also interact with phosphotyrosine proteins (Leung *et al.* 1994, Hall et al. 1993).

Unc-13 from *C. elegans* is another non-kinase phorbol ester/DAG receptor. Unc-13 protein possesses a cysteine-rich domain but also two copies of C2-related domains. This protein binds phorbol esters/DAG in presence of phospholipids only (Kazanietz et al. 1995). Mutation of the *Unc-13* gene in *C. elegans* has been associated with uncoordinated movements, impairment of cholinergic transmission and abnormal neuronal connections. The mammalian homologues, Munc13-1, Munc13-2 and Munc13-3, have a very similar structure to Unc-13 (Brose *et al.* 1995). Munc13 proteins are highly expressed in brain, more specifically in synaptosomes, and have an important role in neurotransmitter release. Indeed, it has been shown that Munc13 interacts with Doc2, syntaxin, synaptobrevin and synaptotagmin (Betz *et al.* 1997, Orita *et al.* 1997, Silinsky & Searl 2003). These proteins are part of the exocytotic machinery involved in neurotransmission process.

Another non-kinase phorbol ester/DAG receptor is RasGRP. This protein also has a cysteine-rich domain and is abundant in hippocampus, but also expressed in thymus, spleen and bone marrow (Ebinu *et al.* 1998). In addition, RasGRP binds calcium. RasGRP also has a CDC25 box in its catalytic region and a Ras exchange motif. These motifs contribute to the RasGRP-mediated dissociation of the Ras-GDP complex, which favors GTP association with Ras, leading to its activation. Indeed, it was shown that phorbol ester treatment triggers recruitment of RasGRP to the plasma membrane, leading to activation of the MAPK pathway (downstream pathway of Ras activation) in fibroblast cells (Tognon *et al.* 1998). Thus, RasGRP represents a link between PLCβ activation and the MAPK pathway.

## 3.6. PKC knockout mice and human diseases

PKC knockout mice represent a good tool to identify the physiological role of individual PKC isoforms. Knockout mice have been made for all isoforms, but this section will focus on DAG/phorbol esters-dependent PKC isoforms (cPKCs and nPKCs). Genetic analysis of patients having disease can also be informative on the role of PKC mutation on certain pathological conditions.

### 3.6.1. Conventional PKC isoforms

PKCα has been reported to be upregulated in ovarian, breast and gastric cancers (Lahn *et al.* 2004, Lin *et al.* 2008). In contrast, PKCα has been reported to have a protective effect on endocrine tumors. Hence, in mouse models of colon carcinoma, mice lacking PKCα develop more aggressive tumors and die earlier than their PKCα-proficient

littermates suggesting PKCα acts as a tumor suppressor (Oster & Leitges 2006). A role of this PKC isoform in the regulation of EGFR has been proposed to explain its protective effect on colorectal cancer. Additionally, a point mutation of PKCα has been linked to invasive human pituitary and thyroid tumors (Alvaro *et al.* 1993, Prevostel *et al.* 1995). Impairment in membrane cellular binding has been associated with this mutant form of PKC (Zhu *et al.* 2005). This loss-of-function mutation points out an important antitumorigenic role in the progression of these cancers. Interestingly, outside of the cancer field, using magnetic resonance, a polymorphic variant of PKCα has been linked to a reduction of hippocampus and parahippocampal gyrus activities during tasks requiring use of episodic memory (de Quervain & Papassotiropoulos 2006). This study suggests that PKCα could be involved in memory performance. Additionally, knockout of PKCα has been associated with an increased insulin signaling (Leitges *et al.* 2002). This suggests that PKCα serves as a physiological feedback inhibitor of insulin signaling. PKCβI and βII knockout mice have impaired immune response, pointing out a significant positive role of these PKC isoforms in immune system function (Leitges *et al.* 1996). In contrast, it is reported that PKCβII knockout mice show a significantly decreased infarct size and enhanced recovery after myocardial ischemia, suggesting this PKC isoform has a negative impact on cellular stress responses in the heart (Kong *et al.* 2008). A carcinogenic role in colonic epithelial cells is also associated with increased expression of PKCβII. Indeed, transgenic mice overexpressing this isoform of PKC in intestinal epithelium exhibit hyperproliferation of colonic epithelium and an increased susceptibility to develop aberrant carcinogenic lesions (Murray *et al.* 1999). Clinical studies also report that specific blockade of PKCβ with ruboxistaurin treatment

normalizes endothelial dysfunction, renal filtration rate and prevents loss of visual acuity associated with diabetic patients (Das Evcimen & King 2007).

Incontrast to the other cPKC isoforms that are ubiquitously expressed, PKCγ is mainly present in brain and spinal cord (Battaini 2001). Hence, PKCγ knockout mice have several defects in their central nervous system. These mice have impaired hippocampal LTP and mild learning deficits, suggesting this PKC isoform is a key regulatory component of memory (Abeliovich *et al.* 1993a, Abeliovich *et al.* 1993b). Additionally, mice lacking PKCγ display impaired motor coordination due to an impaired cerebellar long-term depression (Chen *et al.* 1995). Moreover, knockout of this PKC isoform results in decreased anxiety (Bowers *et al.* 2000). This links PKCγ to the neurobiology of anxiety. It is also reported that mice lacking PKCγ display normal response to acute pain but completely fail to develop neuropathic pain, the persistent and exaggerated pain that can be produced by nonpainful stimuli (Malmberg *et al.* 1997). Interestingly, the neurodegenerative disorder spinocerebellar ataxia type 14 is caused by mutations into the C1B regulatory domain of the neuronal-specific PKCγ. This mutation reduces kinase activity of this PKC isoform and tends to produce an enzyme forming cytoplasmic aggregates causing cytotoxicity (Verbeek *et al.* 2008, Seki *et al.* 2005).

### 3.6.2. Novel PKC isoforms

PKCδ is the only nPKC isoform to be expressed in almost all tissues (Battaini 2001). Knockout of PKCδ results in mice having impaired smooth muscle cells homeostasis and abrogation of self-reactive B cells involved in immune response (Leitges *et al.* 2001, Mecklenbrauker *et al.* 2002). Furthermore, these mice have an impaired

ischemic preconditioning leading to increased cardiac damages during subsequent ischemia (Mayr *et al.* 2004). PKCδ is also known to be a critical pro-apoptotic signal in many cell types (Basu 2003, Brodie & Blumberg 2003). Indeed, caspase cleavage of this PKC isoform leads to constitutive activation of the kinase. Nuclear accumulation of this constitutive form of PKCδ has been reported to be essential for apoptosis (Reyland 2007). Indeed, it was shown that PKCδ is cleaved during dopaminergic cells apoptosis (Kitazawa *et al.* 2003). Treatment of dopaminergic cells with the pesticide methylcyclopentanedienyl manganese tricarbonyl causing Parkinson's disease induces apoptosis via caspase 3-dependent cleavage of PKCδ (Anantharam *et al.* 2002).

The ε isoform of PKC is mainly expressed in hematopoietic tissues but also in brain regions such as neocortex, hippocampus, cerebellar cortex and nucleus accumbens and caudate-putamen (Battaini 2001, Tanaka & Nishizuka 1994, Saito *et al.* 1993). PKCε knockout mice have been reported to have reduced hyperalgesia (Khasar *et al.* 1999). In addition, this hyperalgesia is reduced in normal rats when PKCε activity is inhibited using a selective peptidic inhibitor. This peptide inhibits PKCε translocation by blocking the interaction between PKCε and its specific PKC anchoring protein called beta cotamer protein (β'COP) (Csukai *et al.* 1997). These results strongly suggest that PKCε isoform is involved in nociception due to hyperalgesia. Additionally, it is reported that these PKCε null mice have abnormal macrophages with severely attenuated response to lipopolysaccharide and interferon γ (Castrillo *et al.* 2001). Intravenous administration of Gram-negative or Gram-positive bacteria to PKCε knockout mice results in a significant shorter period of survival, compared to wild type mice. These results provide evidence that PKCε is involved in host defense against bacterial infection. Similarly to PKCδ

knockout mice, PKCε knockout mice also have impaired ischemic preconditioning effect on the heart during subsequent ischemia (Saurin *et al.* 2002). Additionally, these mice show reduced preference to ethanol and the hypnotic response associated to ethanol intake is also reduced (Newton & Messing 2006). Interestingly, studies using brain from Alzheimer's disease (AD) patients have shown that both expression level and activity of PKCε are decreased in the temporal cortex (Matsushima *et al.* 1996).

PKCη-deficient mice exhibit increased risks of developing tumors and wound healing is delayed and impaired in structure, suggesting a role of this PKC isoform in maintenance of epithelial tissues (Chida *et al.* 2003). Indeed, this PKC isoform is only expressed in heart, lungs and skin (Battaini 2001).

Finally, PKCθ knockout mice have impaired T-cell antigen receptor-mediated T-cell activation (Sun *et al.* 2000). Indeed, mature T lymphocytes from these mutant mice fail to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor regulating genes involved in immune response, upon T-cell antigen receptor activation. Interestingly, as opposed to wild type mice, insulin signaling and glucose transport in skeletal muscle from PKCθ-deficient mice are insensitive to lipid infusion treatment, suggesting that this PKC isoform may be involved in development of insulin resistance associated with type 2 diabetes (Kim *et al.* 2004a). It is also reported that PKCθ is expressed in gastrointestinal stromal tumors but is not detectable in other mesenchymal or epithelial tumors (Blay *et al.* 2004). Consequently, PKCθ represents a specific marker for the diagnostic of this type of cancer (Motegi *et al.* 2005). Interestingly, a recent study indicates that PKCθ deficient mice undergo a reduced

phosphorylation of their nicotinic acetylcholine receptors resulting into a delayed maturation of neuromuscular synapses (Lanuza *et al.* 2010).

## 4. Dopaminergic system

Dopamine is the most abundant catecholamine neurotransmitter in the mammalian brain. Cognition, emotions, food intake, locomotor activity, positive reinforcement and endocrine regulation are all controlled by this neurotransmitter. Dopamine also has peripheral roles in renal function, vascular tone, cardiovascular functions, hormone secretion and gastrointestinal motility (Missale *et al.* 1998). Dopaminergic neurotransmission dysfunctions are involved in several pathological conditions such as Parkinson's disease, schizophrenia, depression, drug and alcohol addiction, bipolar disease, Huntington's disease, Tourette's syndrome and attention deficit/hyperactivity disorder (Lebel *et al.* 2007, Wong *et al.* 2000, Le Foll *et al.* 2009).

### 4.1. Dopaminergic synapse

To mediate effects on the postsynaptic neuron, the presynaptic neuron has to synthesize and release dopamine (**Fig. 7**). Biosynthesis of dopamine occurs in terminals of dopaminergic neurons and can be divided in two steps (Callier *et al.* 2003). The first step is rate limiting and is the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase. The second step is the L-DOPA decarboxylation into dopamine by DOPA-decarboxylase. Once dopamine is synthesized, it is pumped into storage vesicles by the vesicular monoamine transporter. Dopamine present in a free state in the presynaptic terminal can be easily degraded by the enzyme monoamine oxidase.

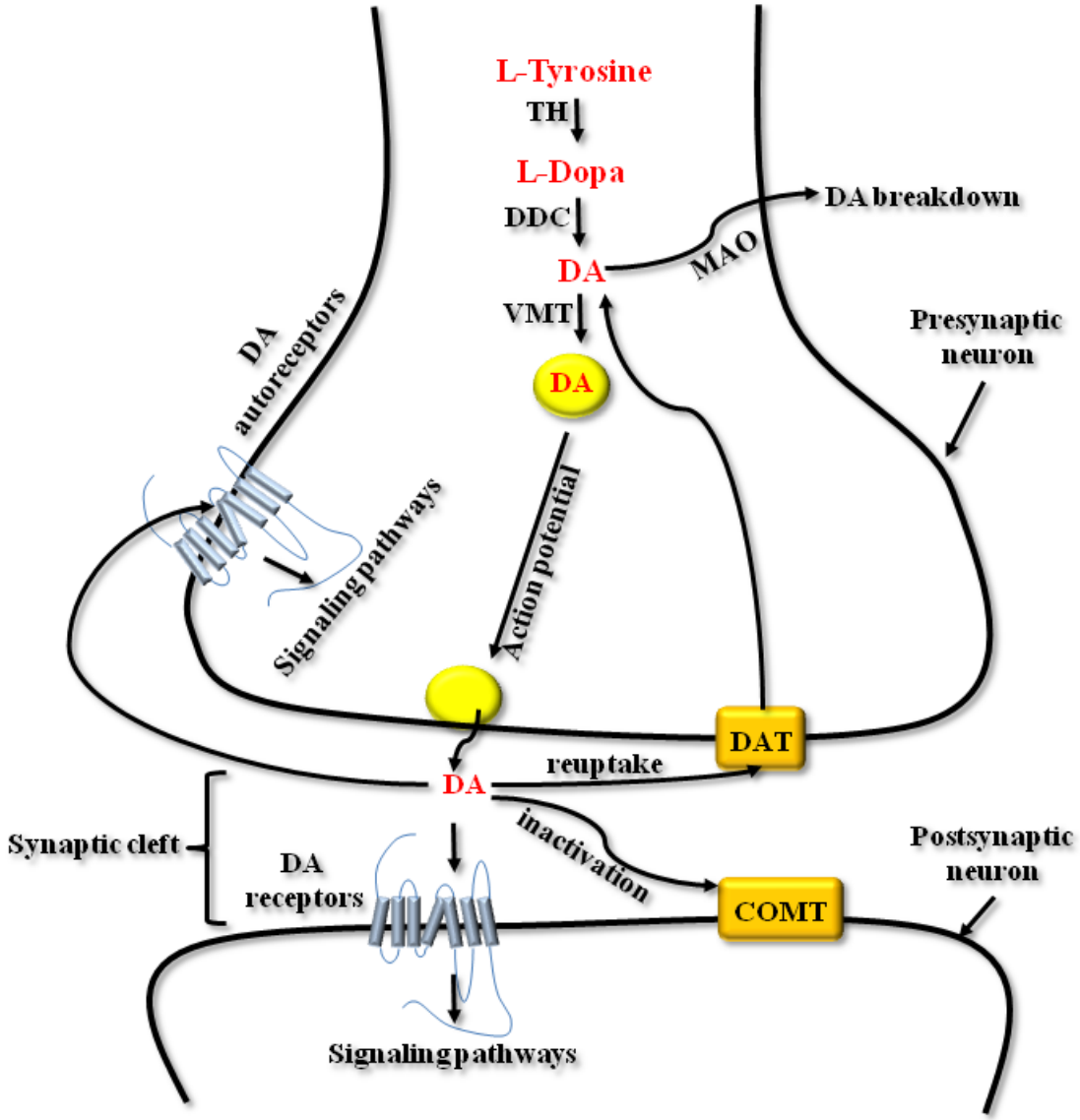


**Figure 7. Dopaminergic synapse.**
Dopamine (DA) is synthesized by the presynaptic neuron. L-tyrosine is converted into Levo-Dihydroxyphenylalanine (L-Dopa) by tyrosine hydroxylase (TH) and then converted into dopamine by dopamine decarboxylase (DDC). Free dopamine is stored into vesicles by vesicular monoamine transporter (VMT) while the free dopamine can be degraded by monoamine oxidase (MAO). Upon an action potential, dopamine is released from vesicles to be in the synaptic cleft where it can bind to postsynaptic DA receptors or presynaptic autoreceptors generating activation of signaling pathways. Excess DA can be inactivated by catechol-O-methyltransferase (COMT) on postsynaptic cell or can be taken up by dopamine transporter (DAT) located on presynaptic cell.

An action potential leading to an increase of calcium concentration in presynaptic terminals induces fusion of dopamine vesicles with the presynaptic membrane. This results in dopamine release into the synaptic cleft. Once in synaptic cleft, dopamine can bind to dopamine receptors located on post synaptic neuron, but also on presynaptic autoreceptors. Dopamine binding to receptors leads to activation of signaling pathways. The nature of these pathways depends on which dopaminergic receptor is activated but also on which neuron these receptors are expressed. Dopaminergic receptors signaling can be blocked by antipsychotic drugs. These drugs are antagonists or inverse agonists. Excess of dopamine in the synaptic cleft can be inactivated by the enzyme catechol-O-methyltransferase located on post synaptic neuron. Furthermore, dopamine can also be taken up into presynaptic terminal by dopamine transporter. Amphetamine and cocaine are potent inhibitors of dopamine transporter function. Use of these drugs lead to an abnormal increase of dopamine concentration into synaptic cleft responsible to psychosis associated with this psychostimulant abuse. Moreover, amphetamine stimulates dopamine release by inducing reverse transport of dopamine from the presynaptic neuron to the synaptic cleft (Kahlig *et al.* 2005).

## 4.2. Dopaminergic neuronal pathways

Dopaminergic neurons are located in specific areas in the brain. They are organized as four main dopaminergic neuronal tracts (Iversen & Iversen 2007, Kienast & Heinz 2006) (**Fig. 8**). The first one is the nigrostriatal pathway. Somas of these dopaminergic neurons are located in the substantia nigra. Dopamine synthesized by these neurons is released in the dorsal striatum. Dopaminergic transmission in this pathway is

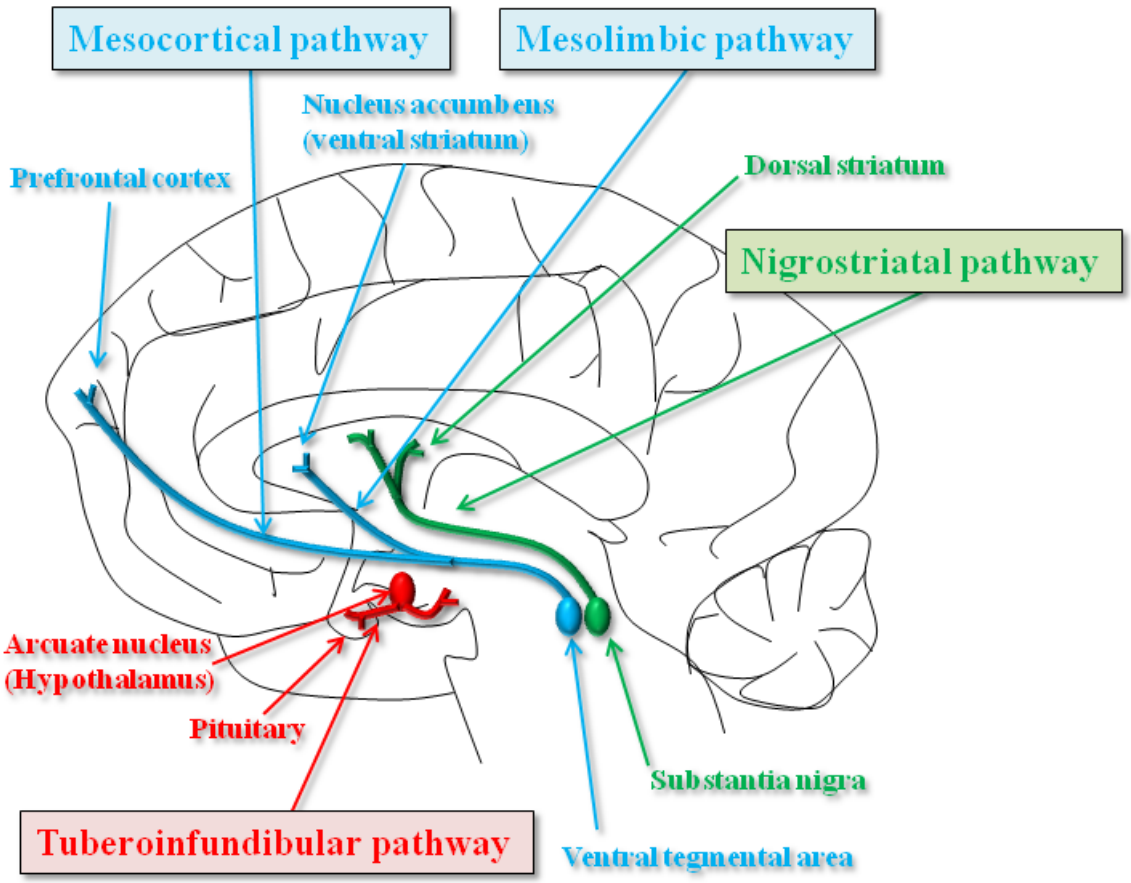


**Figure 8. Dopaminergic neuronal pathways.**
There are four dopaminergic neuronal pathways: nigrostriatal, mesolimbic, mesocortical and tuberoinfundibular. Neurons located in subtantia nigra and projecting to dorsal striatum compose the nigrostriatal pathway. Neurons located in ventral tegmental area that make synaptic contact with nucleus accumbens in the ventral stiatum form the mesolimbic pathway while the one projecting to prefrontal cortex represents the mesocortical pathway. Neurons located in the arcuate nucleus of hypothalamus and sending projections to pituitary form the tuberoinfundibular pathway.

responsible of the motor behavior. Destruction of these neurons is responsible of Parkinson's disease. Abnormalities in dorsal striatum have also been associated with motor tics in Tourette's syndrome.

The second dopaminergic tract is the mesolimbic pathway. Cell bodies of these neurons are originating from ventral tegmental area (VTA) and make synaptic contact with neurons located in the nucleus accumbens (NAc) located in the ventral striatum. This neuronal pathway is involved in motivation, reward anticipation and perception of pleasure. Abnormalities in this dopaminergic tract are responsible for the negative symptoms found in schizophrenic patients (i.e. asociality, lack of motivation, desire and pleasure and blunted emotions). This neuronal dopaminergic pathway is also involved in the reinforcing effects of drug intake and depression (Nestler & Carlezon 2006).

Another dopaminergic tract originates from VTA and is also involved in schizophrenia symptoms: the mesocortical pathway. Neurons from this tract make synaptic contact with other neurons located in the prefrontal cortex. This pathway is essential for working memory and cognitive function. An abnormal prefrontal dopamine release is associated with the positive symptoms of schizophrenic patients (i.e. hallucinations and cognitive deficits).

The last dopaminergic tract is involved in neuroendocrine control. Somas of neuron from the tuberoinfundibular tract are located in the arcuate nucleus of the hypothalamus. Dopamine release from these neurons occurs in the pituitary and inhibits prolactin secretion.

## 4.3. Dopaminergic receptor classification

Dopamine receptors are divided in two classes: D1-like and D2-like receptors. D1-like receptors are composed of D1R or D5R (alternatively named D1A and D1B, respectively) and are mainly coupled to stimulatory G proteins and stimulate AC activity. In addition, D1C and D1D represent two additional D1-like receptors only expressed in nonmammalian vertebrates (Callier et al. 2003). In contrast, D2-like receptors (composed of $D2R_{long}$, $D2R_{short}$, D3R and D4R) are coupled to inhibitory G proteins and consequently inhibit AC activity. In general, D1-like receptors are post-synaptic and D2-like receptors can be both pre- and post-synaptic (Callier et al. 2003, De Mei *et al.* 2009).

### 4.3.1. D1-like receptors

D1-like receptors are characterized by presence of a relatively short IL3 and a long serine and threonine rich CT compared to D2-like receptors. The short IL3 is characteristic of many receptors coupled to stimulatory G proteins. D1R and D5R have very similar TM domains. Overall, they share over 80% identity. However, their IL3 and CT regions display very divergent sequences.

D1R gene is located on chromosome 5q35.1 and is the most abundant receptor subtype among all dopamine receptors (Wong et al. 2000). In vertebrate brains, D1R is particularly abundant in ventral and dorsal striatum where they are mainly located on gamma-amino butyric acid (GABA) medium-sized spiny neurons and co-localize with dynorphin and substance P (De Keyser *et al.* 1988, Palacios *et al.* 1988, Levey *et al.* 1993, Aubert *et al.* 2000). Medium spiny neurons constitute 95% of the striatal neurons (Kemp & Powell 1971). These neurons integrate inputs from glutamatergic cortical

neurons and dopaminergic midbrain neurons. Abnormalities in these neurons to integrate these inputs strongly compromise striatum functions. Furthermore, D1R is also highly expressed in the amydaloid complex and the nucleus tractus solitaries involved in fear conditioning and coordinating the autonomic nervous system, respectively (Callier et al. 2003). In addition, a moderate level of expression has been identified in cortex, olfactory tubercle, globus pallidus, VTA, substantia nigra, optic tract, thalamus, dorsal and ventral hypothalamus, cerebellum and retina.

D5R gene is located on chromosome 4p15.1-p15.3 (Wong et al. 2000). Product of this gene is particularly abundant in hippocampus, where D1R expression is low (Callier et al. 2003). D5R is also highly expressed in nucleus accumbens and thalamus and at a moderate level in cortex, in cholinergic neurons of dorsal striatum, substantia nigra, nucleus tractus solitaries, dorsal hypothalamus, amyloid complex and retina (Surmeier *et al.* 1996, Callier et al. 2003). In term of receptor binding and G protein-coupling properties, D5R has a higher constitutive activity, agonist affinity and potency, but lower inverse agonist affinity and agonist efficacy than D1R (Tiberi & Caron 1994, Charpentier *et al.* 1996, Tiberi *et al.* 1991, Sunahara *et al.* 1991).

### 4.3.2. D2-like receptors

D2-like receptors have a long IL3, a common feature of GPCRs coupled to inhibitory G protein. Furthermore, the CT of D2-like receptors is approximately seven times shorter than CT of D1-like receptors (Missale et al. 1998). While D1-like receptors possess two N-glycosylation sites (on NT and EL2 regions), D2R, D3R and D4R have four, three and one glycosylation sites, respectively.

D2R gene is located on chromosome 11q22-23 and is the most abundant D2-like receptor (Wong et al. 2000). D2R exists as two alternatively sliced isoforms: $D2R_{short}$ and $D2R_{long}$ (Dal Toso *et al.* 1989, Giros *et al.* 1989). $D2R_{long}$ has a 29 amino acids insertion in IL3 absent from $D2R_{short}$. Distribution of D2R in the brain is similar to that of D1R. Similarly to D1R, striatal D2Rs are expressed in GABAergic medium spiny neurons, but co-localize with enkephalin (Aubert et al. 2000, Le Moine & Bloch 1995, Gerfen *et al.* 1995, Surmeier et al. 1996). Striatal D2Rs are also expressed in cholinergic interneurons.

D3R gene is located on chromosome 3q13.3 and D3R and D2R share 75% identity within their TM domains (Wong et al. 2000, Missale et al. 1998). D3R is mainly expressed in limbic areas. GABAergic medium spiny neurons of the nucleus accumbens (rostral and ventrolateral shell) and granule cells of the islands of Calleja have the largest D3R densities (Levesque *et al.* 1992, Landwehrmeyer *et al.* 1993, Murray *et al.* 1994). Importantly, D1R and D3R co-localize in single neurons located in nucleus accumbens. Indeed, their interaction at cellular and behavioral levels has been demonstrated (Ridray *et al.* 1998, Schwartz *et al.* 1998). An interesting particularity of D3R is its ability to inhibit AC5 activity but not other AC isoforms (Robinson & Caron 1997). Furthermore, it is worth mentioning that D3R dopamine affinity is about twenty times higher than that for D2R (Sokoloff *et al.* 1990). This difference of affinity has been related to differences between IL3 of D2R and D3R (Robinson *et al.* 1994). However, quinpirole is the most discriminating drug between D2R and D3R with over 100 times higher affinity for D3R (Sokoloff et al. 1990, Robinson et al. 1994).

Finally, D4R, located on chromosome 11p15.5, is mostly expressed in the same brain regions that D2R (Wong et al. 2000). The highest densities of D4R are located in

dorsomedial thalamus and in the lateral septal nucleus. Comparison between amino acid sequences of D2-like receptors reveals that D4R is the most distantly related of the dopamine receptors coupled to inhibitory G proteins. Indeed, TM domains of D4R share only 53% identity with those of D2R (Missale et al. 1998). Notably, clozapine is particularly useful to distinguish D4R from other D2-like receptors (Van Tol *et al.* 1991). Indeed, clozapine exhibits about 15 times higher affinity for D4R than for D2R and D3R.

## 4.4. Dopaminergic receptor signaling

D1-like and D2-like dopaminergic receptors have different structural features. These differences determine their preferential coupling with stimulatory or inhibitory G protein, respectively (Missale et al. 1998). The nature of stimulated G proteins determine signaling pathways activated downstream of dopaminergic receptors upon ligand binding to receptor. Additionally, the proteins expressed in the cell and the location of dopaminergic receptors dictates which events occur upon receptor activation. The following section describes the most common signaling pathways associated with activation of D1-like and D2-like receptors.

### 4.4.1. D1-like receptor signaling pathways

D1-like receptor signaling is mainly mediated by the production of intracellular cAMP through AC activation by the stimulatory G proteins $G\alpha_s$ or $G\alpha_{olf}$ (**Fig. 9**). AC activation leads to disinhibition of regulatory subunits of PKA. This kinase phosphorylates several proteins involved in gene expression and important signal transduction signals (Neve *et al.* 2004).

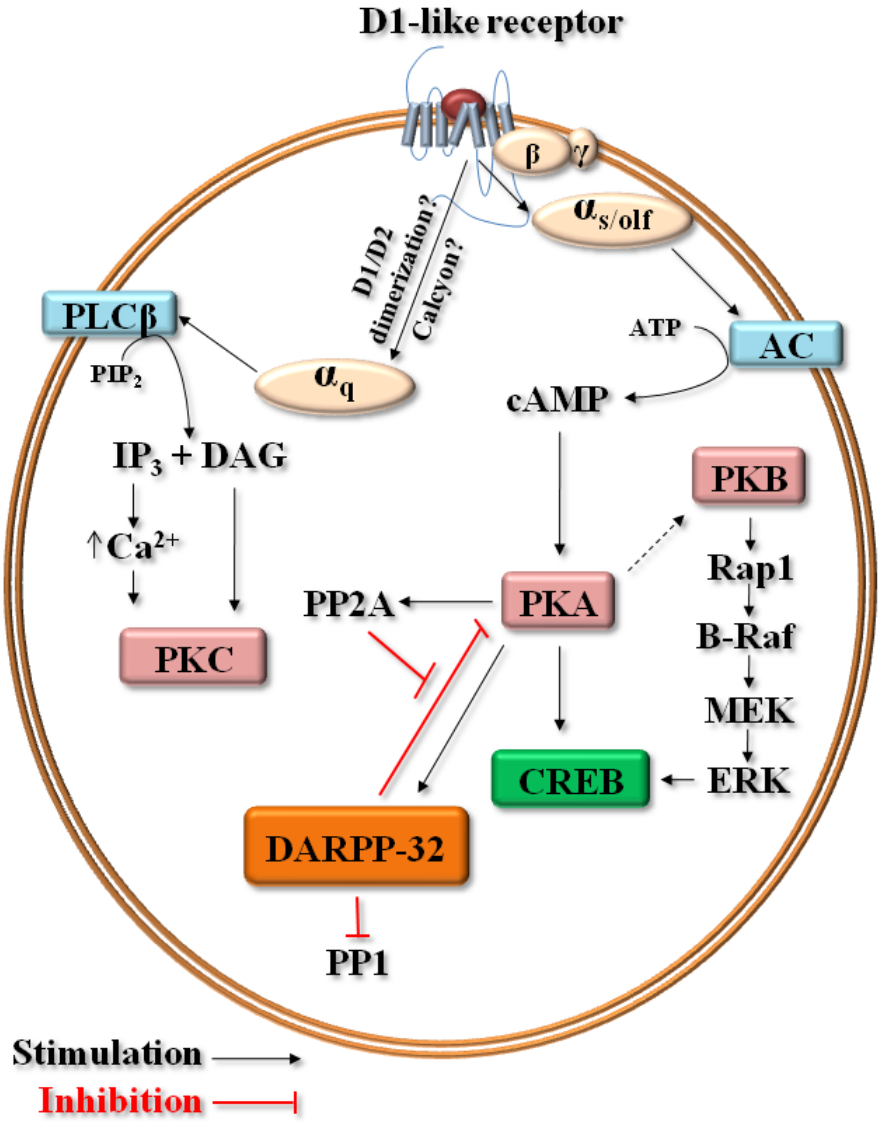


**Figure 9. D1-like receptor signaling pathways.**
The main D1-like receptor signaling pathways are mediated by production of intracellular cAMP via activation of adenylyl cyclase (AC) by stimulatory G proteins ($\alpha_s$ or $\alpha_{olf}$). cAMP activates protein kinase A (PKA) activity leading to dopamine and cAMP-regulated phosphoprotein 32 kDa (DARPP-32)-mediated inhibition of protein phosphatase 1 (PP1). Inhibition of PKA by DARPP-32 is prevented by PKA-mediated stimulation of PKA-stimulated protein phosphatase-2A (PP2A) activity. D1-like receptor-mediated activation of PKA also stimulates ERK activity via protein kinase B (PKB) activation by an unknown mechanism. Both PKA and ERK lead to activation of a transcription factor called cAMP response element-binding protein (CREB), responsible of transcription of several genes having a cAMP response element. $G\alpha_q$-coupling with D1-like receptors was also reported. This lead to activation of phospholipase Cβ (PLCβ) resulting in elevation of intracellular calcium concentration and activation of protein kinase C (PKC). The mechanism for which D1-like receptors couple with Gαq is controversed but could be explained by involvement of a protein called calcyon or through heterodimerization of D1R with D2R.

One of the most important proteins activated by PKA is dopamine and cyclic AMP-regulated phosphoprotein 32 kDa (DARPP-32). Phosphorylation of DARPP-32 by PKA occurs on Thr 34. Phosphorylation of that DARPP-32 residue lead to inhibition of protein phosphatase 1 (PP1) (Hemmings *et al.* 1984). This phosphatase catalyzes dephosphorylation of several important proteins in the brain such as voltage-gated ion channels an numerous neurotransmitter receptors (Greengard *et al.* 1999). Attenuated responses to antipsychotic drugs, psychostimulants and dopamine have been observed in DARPP-32 knockout mice. However, when DARPP-32 is phosphorylated on Thr75 (by cyclin-dependent kinase 5), DARPP-32 inhibits PKA (Bibb *et al.* 1999). But this DARPP-32-mediated inhibition of PKA is prevented by dephosphorylation of Thr75 on DARPP-32 by the PKA-stimulated protein phosphatase-2A (PP2A) (Nishi *et al.* 2000).

Another important PKA substrate is the transcription factor cAMP response element-binding protein (CREB) (Konradi *et al.* 1994). Phosphorylation of Ser133 of CREB by PKA allows binding of CREB to CREB-binding proteins leading to transcription of genes with cAMP response element (Cole *et al.* 1995). Activation of gene expression by phosphorylation of CREB has an important role in synaptic plasticity. Gene transcription by CREB can also be mediated by activation of extracellular signal-regulated kinase (ERK) (Brami-Cherrier *et al.* 2002). Activation of ERK via D1-like receptors is mediated by PKA-dependent activation of PKB by an unknown mechanism.

The last main signaling pathway activated by D1-like receptors is activation of PLCβ leading to elevation of intracellular calcium concentration and PKC activation (Mahan *et al.* 1990). However, this pathway is controversial. Increase of intracellular calcium was reported in mouse LTK- and HEK293 cells transfected with human or

goldfish D1R upon receptor stimulation (Liu *et al.* 1992b, Frail *et al.* 1993). Furthermore, evidence has been reported that D1-like receptors are coupled to $PIP_2$ hydrolysis. Intrarenal administration of a D1-like receptor agonist increases PLCβ activity (Jose *et al.* 1995). In addition, incubation with the D1-like receptor specific agonist SKF38393 increases $IP_3$ production and $PIP_2$ hydrolysis in rat brain striatal slices as well as postmortem human prefrontal cortex membranes (Wang *et al.* 1995, Pacheco & Jope 1997). SKF83959, a D1-like receptor agonist that does not stimulate AC activity, also stimulates $PIP_2$ hydrolysis or $IP_3$ production in membranes from cerebellum and hippocampus from rat brain and in rat striatal and macaque caudate nucleus slices (Jin *et al.* 2003, Panchalingam & Undie 2001). Some studies suggest that activation of this signaling pathway occurs through D1-like receptor coupling with $G\alpha_q$ protein. Indeed, a dopamine-dependent $IP_3$ production has been observed in COS-7 cells cotransfected with D1R and $G\alpha_{15}$ or with $G\alpha_{16}$ proteins (Offermanns & Simon 1995). However, these two $G\alpha_q$ proteins are only expressed in a subset of hematopoietic cells (Wilkie *et al.* 1991, Amatruda *et al.* 1991). Surprisingly, incubation of rat frontal cortex and striatal membranes with SKF83959 or SKF38393 increases [$^{35}$S]GTPγS binding to $G\alpha_q$ but not to or at a less extent to $G\alpha_s$ (Jin et al. 2003, Panchalingam & Undie 2000). Two possible mechanisms have been hypothesized. The first one is the involvement of a protein called calcyon (Lidow *et al.* 2001). The second mechanism is via heterodimerization of D1R with D2R (Lee *et al.* 2004).

#### 4.4.1.1. Modulation of ion channels by D1-like receptors

Activation of D1-like signaling pathways can also regulate activity of several ion channels. Potassium channels are generally inhibited upon D1-like receptor activation via stimulation of PKA/DARPP-32 pathway (Neve et al. 2004, Kitai & Surmeier 1993). In a similar fashion to potassium channels, conductance of N- and P/Q-types of calcium channels is also decreased upon D1-like receptor activation via PKA/DARPP-32 pathway (Surmeier *et al.* 1995, Young & Yang 2004). However, in contrast to N- and P/Q-types calcium channels, L-type calcium currents are increased by D1-like receptor activation. Sodium channels are also regulated by D1-like receptors by PKA activation and inhibition of PP1 via activation of DARPP-32. An increased PKA-mediated phosphorylation of specific serine residues of the pore-forming α-subunit of voltage-gated sodium channels leads to a reduction of sodium currents (Cantrell *et al.* 1997, Surmeier *et al.* 1992, Li *et al.* 1992, Smith & Goldin 1997, Murphy *et al.* 1993).

#### 4.4.1.2. Modulation of ligand-gated ion channels by D1-like receptors

In addition to non ligand-gated ion channels, D1-like receptor stimulation also modulates ligand-gated ion channels activity such as N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and γ-amino butyric acid type A ($GABA_A$) receptors. NMDA receptor response is increased upon D1-like receptor activation by phosphorylation of NR1 subunit of NMDA receptor. DARPP-32-mediated inhibition of PP1, activation of PKA, ERK, L-type calcium channels and PKC had been described to be involved in D1-like receptor-mediated NMDA receptor stimulation (Cepeda *et al.* 1998, Chergui & Lacey 1999,

Flores-Hernandez *et al.* 2002, Snyder *et al.* 1998, Sarantis *et al.* 2009, Yang 2000). It was also reported that D1-like receptor stimulation increase NMDA receptors trafficking (Dunah & Standaert 2001). However, physical interaction between CT of D1R and NR2A subunit of NMDA receptor has been shown to inhibit NMDA currents (Lee *et al.* 2002). AMPA receptors are positively modulated by D1-like receptor activation. AMPA current amplitude is modestly enhanced through activation of L-type calcium channels by D1-like-mediated stimulation of PKA/DARPP-32 pathway (Galarraga *et al.* 1997). In addition, D1-like receptors activation stabilizes AMPA receptor current through PKA-mediated phosphorylation of Ser845 of the AMPA receptor GluR1 subunit and by inhibition of PP1-mediated dephosphorylation of this serine residue (Yan *et al.* 1999b, Snyder *et al.* 2000, Chao *et al.* 2002b, Li *et al.* 2003). In hippocampus, it was reported that D1-like receptors stimulation increases AMPA receptors cell surface expression in a PKA-dependent manner (Gao *et al.* 2006). In a similar fashion to NMDA receptors, GluR1 subunit of AMPA receptors trafficking is enhanced by D1-like receptors-mediated activation of AC (Chao *et al.* 2002a). Finally, $GABA_A$ receptors are differently regulated by D1-like receptors depending on the brain area and the cell type in which $GABA_A$ receptors are expressed. In nucleus accumbens and in medium spiny neurons of striatum, activity of $GABA_A$ receptors is decreased via PKA/DARPP-32-mediated phosphorylation of β1/β3 subunits of $GABA_A$ receptor (Flores-Hernandez *et al.* 2000, Nicola & Malenka 1998). In contrast, in a subpopulation of zinc-sensitive $GABA_A$ receptors localized in large striatal cholinergic interneurons, $GABA_A$ currents are enhanced by D5R stimulation via activation PKA and inhibition of PP1 (Yan & Surmeier 1997). This opposite regulation of $GABA_A$ currents by D1-like receptors activation is possibly explained by a

wide variety of α, β and γ subunit isoforms of $GABA_A$ receptors (Neve et al. 2004). Similarly to D1R and NMDA receptor, direct physical interaction has been described between CT of D5R and $\gamma_{2short}$ subunit of $GABA_A$ receptor (Liu *et al.* 2000). This interaction results in mutual inhibition of D5R and $GABA_A$ receptor.

### 4.4.2. D2-like receptors signaling

D2-like receptors are coupled to inhibitory G proteins ($\alpha_i$ or $\alpha_o$) and consequently reduce activity of AC leading to inhibition of cAMP accumulation (De Camilli *et al.* 1979, Stoof & Kebabian 1981) (**Fig. 10**). However, in opposition to $G\alpha_s$ subunits that can activate all AC isoforms, only AC1, AC5 and AC6 had been reported to be inhibited by $G\alpha_{i/o}$ subunits (Sadana & Dessauer 2009). The corresponding reduction of PKA activity induces inhibition of DARPP-32 activity and disinhibition of PP1. Free Gβγ dimer has also been identified to inhibit AC1, AC3 and AC8, but stimulates AC2, AC4, AC5, AC6 and AC7.

Similarly to other $G\alpha_{i/o}$-linked GPCRs, free Gβγ dimers stimulate PLCβ activity leading to increase of intracellular calcium concentration and activation of PKC (Neve et al. 2004). D2-like receptor-mediated calcium mobilization also activates protein phosphatase-2B (PP2B) (also called calcineurin), which also inhibits DARPP-32 activity and consequently D2-like receptors can contribute to PP1 disinhibition (Nishi *et al.* 1997, Nishi *et al.* 1999). Even if D2-like receptor activation leads to inhibition of PKA activity, CREB can also be activated. D2-like receptor-mediated CREB activation occurs via calcium-dependent proteins such as PP2B, PKC or calcium/calmodulin-dependent protein kinase (CAMK) (Kingsbury *et al.* 2007, Yan *et al.* 1999a). In a similar fashion to

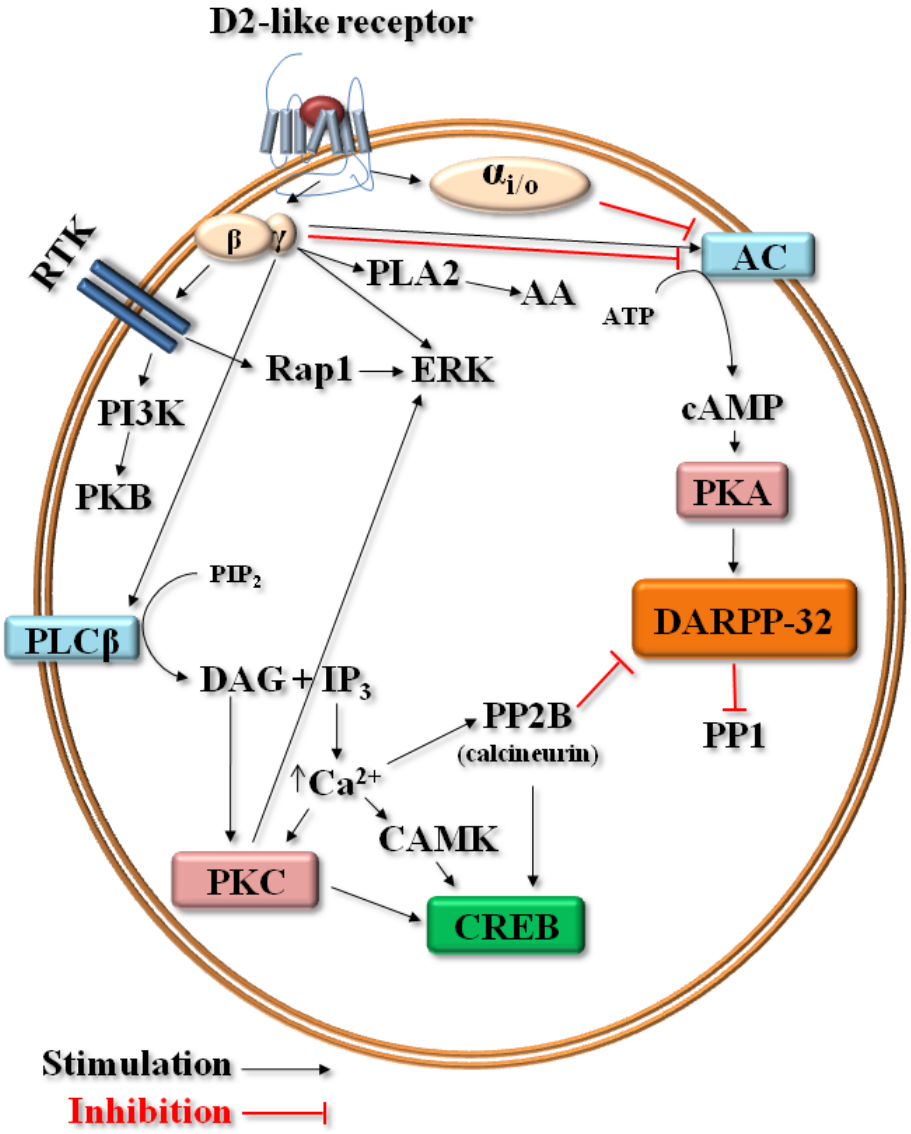


**Figure 10. D2-like receptor signaling pathways.**
D2-like receptor signaling pathways are mediated by $G\alpha_{i/o}$ and $G\beta\gamma$ dimer. $G\alpha_{i/o}$ has an inhibitory effect on specific adenylyl cyclase (AC) isoforms, while $G\beta\gamma$ dimer inhibits or stimulates AC activity depending of AC isoform expressed in the cell. Reduction of AC activity leads to inhibition of dopamine and cAMP-regulated phosphoprotein 32 kDa (DARPP-32) and disinhibition of protein phosphatase 1 (PP1). In addition, $G\beta\gamma$ dimer stimulates phospholipase Cβ (PLCβ) activity leading to mobilization of intracellular calcium and stimulation of protein kinase C (PKC). The increase in calcium concentration also activates calcium/calmodulin-dependent protein kinase (CAMK) and calcineurin that both increase cAMP response element-binding protein (CREB) activity. Calcineurin activation leads to inhibition of DARPP-32. D2-like receptors activate extracellular signal-regulated kinase (ERK) via PKC, $G\beta\gamma$ dimer or through receptor tyrosine kinase (RTK) transactivation. This D2-like receptor-mediated RTK activation can as well activate phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) pathway. Arachidonic acid (AA) is also synthetized through activation of phospholipase $A_2$ ($PLA_2$) by free $G\beta\gamma$.

D1-like receptors, stimulation of D2-like receptors induces ERK phosphorylation (Faure *et al.* 1994, Huff 1996, Luo *et al.* 1998, Welsh *et al.* 1998, Ghahremani *et al.* 2000). However, activation mechanisms are different from D1-like receptors and are PKC- and/or Gβγ-dependent. Additionally, in some cases, D2-like receptor activation of ERK is mediated through transactivation of a receptor tyrosine kinase (RTK) such as epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR) (Kotecha *et al.* 2002, Oak *et al.* 2001, Kim *et al.* 2004b, Wang *et al.* 2005). The specific transactivation mechanism is not clear, but a direct interaction between D2R and PDGFR or EGFR has been observed upon stimulation of D2R (Nair & Sealfon 2003). It is also through D2-like receptor-mediated RTK transactivation that activation of the phosphoinositide 3-kinase (PI3K)/PKB can occur (Nair & Sealfon 2003, Zhen *et al.* 2001). PI3K/PKB pathway mediates neuroprotective effects against oxidative stress.

In addition to activation of PLCβ, D2-like receptors (expect D3R) can activate cytosolic phospholipase $A_2$ ($PLA_2$) through Gβγ dimer (Vial & Piomelli 1995, Nilsson *et al.* 1999, Jelsema & Axelrod 1987, van Tol-Steye *et al.* 1999). This leads to arachidonic acid (AA) release. This compound and its bioactive metabolites are associated with a change in dopamine synthesis and a modulatory effect on DAT activity (L'Hirondel *et al.* 1995, Zhang & Reith 1996).

### 4.4.2.1. Modulation of ion channels by D2-like receptors

D2-like receptors also modulate ion channels activity. In contrast to D1-like receptors, D2-like receptor stimulation increases potassium currents presumably via free Gβγ dimers rather than $G\alpha_i$-mediated inhibition of AC activity (Lacey *et al.* 1987, Liu *et*

*al.* 1994, Greif *et al.* 1995, Wickman *et al.* 1994, Kuzhikandathil *et al.* 1998). There is also evidence that D2-like receptors can form a stable complex with G protein-regulated inwardly rectifying potassium channels (GIRKs) (Lavine *et al.* 2002). Calcium channels are also modulated by D2-like receptors. Activity of L, N and P/Q-type calcium channels are all decreased by D2-like receptors (Seabrook *et al.* 1994a, Seabrook *et al.* 1994b, Kuzhikandathil & Oxford 1999, Okada *et al.* 2003, Yan *et al.* 1997, Zamponi & Snutch 1998, Page *et al.* 1997, Herlitze *et al.* 1997, Qin *et al.* 1997, Hernandez-Lopez *et al.* 2000). This inhibition can be mediated by $G\alpha_i$ protein or via free $G_{\beta\gamma}$ subunit. D2-like receptor activation stimulates or inhibits sodium currents depending of the cellular context (Surmeier & Kitai 1993). Classically, D2-like receptor stimulation induces inhibition of sodium currents possibly via binding of free $G\beta\gamma$ dimer to sodium channels (Surmeier & Kitai 1993). However, in some neostriatal neurons, stimulation of D2-like receptors increases sodium currents. It is hypothesized that prevention of sodium channels phosphorylation via D2-like receptor-mediated PKA inhibition would explain this sodium currents increase.

### 4.4.2.2. Modulation of ligand-gated ion channels by D2-like receptors

Ligand-gated ion channels are negatively modulated by D2-like receptor activity. In D2R knockout mice, glutamatergic neurotransmission is enhanced (Cepeda *et al.* 2001). In contrast to D1-like receptors, NMDA currents are decreased by D2-like receptors. This inhibition of NMDA currents by D2-like receptors relies on different mechanisms depending of the neuron localization in the brain (Snyder et al. 1998, Cepeda *et al.* 1993, Wang *et al.* 2003, Kotecha et al. 2002). Several mechanisms such as

D2-like mediated transactivation of PDGFR, PP1 disinhibition and dephosphorylation of NR1 subunit or removal of the magnesium blockade of NMDA receptor via cell hyperpolarization due to changes in potassium and sodium permeability have been suggested. Reduction of AMPA currents by D2-like receptors is also observed in striatum (Hernandez-Echeagaray *et al.* 2004, Hakansson *et al.* 2006). This inhibition is mediated by D2-like mediated inhibition of PKA/DARPP-32 cascade leading to a dephosphorylation of Ser 845 on GluR1 subunit of AMPA receptor. Recently, in the prefrontal cortex, D4R had been shown to inhibit AMPA-mediated synaptic transmission via PP2B activation (Yuen & Yan 2009). Furthermore, GABAergic currents are inhibited by D2-like receptor agonists in striatum (Cooper & Stanford 2001, Sciamanna *et al.* 2009). In prefrontal cortex, D2R agonists decrease presynaptic GABAergic release and post-synaptic $GABA_A$ receptor responsiveness (Seamans *et al.* 2001, Valenzuela *et al.* 1995). This post-synaptic effect is mediated by D2-like receptor-mediated transactivation of PDGFR.

## 4.5. D1-like receptor knockout mice

Knockout mice exist for each dopaminergic receptor. Behavioral studies and electrophysiology experiments using these mice considerably helped understanding the in vivo functionality controlled by these GPCRs, which was hampered by the lack of highly selective dopaminergic ligands. The following section will focus solely on D1R and D5R knockout mice as topic of my thesis deals with D1-like receptors.

D1R null mutant mice highlight the importance of D1R in motor control, reward and cognition. These mice exhibit several motor abnormalities. D1R knockout mice have

growth retardation unless hydrated food is provided (Drago *et al.* 1994). This observation potentially suggests a motor deficit in the dopamine-controlled masticatory function. Interestingly, non-functional masticatory activity is observed in patients with advanced Parkinson's disease. Additionally, a reduction of orofacial movements, impaired motor coordination and reduced spontaneous grooming behavior had been observed in D1R null mutant mice (Tomiyama *et al.* 2002, Cromwell *et al.* 1998, Drago *et al.* 1999). Acute or chronic treatment with psychostimulants induces hyperactivity via phosphorylation of DARPP-32. Both hyperactivity and phosphorylation of DARPP-32 are attenuated in D1R knockout mice (Crawford *et al.* 1997, Svenningsson *et al.* 2003). These results are in agreement with an increased phosphorylation of DARPP-32 upon stimulation of D1-like receptors (see section 4.4.2.1). Absence of D1R expression also reduces reinforcing properties of rewarding stimuli. It was reported that D1R knockout mice have reduced voluntary ethanol consumption as well as a reduced operant responding to sucrose (El-Ghundi *et al.* 1998, El-Ghundi *et al.* 2003). These observations are in agreement with a previous study showing that administration of D1R/D5R antagonist ecopipam to cocaine addicts attenuates euphoric effects associated to cocaine intake (Romach *et al.* 1999). These results confirm the importance of D1R in the mesolimbic dopaminergic pathway. As mentioned above, cognition is also impaired in D1R knockout mice. Indeed, these mice exhibit defective long-term potentiation (LTP), a molecular mechanism underlying learning and memory, in both corticostriatal and hippocampal neurons (Matthies *et al.* 1997, Centonze *et al.* 2003). Delayed extinction of conditioned fear response is also observed in D1R knockout mice confirming that D1R is involved in prefrontal cortex functions (El-Ghundi *et al.* 2001). In fact, abnormalities in LTP of prefrontal cortex

neurons from D1R knockout mice are reported (Huang *et al.* 2004). These results confirmed previous studies performed with rodents and non-human primates in which D1-like receptor agonists or antagonists were administered into prefrontal cortex. Administration of these drugs induced modification of cognitive performances (Williams & Goldman-Rakic 1995, Granon *et al.* 2000, Robbins 2000, Lidow *et al.* 2003). Microinfusions of the D1-like receptor agonists and D1-like receptor antagonists into prefrontal cortex enhances and impairs accuracy of attentional performance, respectively. In opposition, no change on cognitive performances is observed by administration of D2-like receptor antagonist such as sulpiride.

As opposed to D1R, D5R expression is mainly restricted to hippocampus. D5R activation mediates hippocampal acetylcholine release (Hersi *et al.* 2000). It is worth to mention that cholinergic neurotransmission has an important role in mediating learning and memory (Everitt & Robbins 1997). Indeed, D5R null mutant mice display a reduction of acetylcholine release in the hippocampus upon D1R/D5R agonist treatment (Laplante *et al.* 2004). In addition, it was reported that male D5R knockout mice show an antidepressant-like phenotype in a mouse model of behavioral despair (Holmes *et al.* 2001). Indeed, these results are consistent with the role of hippocampus in depression (MacQueen *et al.* 2003). In contrast to D1R knockout mice, D5R null mutant mice are healthy and no growth retardation is observed. D5R null mutant mice also display normal motor functions (Elliot *et al.* 2003). However, these mice exhibit abnormally elevated high blood pressure by 3 months of age (Hollon *et al.* 2002). This is mediated by an increased sympathetic tone mediated by sensitization of V1 vasopressin receptors and non-NMDA glutamatergic receptors in the ventro-lateral medulla.

## 5. Regulation of dopaminergic receptors by PKC

As previously mentioned in section 2.2, GPCR activity can be modulated by second messenger-dependent kinases such as PKA and PKC. Dopamine receptor activity is also regulated by these kinases. The following section focuses on PKC-mediated regulation of dopaminergic receptor activity.

### 5.1. Regulation of D2-like receptor activity by PKC

Several studies report desensitization of D2-like receptors by PKC. D2R is the most studied receptor among D2-like receptors in regard to PKC regulation. It is reported that both rat $D2R_{short}$ and rat $D2R_{long}$ are similarly phosphorylated in transfected human embryonic kidney (HEK) 293T cells upon treatment with phorbol 12-myristate 13-acetate (PMA), a PKC activator (Namkung & Sibley 2004). In this study, PMA-mediated phosphorylation of rat $D2R_{long}$ is mimicked by stimulation of the cotransfected M1 muscarinic receptor (a $G\alpha_q$-coupled receptor). In addition, phosphorylation of rat $D2R_{long}$ by PMA is accompanied by a β-arrestin/dynamin-dependent internalization of the receptor and a reduction of dopamine potency to inhibit forskolin-stimulated cAMP accumulation. Mutagenesis analyses suggest that phosphorylation of rat $D2R_{long}$ upon PMA treatment occurs on 2 regions of IL3 of the receptor: the proximal region involving Ser228 and Ser229 and the distal segment of IL3 mainly on Ser355 but also with a minor contribution of Ser352 and Ser354. Both phosphorylation of proximal and distal regions of IL3 of rat $D2R_{long}$ are required for PMA-mediated internalization of this receptor. Meanwhile, only phosphorylation of Ser 355 located in distal region of IL3 is involved in the reduction of dopamine potency to inhibit forskolin-stimulated cAMP accumulation

upon PKC stimulation. Namkung *et al*. hypothesized that phosphorylation of the receptor by PKC would enhance GRK affinity for the neighboring Ser/Thr and that this mechanism would be responsible of β-arrestin/dynamin-dependent internalization of rat $D2R_{long}$. Interestingly, another study shows that activation of PKC by PMA treatment decreases the rat $D2R_{long}$-dependent inhibition of cAMP accumulation but also potentiate the facilitation of arachidonic acid release associated with $D2R_{long}$ activation in transfected Chinese hamster ovary (CHO) cells (Di Marzo *et al.* 1993). Both human $D2R_{short}$- and human $D2R_{long}$-mediated increase of cytosolic free calcium is reduced by treatment with the PKC activator 12-O-tetra-decanoyl 4β-phorbol 13-acetate (TPA) in transfected mouse $Ltk^-$ fibroblast cells (Liu *et al.* 1992a). However, the extent of PKC-mediated desensitization of the two human D2R isoforms is different. While $D2R_{short}$-mediated mobilization of calcium is completely inhibited by TPA treatment, the corresponding effect on $D2R_{long}$ is partial with the same TPA concentration. Presence of PKC pseudosubstrates sites on the IL3 of human $D2R_{long}$ (on the 29 amino acid sequence absent from $D2R_{short}$) results in partial resistance of PKC-mediated $D2R_{long}$ desensitization (Morris *et al.* 2007). These pseudosubstrate sites on human $D2R_{long}$ mimic the pseudosubstrate site located on N-terminal region of PKC and bind to the PKC substrate-binding pocket. Consequently, these regions of human $D2R_{long}$ compete with the real phosphorylable PKC sites on the receptor. This mechanism explains why phosphorylation of Ser228 and Ser229 is observed for human $D2R_{short}$ but not for human $D2R_{long}$ upon TPA treatment. An interesting study also suggests that the PMA-mediated inhibition of forskolin-stimulated AC activity by human $D2R_{long}$ is mediated by disruption of the interaction between actin-binding protein 280 (ABP-280) and $D2R_{long}$

(Li *et al.* 2000). ABP-280, also called filamin A, is a cytoskeleton-associated protein believed to cluster D2R with important components of signal transduction. Phosphorylation of a PKC site located on distal segment of IL3 prohibits binding of the receptor with ABP-280. This PKC site corresponds to Ser355 in rat $D2R_{long}$ that has been identified as a key site in the reduction of dopamine potency to inhibit forskolin-stimulated cAMP accumulation upon PKC stimulation (Namkung & Sibley 2004). Li *et al*. hypothesized that presence or absence of ABP-280 could account for the different agonist sensitivity observed for pre-synaptic and post-synaptic D2R. In fact, it is reported that D2R autoreceptors expressed on nigral dopaminergic cell bodies and dendrites are at least 6 times more sensitive to agonists than are postsynaptic D2R in the caudate nucleus (Skirboll *et al.* 1979). Interestingly, it was recently shown that neurotensin-mediated increase of dopaminergic neuron excitability is mediated by desensitization of D2R autoreceptors (Thibault *et al.* 2011a). Similarly to PMA, stimulation of the $G\alpha_q$-coupled neurotensin type 1 receptor (NTR1) induces activation of PKC and consequently D2R internalization. Through this heterologous regulation of D2R, neurotensin can modulate dopamine release in the striatum.

In a similar fashion to D2R, D3R-mediated response is also dampened by PMA treatment. As opposed to D2R, D3R undergoes only subtle homologous desensitization and GRK/β-arrestin-dependent sequestration (Kim *et al.* 2001). Instead of mediating D3R sequestration, phosphorylation of human D3R by GRK2 or GRK3 reduces D3R coupling to G protein by disrupting D3R/ABP-280/β-arrestin complex (Kim *et al.* 2005). In fact, in a similar fashion to D2R, ABP-280 is an important binding partner of D3R by linking the receptor to downstream signaling components (Lin *et al.* 2001, Li *et al.* 2002). ABP-280

is also required for PMA-induced D3R sequestration (Cho *et al.* 2007b). Results from this study suggest that PMA-mediated phosphorylation of serine residue 257 located in the middle of IL3 of human D3R enables interaction between ABP-280 and D3R and consequently sequestration upon stimulation of PKC.

As opposed to D2R and D3R, only a few studies have been performed on D4R regulation. It is reported that D4R stimulation mediates phospholipid methylation in SK-N-MC human cultured neuroblastoma cell lines (Sharma *et al.* 1999). By increasing the distance between phospholipid headgroups, this D4R-mediated lipid modification reduces membrane-packing density and increase membrane fluidity. Upon dopamine-induced conformation changes of D4R, methionine residue located on TM6 of D4R serves as a substrate for an enzyme facilitating transfer of a methyl group to phospholipids. It was showed that PKC activity is required to observe this D4R-mediated phospholipid methylation (Sharma *et al.* 2001). However, the specific mechanism remains unknown.

## 5.2. Evidence of D1R potentiation by PKC in striatum

Similarly to D2R and D3R, it is reported that D1R is also desensitized by PKC in HEK293T cells (Rex *et al.* 2008). However, in contrast, in rat primary striatal cultures, known to express predominantly D1R over D5R, PMA treatment potentiates dopamine-induced cAMP formation (Surmeier et al. 1996, Schinelli *et al.* 1994). Interestingly, increase of the intracellular calcium concentration by treatment with the calcium ionophore A23187 does not boost PMA-mediated potentiation of cAMP production in these cells. This suggests that conventional PKC isoforms may not be involved in PMA-

mediated D1-like receptor potentiation (calcium is a cofactor for these isoforms) in striatal cells. A subsequent study demonstrated that group I metabotropic glutamate receptor (mGluR) activation potentiates also dopamine-induced cAMP formation via PKC in cultured striatal neurons (Paolillo *et al.* 1998). Group I mGluRs (composed of mGluR1 and mGluR5) are GPCRs coupled to $G\alpha_{q/11}$ and thus stimulate PLCβ activity. Consequently, stimulation of PKC activity is a major signaling pathway associated with mGluR1 and mGluR5 activation. Several studies suggest that mGluR5 but not mGluR1 is expressed in striatal neurons (Paolillo et al. 1998, Prezeau *et al.* 1994, Ghasemzadeh *et al.* 1996, Testa *et al.* 1994, Shigemoto *et al.* 1993). Thus, it is hypothesized that potentiation of D1-like receptor-mediated cAMP formation is mediated by PKC activation via mGluR5 activation in striatal neurons. GABAergic striatal spiny neurons represent the majority of striatal neurons (Kemp & Powell 1971). It is where dopaminergic nigrostriatal pathway and glutamatergic corticostriatal pathway converge and where those signals are integrated by striatal neurons. Consequently, activation of glutamatergic corticostriatal neurons would represent a way to accentuate the effects of nigrostriatal neurons stimulation in striatum. Meanwhile, the major caveat with studying D1-like receptors in vivo is the lack of ligands discriminating between D1R and D5R, which has not allow concluding whether the PMA/PKC potentiation mechanism operates through D1R and/or D5R. Most importantly, the underlying molecular mechanisms involved in this PKC-mediated potentiation of striatal D1-like receptor signaling are unknown.

## 6. Rationale, hypotheses and objectives

Previously, our lab used heterologous cells (HEK293 cells) transfected with hD1R or hD5R to address if the increase in DA-induced intracellular cAMP observed in striatal neurons upon PMA treatment is mediated through an effect on D1R or D5R potentiation. HEK293 cells do not express dopaminergic receptors but do have all conventional and novel PKC isoforms (Jackson *et al.* 2005). Quantification of intracellular cAMP produced by receptor activation in the presence or absence of dopamine in intact cells pretreated with or without PMA was assessed to determine the effect of PKC stimulation on each human D1-like receptor. Our lab demonstrated that PMA treatment mediates an opposite regulation of human D1-like receptors culminating in a robust hD1R sensitization and hD5R desensitization, respectively (Jackson et al. 2005). Moreover, using several pharmacological PKC blockers, our lab showed that Gö6983 and bis-indolylmaleimide I (two drugs blocking conventional and novel PKCs) but not Gö6976 (a drug mediating blockade of conventional PKCs) inhibit PMA-mediated hD1R sensitization and hD5R desensitization. Based on these findings, our lab suggested that novel PKC isoforms are potentially involved in the PMA regulation of human D1-like receptors. This view agrees with previous studies performed in striatal neurons discussed above in which the PMA-mediated increase of dopamine-induced cAMP production was not changed by an elevation of intracellular calcium levels (Schinelli et al. 1994, Paolillo et al. 1998). Preliminary results obtained from our lab also show that a treatment of cells with BAPTA-AM (a calcium chelator) does not have an effect on PMA-mediated regulation of hD1R and hD5R (Jackson and Tiberi, unpublished data). In addition, our lab showed that inhibition of PKC by Gö6983 and bis-

indolylmaleimide I completely abrogate PMA-mediated hD5R desensitization while these two drugs block the PMA-induced hD1R sensitization by 50%. These results suggest that PMA-induced hD1R sensitization is also controlled by a PKC inhibitor-resistant or PKC-independent mechanism.

The existence of several AC isoforms displaying either stimulatory or inhibitory regulation by PKC raises a question about whether the differential PMA regulation of D1-like receptors is linked to the coupling of hD1R and hD5R to different AC isoforms (Jacobowitz *et al.* 1993, Jacobowitz & Iyengar 1994, Beazely & Watts 2005, Kawabe *et al.* 1996, Lai *et al.* 1997, Zimmermann & Taussig 1996). Unpublished studies from our lab suggest an hD1R and hD5R coupling to multiple and same AC isoforms in transfected HEK293 cells. Thus, it is unlikely that differential regulation of hD1R and hD5R by PMA is explained by the formation of distinct complexes between D1-like receptors and AC isoforms. Moreover, as opposed to $G\alpha_z$ and $G\alpha_{12}$ subunits, $G\alpha_s$ subunit is not a substrate for PKC (Kozasa & Gilman 1996). This also excludes the possibility that hD1R or hD5R activity could be mediated by phosphorylation of their $G\alpha_s$ subunit by PKC. This leads to my **first hypothesis** that PKC does not mediate its effect by modulating hD1R and hD5R activation profile through direct regulation of AC or $G\alpha_s$ activities, but via regulation of the receptor itself.

Bioinformatics analyses using human D1-like receptors suggest the presence of PKC sites located on all cytoplasmic regions, but more prominently on IL3 and CT. This may point to a possible involvement of IL3 and/or CT in the opposed regulation of these two subtypes by PMA treatment. Indeed, the small size of IL1 and IL2 and their high degree of conservation between hD1R and hD5R relative to IL3 and CT suggest that IL1

and IL2 are not the key cytoplasmic regions involved in PMA-mediated regulation of human D1-like receptors. Interestingly, a study using fusion proteins demonstrated that CT of human D1R and CT of mouse D5R are not phosphorylated by PKC in vitro (Zamanillo *et al.* 1995). Altogether, these findings lead to my **second hypothesis** that IL3 is the major structural determinant involved in PKC-mediated regulation of hD1R sensitization and hD5R desensitization.

The most interesting feature in the regulation of human D1-like receptors by PMA is the opposite effects we observed on hD1R and hD5R. This suggests that different regulation mechanisms govern the modulation of hD1R and hD5R by PKC. One possible mechanism is that these receptors are differentially phosphorylated by PKC upon PMA treatment. This represents my **third hypothesis**. In that case, different PKC phosphorylation sites on IL3 of hD1R and hD5R could explain the opposite effect of PMA treatment observed for these two receptors. The presence of different PKC phosphorylation sites may also suggest that distinct nPKC isoforms control PMA-mediated regulation of hD1R and hD5R.

Desensitization is not always mediated by receptor phosphorylation. Indeed, several recent publications have implicated GRKs in phosphorylation-independent desensitization of various GPCRs such as endothelin A and B, thromboxane A2, $\alpha_{1b}$-adrenergic, M1 and M3 muscarinic cholinergic, parathyroid hormone, thyrotropin-releasing hormone, serotoninergic type 2C, mGluR1a, angiotensin type 1A and H1 histamine receptors (Sterne-Marr *et al.* 2004, Willets *et al.* 2005, Dhami *et al.* 2005, Day *et al.* 2003). For many of these receptors, expression of GRK2 RH domain (known to interact directly with $G_{\alpha q}$ protein members) is sufficient to attenuate signaling in the

absence of receptor phosphorylation. GRK2 and GRK3 are also well known to be targeted to the cell membrane by binding free Gβγ subunits (Pitcher *et al.* 1992b). Furthermore, our lab observed that CT truncation of D1R abolished its dopamine-induced phosphorylation while undergoing agonist-induced desensitization (Jackson *et al.* 2002). These observations lead to my **fourth hypothesis** that hD1R or hD5R are respectively sensitized or desensitized by a receptor phosphorylation-independent mechanism through a physical interaction with PKC as seen with GRKs.

These hypotheses will be addressed in my thesis with the following objectives:

1) Demonstration that PMA-mediated regulation of human D1-like receptors occurs at the receptor level.
2) Delineation of the structural determinants of D1-like receptors responsible for hD1R sensitization and hD5R desensitization by PKC activation.
3) Characterization of the PKC-mediated mechanisms controlling the hD1R and hD5R function.
4) Identification of the novel PKC isoform(s) involved in the opposite regulation hD1R and hD5R.

# Chapter 2

# Manuscript 1

# The third intracellular loop of D1 and D5 dopaminergic receptors dictates their subtype-specific PKC-induced sensitization and desensitization in a receptor conformation-dependent manner.

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Abbreviations: AC, adenylyl cyclase; $B_{max}$, maximal binding capacity; BSA, bovine serum albumin; β2-AR, β2-adrenergic receptor; CA, [$^3$H]-cAMP formed; CT, cytoplasmic tail; DA, dopamine; DMSO, dimethyl sulfoxide; EL3, third extracellular loop; $EC_{50}$, effective concentration producing half-maximal response; $E_{max}$, maximum efficacy; FBS, fetal bovine serum; FSK, forskolin; GPCR, G protein-coupled receptor; hD1R; human D1 receptor; hD5R; human D5 receptor; HEK293, human embryonic kidney 293; IL3, third intracellular loop; MEM, minimum essential medium; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; TM, transmembrane; TR, terminal region; TU, total uptake.

**Statement of author contributions:**

BP and MT conceived and designed research. BP and XY performed research. BP and MT analyzed data, wrote and edited the manuscript.

## Abstract

We previously showed that phorbol-12-myristate-13-acetate (PMA) mediates a robust PKC-dependent sensitization and desensitization of the highly homologous human Gs protein and adenylyl cyclase (AC)-linked D1 (hD1R) and D5 (hD5R) dopaminergic receptors, respectively. Here, we demonstrate using forskolin-mediated AC stimulation that PMA-mediated hD1R sensitization and hD5R desensitization is not associated with changes in AC activity. We next employed a series of chimeric hD1R and hD5R to delineate the underlying structural determinants dictating the subtype-specific regulation of human D1-like receptors by PMA. We first used chimeric receptors in which the whole terminal region (TR) spanning from the extracellular face of transmembrane domain 6 to the end of cytoplasmic tail (CT) or CT alone were exchanged between hD1R and hD5R. CT and TR swaps lead to chimeric hD1R and hD5R retaining PMA-induced sensitization and desensitization of wild type parent receptors. In striking contrast, hD1R sensitization and hD5R desensitization mediated by PMA are correspondingly switched to PMA-induced receptor desensitization and sensitization following the IL3 swap between hD1R and hD5R. Cell treatment with the PKC blocker, Gö6983, inhibits PMA-induced regulation of these chimeric receptors in a similar fashion to wild type receptors. Further studies with chimeras constructed by exchanging IL3 and TR show that PMA-induced regulation of these chimeras remains fully switched relative to their respective wild type parent receptor. Interestingly, results obtained with the exchange of IL3 and TR also reveal that the D1-like subtype-specific regulation by PMA, while fully dictated by IL3, can be modulated in a receptor conformation-dependent manner. Overall, our results

strongly suggest that IL3 is the critical determinant underlying the subtype-specific regulation of human D1-like receptor responsiveness by PKC.

## Introduction

Dopamine (DA) is an important catecholamine neurotransmitter regulating numerous physiological effects in the central nervous system and peripheral tissues through the binding to and activation of cell-surface seven-transmembrane proteins (Missale et al. 1998, Beaulieu & Gainetdinov 2011). Dysfunction of central dopaminergic receptor neurotransmission has been implicated in multiple brain disorders, which include Parkinson's disease, schizophrenia and drug addiction (Papakostas 2006, Goto & Grace 2007, Tang *et al.* 2007, Kienast & Heinz 2006, Chao & Nestler 2004, Pantazopoulos *et al.* 2004). DA receptors are G protein-coupled receptors (GPCRs) classified as D1-like (D1R and D5R) and D2-like ($D2R_{short}$, $D2R_{long}$, D3R and D4R) subtypes (Missale et al. 1998, Beaulieu & Gainetdinov 2011). This classification is based on the primary sequence identity shared between subtypes and receptor ability to stimulate or inhibit adenylyl cyclase (AC) activity, respectively (Missale et al. 1998, Beaulieu & Gainetdinov 2011). Studies suggest that D1R and D5R, while sharing over 80% identity in their transmembrane (TM) regions, exhibit distinguishing ligand binding and G protein coupling properties in human embryonic kidney 293 (HEK293) cells (Tiberi & Caron 1994, Plouffe *et al.* 2010). Indeed, D5R has higher constitutive activity, decreased inverse agonist affinity, increased agonist affinity, potency and intrinsic activity relative to D1R. Interestingly, these natural differences between D1R and D5R ligand binding and G protein coupling properties are highly reminiscent of functional properties distinguishing wild type and constitutively active mutant forms of GPCRs (Tiberi & Caron 1994, Costa & Cotecchia 2005, Cotecchia 2007, Plouffe et al. 2010). Mutagenesis studies indicate that the third intracellular loop (IL3) and cytoplasmic tail

(CT) of D1R and D5R play a central role in regulating D1-like subtype-specific ligand binding and G protein coupling properties (Charpentier et al. 1996, Iwasiow *et al.* 1999, Jackson *et al.* 2000, Tumova *et al.* 2003, Tumova *et al.* 2004).

Additionally, we have recently demonstrated that intracellular cAMP formation by human D1R (hD1R) and D5R (hD5R) is regulated in an opposite fashion following phorbol-12-myristate-13-acetate (PMA) treatment (Jackson et al. 2005). Indeed, in PMA-treated HEK293 cells, hD1R and hD5R undergo robust protein kinase C (PKC)-mediated sensitization and desensitization, respectively (Jackson et al. 2005). This subtype-specific PKC-dependent regulation of D1R and D5R responsiveness by PMA is unique among GPCRs. For instance, the closely related human β1- and β2-adrenergic receptors are desensitized in a distinctive manner by PKC isoforms (Guimond *et al.* 2005). Studies also demonstrated that PKC-induced desensitization is mediated through phosphorylation of IL3 and CT regions (Sato *et al.* 2004, Airas *et al.* 2001, Bailey *et al.* 2009, Pin & Bahr 2008, Kilianova *et al.* 2006, Diviani *et al.* 1997, Liang *et al.* 1998, Yuan *et al.* 1994). Likewise, PKC-mediated desensitization of D2-like dopaminergic receptor is controlled through specific residues located on their large IL3 region (Namkung & Sibley 2004, Morris et al. 2007, Cho *et al.* 2007a, Thibault *et al.* 2011b). Interestingly, PMA-induced activation of PKC potentiates the extent of D1-like receptor-mediated cAMP formation by DA in primary striatal cultures (Schinelli et al. 1994). Additionally, stimulation of striatal Gq-linked metabotropic glutamate and M1 muscarinic receptors also leads to PKC-dependent potentiation of D1-like receptor-induced cAMP formation (Paolillo et al. 1998, Sanchez-Lemus & Arias-Montano 2006). Importantly, striatal neurons express predominantly the D1R subtype (Sunahara *et al.* 1990, Dearry *et al.* 1990, Tiberi et al.

1991, Surmeier et al. 1996). In agreement with these findings, our studies in HEK293 cells clearly suggest that PKC-induced D1-like receptor sensitization is mediated through the D1R but not D5R subtype (Jackson et al. 2005). In fact, adenovirus-transformed HEK293 cells, which are used in our studies, exhibit a molecular phenotype similar to early differentiating neurons (Shaw *et al.* 2002). Therefore, PKC-mediated D1R sensitization reported in our HEK293 cells seems consistent with findings using native preparations from striatum. We also observe that D1R is phosphorylated in HEK293 cells following PMA treatment (unpublished data). This is in agreement with previous studies reporting PKC-induced D1R phosphorylation in C6 glioma and HEK293-tsa201 (referred to as HEK293T) cells (Gardner *et al.* 2001, Rex et al. 2008, Rankin & Sibley 2010). However, in striking contrast to our study, D1R is desensitized by PKC activation in HEK293T cells (Rex et al. 2008). The use of different HEK293 cell lines and experimental approach may explain the discrepancies between PKC-mediated D1R regulation studies. Most importantly, the molecular mechanisms implicated in the opposite regulation of hD1R and hD5R remains to be elucidated.

Herein, we first investigate whether the opposite regulation of D1R and D5R by PMA is mediated through a direct modulation of cellular AC activity. Furthermore, the central objective of the present study is to delineate the structural determinants dictating the opposite regulation of D1R and D5R-induced cAMP formation by PMA using multiple chimeric D1-like receptors.

## Materials and methods

### Materials

Minimal essential media (MEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), gentamicin, trypsin-EDTA, and HEPES buffer were from Invitrogen (Burlington, ON, Canada). N-[methyl-$^{3}$H]-SCH23390 (65-91 Ci/mmol) and [$^{14}$C]-cAMP (252 mCi/mmol) were purchased from GE Healthcare (Baie d'Urfé, QC, Canada). [$^{3}$H]-adenine (20 Ci/mmol) was from PerkinElmer (Boston, MA, USA). DA, *cis*-flupenthixol, dimethyl sulfoxide (DMSO), forskolin (FSK), and 1-methyl-3-isobutylxanthine (IBMX) were from Sigma-Aldrich (Oakville, ON, Canada). Ionomycin, PMA and Gö6983 were obtained from Calbiochem (La Jolla, CA, USA).

### Receptor and adenylyl cyclase constructs

Human wild type (hD1R and hD5R) and chimeric receptor DNA constructs were cloned in the expression vector pCMV5. In this study, eight chimeric receptors were used (hD1-$CT_{D5}$, hD5-$CT_{D1}$, hD1-$TR_{D5}$, hD5-$TR_{D1}$, hD1-$IL3_{D5}$, hD5-$IL3_{D1}$, hD1-$IL3TR_{D5}$ and hD5-$IL3TR_{D1}$). hD1-$CT_{D5}$ and hD5-$CT_{D1}$ chimeras were constructed by exchanging the whole CT using a PCR approach as detailed elsewhere (Jackson et al. 2000). hD1-$TR_{D5}$ and hD5-$TR_{D1}$ chimeras were generated by swapping the terminal region (TR) encompassing the extracellular face of transmembrane 6 (TM6) up to the end of CT as described previously (Iwasiow et al. 1999). The hD1-$IL3_{D5}$, hD5-$IL3_{D1}$, hD1-$IL3TR_{D5}$ and hD5-$IL3TR_{D1}$ chimeras were prepared by switching IL3 alone or with TR using a strategy based on endonuclease digestions of the conserved restriction sites *BspH*I and *Bcl*I located within the TM5 and TM6, respectively. Integrity of DNA sequences of

chimeras were confirmed by automated DNA sequencing done at the StemCore Laboratories of the Ottawa Genomics Innovation Centre (Ottawa, ON, Canada, www.stemcore.ca). Bovine AC type 1 (AC1), rat AC type 2 (AC2), human AC type 5 (AC5) and 7 (AC7) were constructed in pCMV5. Human AC type 6 (AC6) was in pcDNA3.

**Cell culture and transfection**

HEK293 cells (American Type Culture Collection, Manassas, VA, USA; CRL-1573) from 40 to 52 passages were grown in MEM supplemented with 10% v/v heat-inactivated FBS and gentamicin (20 μg/ml) at 37 °C in an humidified 5% $CO_2$ environment and seeded into 100-mm dishes ($2.5 \times 10^6$ cells/dish). For studies using FSK and dose-response curves of DA, cells were transiently transfected using the following amounts of receptor construct DNA/dish: hD1R (0.02 μg), hD5R (0.02 μg), hD1-$CT_{D5}$ (5 μg), hD5-$CT_{D1}$ (0.02 μg), hD1-$TR_{D5}$ (0.02 μg), hD5-$TR_{D1}$ (0.04 μg), hD1-$IL3_{D5}$ (0.02 μg), hD5-$IL3_{D1}$ (0.04 μg), hD1-$IL3TR_{D5}$ (5 μg) and hD5-$IL3TR_{D1}$ (0.04-0.08 μg) using a modified calcium phosphate precipitation procedure (Plouffe et al. 2010). For studies using co-transfections of receptor and AC isoforms, cells were transfected with 1 μg DNA/dish of AC isoform plasmids with 0.015-0.15 μg DNA/dish of hD1R or hD5R. Empty pCMV5 vector was added to normalize the total amount of DNA to 5 μg per dish when less than 5 μg per dish of receptor construct DNA was used. To study the effect of PMA on constitutive activity, cells were transfected with 5 μg of receptor construct DNA/dish, which in our hands give the maximal receptor expression achievable in HEK293 cells (Plouffe et al. 2010). After an overnight incubation with the DNA-calcium

phosphate precipitate, HEK293 cells were washed with PBS, trypsinized and reseeded in 6-well plates (FSK and constitutive activity experiments) or 12-well plates (AC studies and DA dose-response curves) and 100-mm dishes (radioligand binding) as described (Plouffe et al. 2010). Cells were then grown for an additional 48 h.

**Whole cell cAMP and radioligand binding assays**

Regulation of AC activity by human wild type and chimeric D1-like receptors was assessed using metabolic labeling with [$^3$H]-adenine (1-2 μCi/ml) and whole cell cAMP assays in the presence of IBMX (1 mM) as described (Plouffe et al. 2010). Control (DMSO 0.02% v/v) and PMA (1 μM) treatments were done essentially as previously published (Jackson et al. 2005). To assess the role of PKC in PMA-induced regulation, experiments were performed with the PKC inhibitor Gö6983. For these studies, cells in labeling media were treated with DMSO (0.006% v/v; control) and Gö6983 (300 nM) for 15 min at 37°C. At the end of treatment period, media was aspirated and cells incubated in 20 mM HEPES-buffered MEM with IBMX (cAMP assay media) in the absence and presence of PMA with increasing concentrations of DA. It is worth mentioning that for other experiments, all drugs were added at the same time (no pretreatment) and cells bathing in cAMP assay media treated for 30 min at 37°C in a humidified 5% $CO_2$ environment. The amount of intracellular [$^3$H]-cAMP was determined from samples purified by sequential chromatography using Dowex and alumina columns (Johnson *et al.* 1994, Plouffe et al. 2010). The levels of intracellular [$^3$H]-cAMP formed (CA) over the total amount of [$^3$H]-adenine uptake (TU) were calculated to establish the relative AC activity and expressed as CA/TU × 1000 values (Plouffe et al. 2010). For radioligand

assays, membrane preparations made from transfected cells seeded in a 100-mm dish were incubated with a saturating concentration (6-8 nM) of N-[methyl-$^3$H]-SCH23390 in the absence or presence of 10 μM *cis*-flupenthixol (to measure total and nonspecific binding) to calculate the receptor expression ($B_{max}$) in pmol/mg membrane proteins as described (Plouffe et al. 2010). Membrane protein concentrations were determined using Bio-Rad Protein Dye Reagent (Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin as standard.

**Statistical analysis**

All data are reported as arithmetic means ± standard error unless indicated otherwise. All statistical analyses were performed using GraphPad Prism software, version 5.03 for Windows (GraphPad Software, San Diego; http://www.graphpad.com). Homogeneity of variances was assessed using Hartley's $F_{max}$ test. Dose-response curves of DA were simultaneously analyzed by a four-parameter logistic equation using raw data (CA/TU × 1000). Intracellular cAMP levels obtained for each curve point were then expressed as percent of the best-fitted DA-mediated maximal stimulation value ($E_{max}$) of control treatment (DMSO). Individual curves were averaged and analyzed again to obtain best-fitted parameters for the effective concentration producing 50% of maximal response ($EC_{50}$) and $E_{max}$. Best-fitted values for $EC_{50}$ and $E_{max}$ obtained from control and PMA treatments were compared using unconstrained, shared and constrained curve fitting. Statistical differences between experimental conditions were determined using one-sample *t* test, unpaired *t* test and one-way ANOVA with Newman-Keuls post test as indicated in the text. The level of significance was established at $p < 0.05$.

## Results and discussion

### PMA treatment has no direct effect on AC activity in HEK293 cells

An important issue that remains to be addressed is whether the PMA-induced regulation of hD1R sensitization and hD5R desensitization is mediated through a modulation of cellular AC activity. HEK293 cells express several AC isoforms (AC1, AC2, AC3, AC6 and AC7) (Hellevuo *et al.* 1993, Premont 1994, Zaworski *et al.* 1999). Furthermore, these AC isoforms have been reported to undergo positive (AC1, AC2, AC3, AC7) and negative (AC6) regulation by PKC (Sadana & Dessauer 2009). To test whether the distinct PMA-induced regulation of human D1-like receptors could be linked to a subtype-specific coupling to AC isoforms, we performed studies using a co-transfection of hD1R and hD5R with prototypical members belonging to different groups of AC isoforms (AC1, Group I; AC2 and AC7, Group II; AC5 and AC6, Group III) (Sadana & Dessauer 2009). Our co-transfection studies demonstrate that hD1R and hD5R do not couple to AC in an isoform-dependent manner in HEK293 cells (**Fig. 1**). Therefore, the opposite regulation of hD1R and hD5R responsiveness mediated by PMA in HEK293 cells cannot be explained by PKC-induced modulation of AC isoforms as previously described (Sadana & Dessauer 2009).

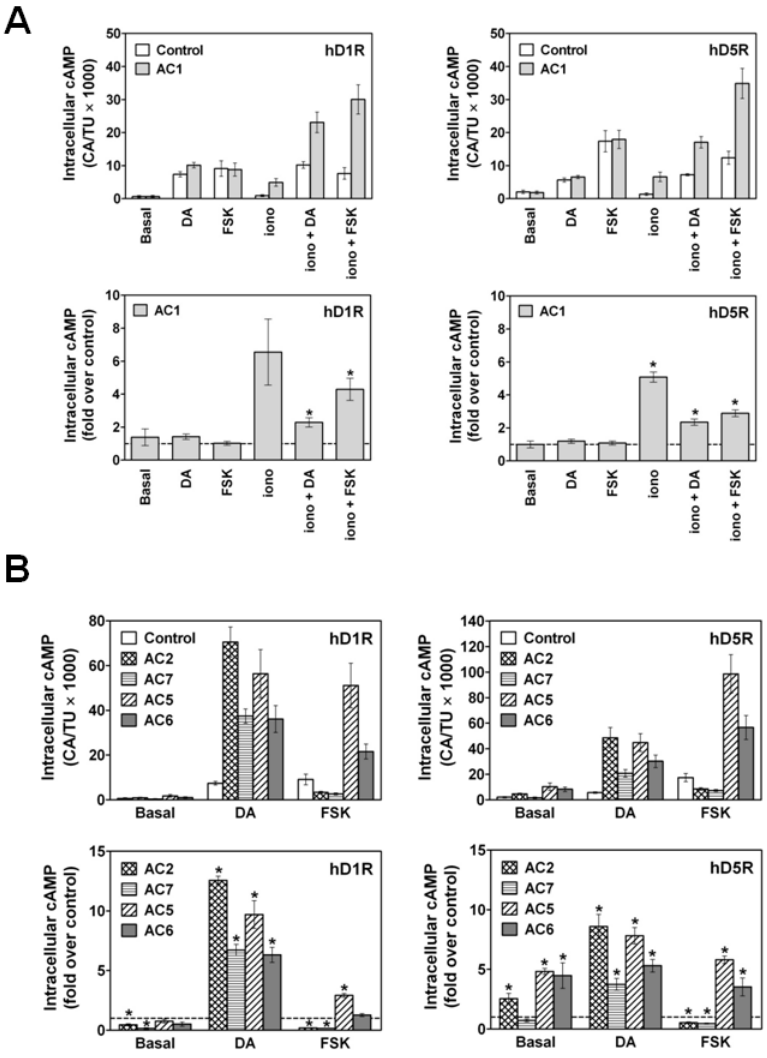


**Figure 1. Stimulation of AC isoforms by hD1R and hD5R in HEK293 cells.**
HEK293 cells transfected with hD1R or hD5R along with AC1 (**A**) or with AC2, AC7, AC5 or AC6 (**B**) were seeded in 12-well plates and labeled with 1 μCi/ml of [$^3$H]-adenine overnight. Cells were incubated in the absence or presence of DA (10 μM), FSK (1 μM) or iono (1 μM) for 30 min at 37°C. Results are expressed as the arithmetic mean ± S.E. of 4 experiments done in triplicate. Intracellular cAMP levels expressed as [$^3$H]-cAMP formed (CA) over the total uptake of [$^3$H]-adenine (TU) × 1000 in cells expressing hD1R (***left***) or hD5R (***right***) are reported using raw data (***upper panels***). For each treatment, results are also expressed as fold over respective control condition (without AC transfection) (***bottom panels***). $B_{max}$ values in pmol/mg membrane proteins for N-[methyl-$^3$H]-SCH23390 (expressed as arithmetic means ± S.E.) were 1.4 ± 0.2 (hD1R control), 1.3 ± 0.1 (hD1R + AC1), 1.4 ± 0.2 (hD1R + AC2), 1.6 ± 0.2 (hD1R + AC7), 1.5 ± 0.4 (hD1R + AC5), 1.4 ± 0.2 (hD1R + AC6), 1.1 ± 0.1 (hD5R control), 1.2 ± 0.2 (hD5R + AC1), 1.3 ± 0.2 (hD5R + AC2), 1.3 ± 0.3 (hD5R + AC7), 1.2 ± 0.2 (hD5R + AC5) and 1.3 ± 0.2 (hD5R + AC6). $B_{max}$ values were not statistically different as assessed using one-way ANOVA (with Newman-Keuls post test).
*$p < 0.05$ when compared to control values set to 1 (dashed line) using one-sample *t* tests.

To assess further whether PKC-induced regulation of hD1R and hD5R responsiveness is mediated through a direct effect on AC activity, we tested the impact of PMA treatment on the extent of FSK stimulation of endogenous ACs in HEK293 cells transfected with human wild type D1-like receptors. Our results show that FSK-mediated AC stimulation remains unchanged while DA-mediated maximal activation of AC is potentiated in cells transfected with hD1R following PMA treatment (**Fig. 2**). We next assessed the extent of AC stimulation by FSK in cells transfected with hD5R. Importantly, Gs and FSK have been shown to activate different AC isoforms in a synergistic or additive manner (Sadana & Dessauer 2009). Additionally, the extent of constitutive activity of Gs-linked GPCRs modulates the level of FSK-induced stimulation (Alewijnse *et al.* 1997, Sheng *et al.* 2005). As previously reported by our group (Plouffe et al. 2010), we demonstrate that the extent of FSK stimulation is greater in cells expressing hD5R relative to cells transfected with similar levels of hD1R (2-fold increase), which is in agreement with higher constitutive activity ( 2-fold) of hD5R (**Fig. 2A**). Therefore, the percent reduction of FSK stimulation observed in hD5R-expressing cells is consistent with the significantly reduced level of hD5R constitutive activity following PMA treatment (**Fig. 2B**). As we previously reported, PMA had no major effect on the DA-dependent maximal production of intracellular cAMP promoted by hD5R in PMA-treated cells. In the present study, while AC stimulation by FSK alone was significantly diminished by virtue of reduced hD5R constitutive activity, the activation of AC in cells co-stimulated with DA and FSK is unchanged upon PMA treatment (**Fig. 2A**). Overall, our results suggest that the PMA-induced regulation of human D1-like subtypes is not mediated through a direct modulation of AC activity but is controlled at

the receptor level. In the next series of experiments, we delineate the receptor determinants involved in the opposite regulation of hD1R and hD5R responsiveness by PMA using different chimeric receptors.

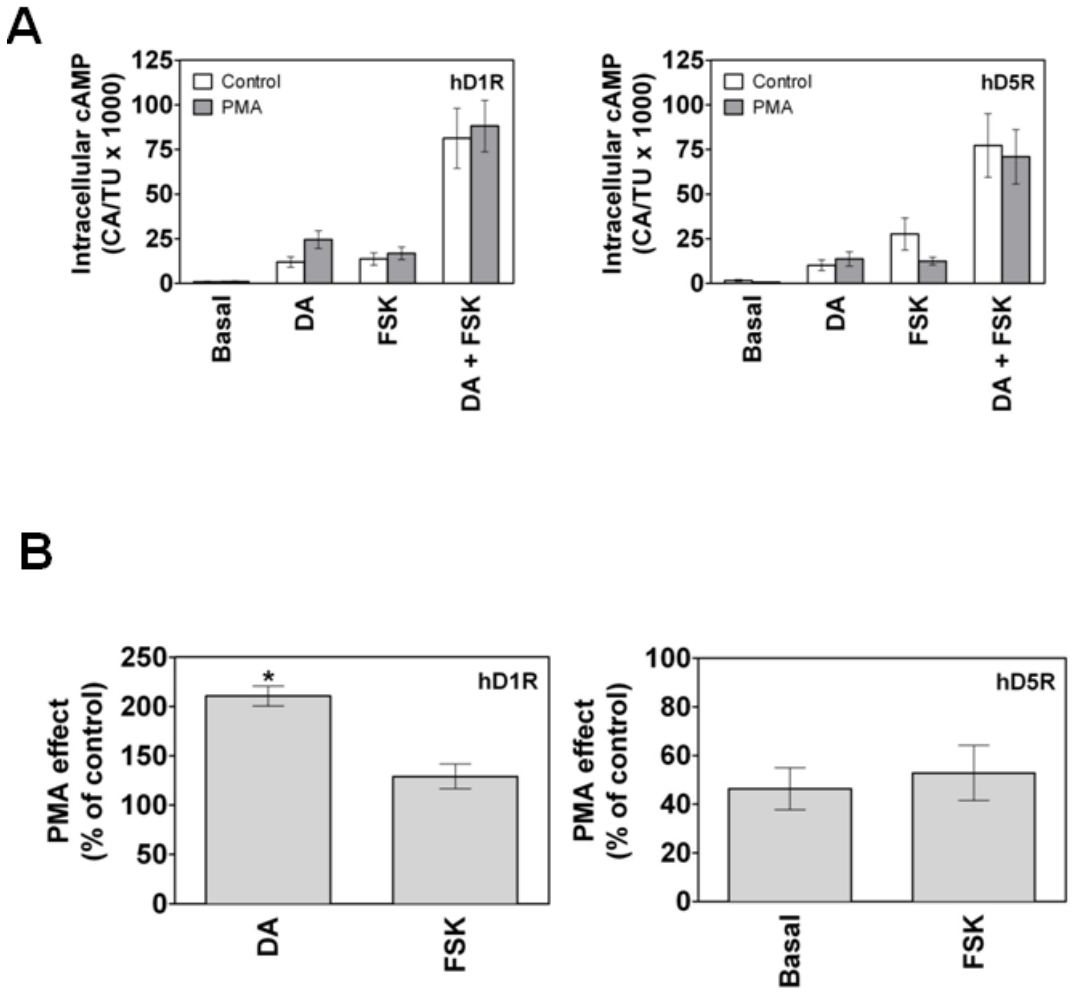


**Figure 2. Effect of PMA on FSK-stimulated AC activity in HEK293 cells.**
HEK293 cells transfected with hD1R and hD5R were seeded in 6-well plates and labeled with 2 μCi/ml of [$^3$H]-adenine overnight. Cells treated with 0.02% (v/v) DMSO (Control) or 1 μM PMA were incubated in the absence or presence of FSK (10 μM) and/or DA (10 μM) for 30 min at 37°C. Results are expressed as the arithmetic mean ± S.E. of 3 experiments done in triplicate. (**A**) Intracellular cAMP levels expressed as [$^3$H]-cAMP formed (CA) over the total uptake of [$^3$H]-adenine (TU) × 1000 in cells expressing hD1R (***left***) or hD5R (***right***) are reported using raw data. (**B**) PMA-induced effect on AC stimulation by DA and FSK expressed as percent of control condition (DMSO) in cells expressing hD1R (***left***) and hD5R (***right***). $B_{max}$ values in pmol/mg membrane proteins for N-[methyl-$^3$H]-SCH23390 (expressed as arithmetic means ± S.E.) were 1.8 ± 0.2 (hD1R) and 2.2 ± 0.1 (hD5R). $B_{max}$ values were not statistically different using an unpaired *t* test.
*$p < 0.05$ using unpaired *t* tests.

**CT of hD1R and hD5R does not dictate the opposite PKC-induced regulation of DA-dependent receptor responsiveness**

The CT of human D1-like receptors displays a low degree of primary sequence identity (**Fig. 3A** and **B**). We then reasoned that differences in CT sequences among hD1R and hD5R may underlie the opposite regulation of human D1-like receptor responsiveness by PMA. In support of this view, a recent study reported that mutations in CT of rat D1R abrogate its PKC-induced receptor phosphorylation (Rankin & Sibley 2010). However, fusion protein studies using the full-length CT of D1-like receptors suggest that PKC does not phosphorylate CT of hD1R and hD5R (Zamanillo et al. 1995). To assess the possible role of CT of human D1-like receptors, we compared the effect of PMA on wild type and chimeric receptors harboring a swapped CT (hD1-$CT_{D5}$ and hD5-$CT_{D1}$). PMA causes a robust increase of $E_{max}$ (~200%) associated with a small rightward shift in $EC_{50}$ for DA (~2.0 fold) in cells expressing hD1R (**Fig. 3A**, **Table 1**). In contrast, PMA promotes a strong rightward shift in $EC_{50}$ (~40 fold) for DA with a weak effect on $E_{max}$ value in cells transfected with hD5R (**Fig. 3B**, **Table 1**) as previously reported (Jackson et al. 2005). PMA-treated cells expressing hD1-$CT_{D5}$ and hD5-$CT_{D1}$ essentially retain the regulatory properties of cells transfected with hD1R and hD5R, respectively (**Fig. 3**). Altogether, our data suggest that the CT of hD1R and hD5R does not dictate the opposite regulation of human D1-like receptor responsiveness by PMA.

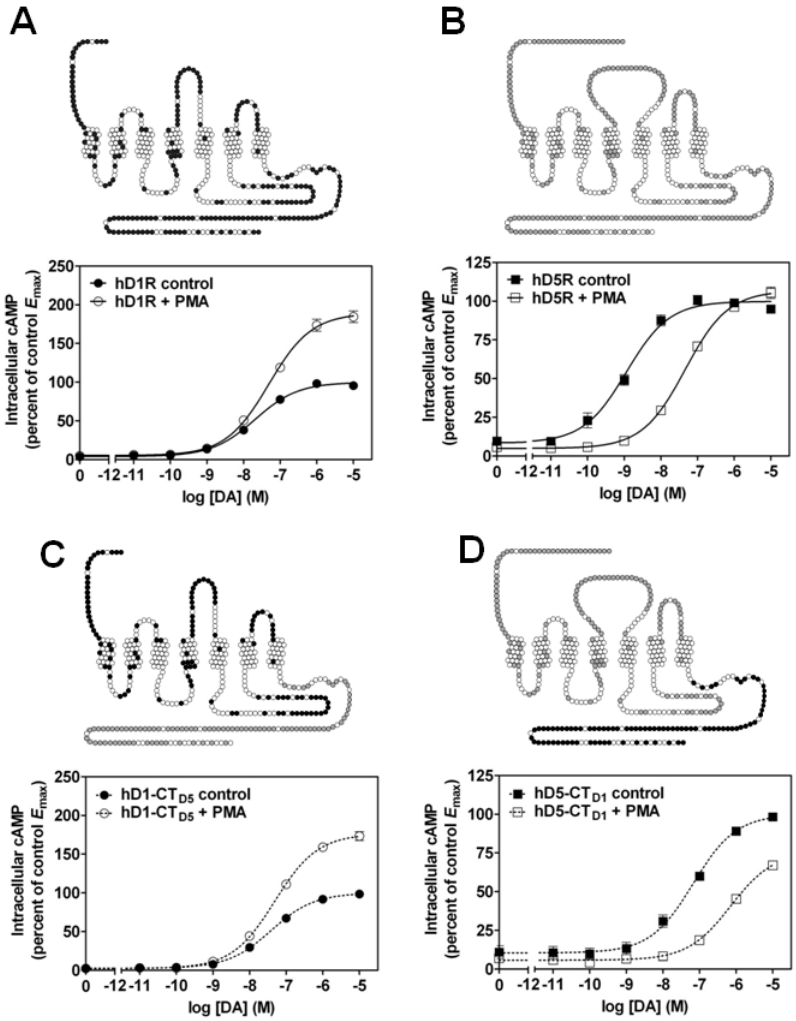


**Figure 3. Dose-response curves of DA for AC stimulation in HEK293 cells transfected with hD1R, hD5R, hD1-CT$_{D5}$ and hD5-CT$_{D1}$ in the absence and presence of PMA.**

Schematic diagrams of hD1R, hD5R, hD1-CT$_{D5}$ and hD5-CT$_{D1}$ are represented. White circles depict identical residues between hD1R and hD5R while black circles and shaded circles represent unique residues found in hD1R and hD5R, respectively. HEK293 cells transfected with hD1R (**A**), hD5R (**B**) hD1-CT$_{D5}$ (**C**) and hD5-CT$_{D1}$ (**D**) were seeded in 12-well plates and labeled with 1 μCi/ml of [$^3$H]-adenine overnight. Cells expressing similar levels of wild type (solid line) and chimeric (dashed line) receptors were incubated in the absence (0.1 mM ascorbic acid) or presence of increasing concentrations of DA with 0.02% (v/v) DMSO (Control, filled symbols) or 1 μM PMA (open symbols) for 30 min at 37°C. Each point is expressed as the arithmetic mean ± S.E. of 3 experiments done in triplicate and plotted as a function of log of DA concentrations. Each point for control and PMA treatments are expressed as percent of the best-fitted $E_{max}$ determined from the corresponding averaged control curve using raw data (CA/TU × 1000) and simultaneous curve fitting with GraphPad Prism. DA-mediated maximal activation ($E_{max}$) and $EC_{50}$ obtained from best-fitted averaged curves (expressed as percent of control $E_{max}$) using a shared value of Hill slope and $B_{max}$ values are reported in **Table 1**.

**Table 1. Best-fitted parameters for dose-response curves of DA in HEK293 cells transfected with hD1R, hD1-$CT_{D5}$, hD5R and hD5-$CT_{D1}$ in the absence or presence of PMA.**

| Receptor | Treatment | $E_{max}$ (% of Control) | $EC_{50}$ (nM) | $EC_{50}$ shift (PMA/Control) |
|---|---|---|---|---|
| **hD1R** | Control | 100 ± 2 | 21 (15 – 28) | |
| | PMA | 189 ± 2[a,c] | 49 (41 – 57)[a] | 2.3 (1.6 – 3.3)[c] |
| **hD1-$CT_{D5}$** | Control | 100 ± 2 | 39 (29 – 52) | |
| | PMA | 176 ± 2[a,b,c] | 49 (41 – 58) | 1.3 (0.6 – 2.7)[b,c] |
| **hD5R** | Control | 100 ± 2 | 1.2 (0.9 – 1.6) | |
| | PMA | 107 ± 2[a,b] | 46 (35 – 61)[a] | 41 (12 – 138)[b] |
| **hD5-$CT_{D1}$** | Control | 100 ± 2 | 67 (48 – 94) | |
| | PMA | 76 ± 4[a,b,c] | 711 (416 – 1216)[a] | 11 (8 – 15)[b,c] |

Averaged dose-response curves (shown in **Fig. 3**) were analyzed by simultaneous curve fitting with a four-parameter logistic equation using GraphPad Prism. Best-fitted parameters for DA-mediated maximal activation of AC ($E_{max}$) are expressed as percentage of control ± S.E. while best-fitted values for $EC_{50}$ are reported with 95% confidence intervals obtained using simultaneous fitting of averaged curves from 3 experiments. $EC_{50}$ shift (PMA/control) values (expressed as the geometric mean with 95% confidence intervals) were computed from individual best-fitted dose-response curves. Statistical analysis of log transform of $EC_{50}$ shift values was performed using one-way ANOVA with Newman-Keuls post test. $B_{max}$ values for N-[methyl-$^3$H]-SCH23390 in pmol/mg membrane proteins (expressed as arithmetic means ± S.E.) were 1.2 ± 0.44 (hD1R), 1.1 ± 0.09 (hD1-$CT_{D5}$), 1.4 ± 0.37 (hD5R) and 0.90 ± 0.20 (hD5-$CT_{D1}$). $B_{max}$ values were not statistically different as assessed using one-way ANOVA (with Newman-Keuls post test).

[a]$p < 0.05$, control vs. PMA; [b]$p < 0.05$, PMA vs. PMA (hD1R); [c]$p < 0.05$, PMA vs. PMA (hD5R).

Meanwhile, the sensitization and desensitization parameters of hD1-$CT_{D5}$ and hD5-$CT_{D1}$ were somewhat different (**Table 1**). For instance, PMA treatment of cells expressing hD5-$CT_{D1}$ leads to a reduced $EC_{50}$ shift for DA when compared with cells expressing wild type hD5R. Additionally, PMA-induced increase of $E_{max}$ in cells expressing hD1-$CT_{D5}$ was slightly lower than $E_{max}$ augmentation in PMA-treated cells harboring hD1R (**Table 1**). Likewise, PMA promotes a greater reduction of $E_{max}$ in cells expressing hD5-$CT_{D1}$ relative to cells harboring hD5R (**Table 1**). Differences in the sensitization and desensitization parameters of hD1-$CT_{D5}$ and hD5-$CT_{D1}$ as compared with their respective wild type parent receptor are potentially explained by changes in DA affinity and potency (as indexed using $EC_{50}$) of these chimeras. Wild type hD5R exhibits a significantly higher affinity and potency for DA relative to that of wild type hD1R (Sunahara et al. 1991, Tiberi et al. 1991, Tiberi & Caron 1994). Intriguingly, while DA affinity of wild type D1-like receptors is fully switched upon exchanging CT between D1R and D5R, DA potency ($EC_{50}$ value) in cells expressing hD1-$CT_{D5}$ was not increased accordingly (Jackson et al. 2000, Demchyshyn *et al.* 2000). Importantly, the exchange of EL3 along with CT of hD1R and hD5R generates chimeric receptors that exhibit a full switch in both DA affinity and $EC_{50}$ suggesting an important molecular interplay between EL3 and CT in shaping D1-like subtype-specific conformations for ligand binding and G protein coupling properties (Tumova et al. 2003, Iwasiow et al. 1999). Therefore, we hypothesized that chimeric receptors with swapped EL3 and CT regions may display a full switch in PMA-mediated regulation of receptor responsiveness. To address this issue, we used two chimeras, hD1-$TR_{D5}$ and hD5-$TR_{D1}$, in which the terminal region spanning from the extracellular face of TM6 and EL3 to the

end of CT was exchanged between hD1R and hD5R (**Fig. 4C** and **D**) (Iwasiow et al. 1999).

Studies using hD1-$TR_{D5}$ and hD5-$TR_{D1}$ clearly demonstrate that a molecular interplay between EL3 and CT does not impart the PMA-induced hD1R sensitization and hD5R desensitization (**Fig. 4**). In contrast to cells transfected with hD5-$CT_{D1}$, cells expressing hD5-$TR_{D1}$ undergoes similar PMA-induced $EC_{50}$ shift when compared with hD5R (**Table 2**). Meanwhile, as seen with hD5-$CT_{D1}$, PMA treatment of cells expressing hD5-$TR_{D1}$ still promotes a reduction (~25 %) of $E_{max}$ relative to cells transfected with hD5R (**Fig. 4B, D** and **Table 2**). Moreover, in similar fashion to studies using hD1-$CT_{D5}$, a reduced $E_{max}$ potentiation was also observed in cells transfected with hD1-$TR_{D5}$ relative to cells expressing hD1R.

Overall, results obtained with hD1-$CT_{D5}$, hD5-$CT_{D1}$, hD1-$TR_{D5}$ and hD5-$TR_{D1}$ chimeras suggest that structural determinants located within CT of human D1-like receptors are not implicated in dictating PMA-induced hD1R sensitization and hD5R desensitization. Alternatively, our findings reveal that the shaping of PMA-induced hD1R sensitization and hD5R desensitization parameters are potentially dependent on receptor subtype-specific conformations. We next investigated the role of IL3 region in controlling the PMA-induced regulation of hD1R and hD5R responsiveness using a new set of chimeras. It is worth mentioning that IL3 of hD1R and hD5R shares a higher degree of identity than that displayed between their CT regions (**Fig. 5A** and **B**).

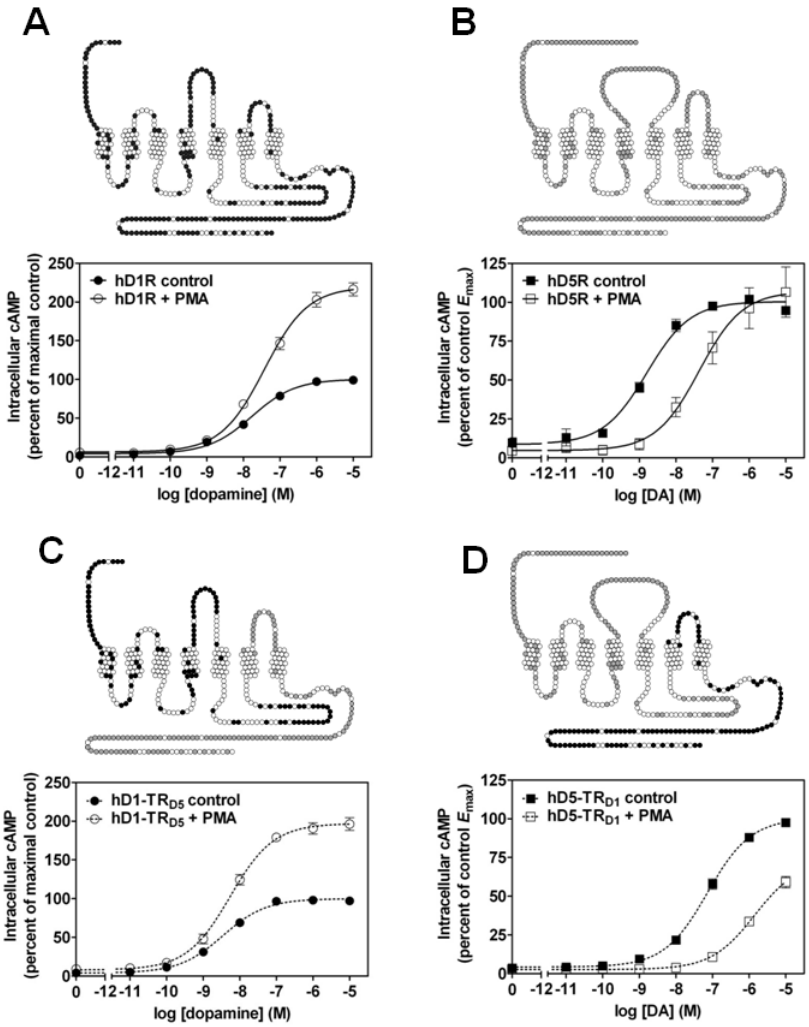


**Figure 4. Dose-response curves of DA for AC stimulation in HEK293 cells transfected with hD1R, hD5R, hD1-TR$_{D5}$ and hD5-TR$_{D1}$ in the absence and presence of PMA.**

Schematic diagrams of hD1R, hD5R, hD1-TR$_{D5}$ and hD5-TR$_{D1}$ are represented. White circles depict identical residues between hD1R and hD5R while black circles and shaded circles represent unique residues found in hD1R and hD5R, respectively. HEK293 cells transfected with hD1R (**A**), hD5R (**B**) hD1-TR$_{D5}$ (**C**) and hD5-TR$_{D1}$ (**D**) were seeded in 12-well plates and labeled with 1 μCi/ml of [$^3$H]-adenine overnight. Cells expressing similar levels of wild type (solid line) and chimeric (dashed line) receptors were incubated in the absence (0.1 mM ascorbic acid) or presence of increasing concentrations of DA with 0.02% (v/v) DMSO (Control, filled symbols) or 1 μM PMA (open symbols) for 30 min at 37°C. Each point is expressed as the arithmetic mean ± S.E. of 3-5 experiments done in triplicate and plotted as a function of log of DA concentrations. Each point for control and PMA treatments are expressed as percent of the best-fitted $E_{max}$ determined from the corresponding averaged control curve using raw data (CA/TU × 1000) and simultaneous curve fitting with GraphPad Prism. DA-mediated maximal activation ($E_{max}$) and $EC_{50}$ obtained from best-fitted averaged curves (expressed as percent of control $E_{max}$) using a shared value of Hill slope and $B_{max}$ values are reported in **Table 2**.

**Table 2. Best-fitted parameters for dose-response curves of DA in HEK293 cells transfected with hD1R, hD1-$TR_{D5}$, hD5R and hD5-$TR_{D1}$ in the absence or presence of PMA.**

| Receptor | Conditions | $E_{max}$ (% of Control) | $EC_{50}$ (nM) | $EC_{50}$ shift (PMA/Control) |
|---|---|---|---|---|
| hD1R | Control | 100 ± 3 | 17 (11 – 27) | |
| | PMA | 219 ± 4[a,c] | 37 (30 – 46)[a] | 2.4 (1.2 – 4.5)[c] |
| hD1-$TR_{D5}$ | Control | 100 ± 3 | 3.4 (2.2 – 5.4) | |
| | PMA | 197 ± 3[a,b,c] | 5.4 (4.3 – 6.9) | 1.6 (1.2 – 2.1)[c] |
| hD5R | Control | 101 ± 3 | 1.6 (0.9 – 3.0) | |
| | PMA | 107 ± 4[b] | 43 (24 – 77)[a] | 25 (12 – 50)[b] |
| hD5-$TR_{D1}$ | Control | 100 ± 4 | 73 (45 – 120) | |
| | PMA | 73 ± 8[a,b,c] | 1403 (521 – 3777)[a] | 20 (13 – 31)[b] |

Averaged dose-response curves (shown in **Fig. 4**) were analyzed by simultaneous curve fitting with a four-parameter logistic equation using GraphPad Prism. Best-fitted parameters for DA-mediated maximal activation of AC ($E_{max}$) are expressed as percentage of control ± S.E. while best-fitted values for $EC_{50}$ are reported with 95% confidence intervals obtained using simultaneous fitting of averaged curves from 3 to 5 experiments. $EC_{50}$ shift (PMA/control) values (expressed as the geometric mean with 95% confidence intervals) were computed from individual best-fitted dose-response curves. Statistical analysis of log transform of $EC_{50}$ shift values was performed using one-way ANOVA with Newman-Keuls post test. $B_{max}$ values for N-[methyl-$^3$H]-SCH23390 in pmol/mg membrane proteins (expressed as arithmetic means ± S.E.) were 0.87 ± 0.21 (hD1R), 0.54 ± 0.20 (hD1-$TR_{D5}$), 0.98 ± 0.22 (hD5R) and 0.74 ± 0.13 (hD5-$TR_{D1}$). $B_{max}$ values were not statistically different as assessed using one-way ANOVA (with Newman-Keuls post test).

[a]$p < 0.05$, control vs. PMA; [b]$p < 0.05$, PMA vs. PMA (hD1R); [c]$p < 0.05$, PMA vs. PMA (hD5R).

**Opposite PKC-induced regulation of DA-dependent hD1R and hD5R responsiveness is mediated through IL3 region**

To explore the role of IL3 in PMA-mediated hD1R sensitization and hD5R desensitization, we exchanged the IL3 between hD1R and hD5R to generate two chimeras referred to as hD1-$IL3_{D5}$ and hD5-$IL3_{D1}$ (**Fig. 5C** and **D**). The DA affinity of hD1-$IL3_{D5}$ (~4 μM) and hD5-$IL3_{D1}$ (~1.5 μM) is partially switched when compared to hD1R (~9 μM) and hD5R (~0.7 μM), respectively (Iwasiow et al., in preparation). Interestingly, PMA-induced regulation of DA-dependent AC activation by hD1-$IL3_{D5}$ (desensitization) and hD5-$IL3_{D1}$ (sensitization) is highly reminiscent of PMA effects observed in cells expressing similar levels of hD5R (desensitization) and hD1R (sensitization) (**Fig. 5**). These findings strongly suggest that IL3 determinants dictate the PKC-induced sensitization and desensitization of DA-dependent hD1R and hD5R responsiveness, respectively. To test further that these chimeras retain similar PMA properties of their wild type counterparts, we used Gö6983, a pharmacological blocker of PKC that inhibits PMA-induced hD1R sensitization and hD5R desensitization in HEK293 cells (Jackson et al. 2005). Studies using Gö6983 (300 nM) show that this PKC inhibitor negates the PMA-induced regulation of hD1-$IL3_{D5}$ (desensitization) and hD5-$IL3_{D1}$ (sensitization) in a similar fashion to their wild type counterparts (**Fig. 6**).

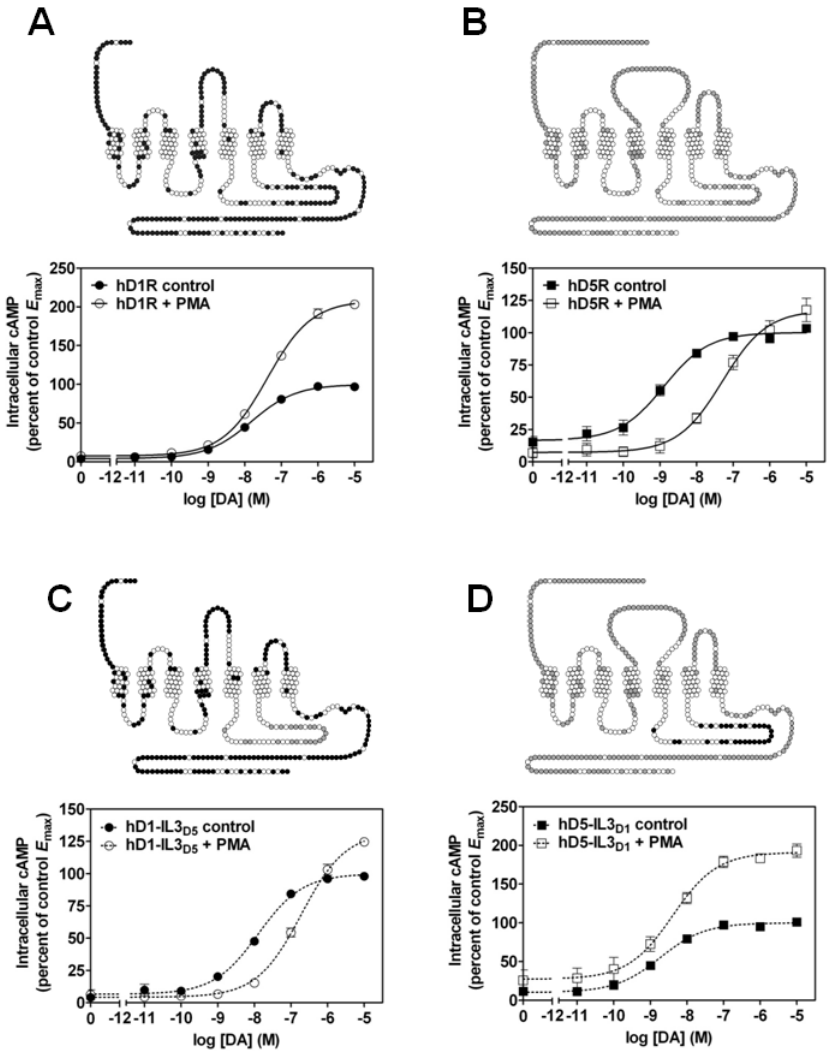


**Figure 5. Dose-response curves of DA for AC stimulation in HEK293 cells transfected with hD1R, hD5R, hD1-IL3$_{D5}$ and hD5-IL3$_{D1}$ in the absence and presence of PMA.**

Schematic diagrams of hD1R, hD5R, hD1-IL3$_{D5}$ and hD5-IL3$_{D1}$ are represented. White circles depict identical residues between hD1R and hD5R while black circles and shaded circles represent unique residues found in hD1R and hD5R, respectively. HEK293 cells transfected with hD1R (**A**), hD5R (**B**) hD1-IL3$_{D5}$ (**C**) and hD5-IL3$_{D1}$ (**D**) were seeded in 12-well plates and labeled with 1 μCi/ml of [$^3$H]-adenine overnight. Cells expressing similar levels of wild type (solid line) and chimeric (dashed line) receptors were incubated in the absence (0.1 mM ascorbic acid) or presence of increasing concentrations of DA with 0.02% (v/v) DMSO (Control, filled symbols) or 1 μM PMA (open symbols) for 30 min at 37°C. Each point is expressed as the arithmetic mean ± S.E. of 5 experiments done in triplicate and plotted as a function of log of DA concentrations. Each point for control and PMA treatments are expressed as percent of the best-fitted $E_{max}$ determined from the corresponding averaged control curve using raw data (CA/TU × 1000) and simultaneous curve fitting with GraphPad Prism. DA-mediated maximal activation ($E_{max}$) and $EC_{50}$ obtained from best-fitted averaged curves (expressed as percent of control $E_{max}$) using a shared value of Hill slope and $B_{max}$ values are reported in **Table 3**.

**Table 3. Best-fitted parameters for dose-response curves of DA in HEK293 cells transfected with hD1R, hD1-IL3$_{D5}$, hD5R and hD5-IL3$_{D1}$ in the absence and presence of PMA.**

| Receptor | Conditions | $E_{max}$ (% of Control) | $EC_{50}$ (nM) | $EC_{50}$ shift (PMA/Control) |
|---|---|---|---|---|
| hD1R | Control | 100 ± 4 | 15 (8.4 – 28) | |
| | PMA | 208 ± 5[a,c] | 41 (31 – 56)[a] | 2.7 (1.9 – 3.8)[c] |
| hD1-IL3$_{D5}$ | Control | 100 ± 4 | 13 (7.0 – 23) | |
| | PMA | 132 ± 6[a,b,c] | 192 (115 – 319)[a] | 16 (11 – 21)[b,c] |
| hD5R | Control | 100 ± 3 | 1.3 (0.7 – 2.5) | |
| | PMA | 118 ± 4[a,b] | 51 (30 – 87)[a] | 42 (28 – 62)[b] |
| hD5-IL3$_{D1}$ | Control | 100 ± 3 | 1.9 (1.0 – 3.5) | |
| | PMA | 191 ± 3[a,b,c] | 4.1 (2.9 – 5.7) | 2.2 (1.0 – 4.8)[c] |

Averaged dose-response curves (shown in **Fig. 5**) were analyzed by simultaneous curve fitting with a four-parameter logistic equation using GraphPad Prism. Best-fitted parameters for DA-mediated maximal activation of AC ($E_{max}$) are expressed as percentage of control ± S.E. while best-fitted values for $EC_{50}$ are reported with 95% confidence intervals obtained using simultaneous fitting of averaged curves from 5 experiments. $EC_{50}$ shift (PMA/control) values (expressed as the geometric mean with 95% confidence intervals) were computed from individual best-fitted dose-response curves. Statistical analysis of log transform of $EC_{50}$ shift values was performed using one-way ANOVA with Newman-Keuls post test. $B_{max}$ values for N-[methyl-$^3$H]-SCH23390 in pmol/mg membrane proteins (expressed as arithmetic means ± S.E.) were 1.0 ± 0.15 (hD1R), 0.76 ± 0.11 (hD1-IL3$_{D5}$), 1.1 ± 0.15 (hD5R) and 1.1 ± 0.22 (hD5-IL3$_{D1}$). $B_{max}$ values were not statistically different as assessed using one-way ANOVA (with Newman-Keuls post test).

[a]$p < 0.05$, control vs. PMA; [b]$p < 0.05$, PMA vs. PMA (hD1R); [c]$p < 0.05$, PMA vs. PMA (hD5R).

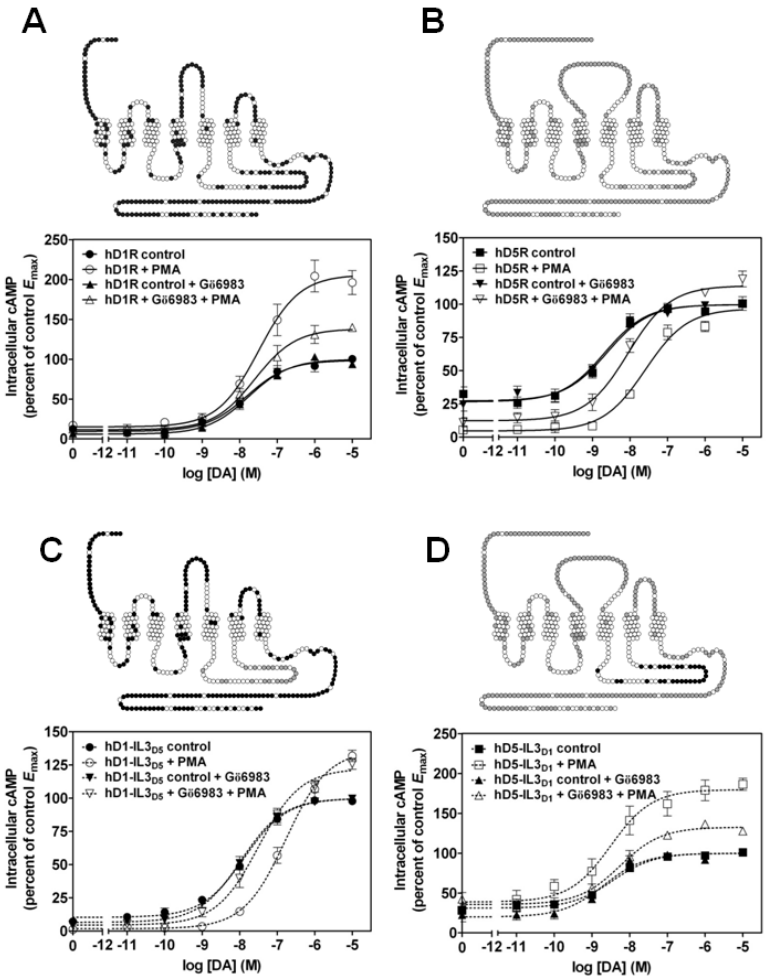


**Figure 6. Dose-response curves of DA for AC stimulation in HEK293 cells transfected with hD1R, hD5R, hD1-IL3$_{D5}$ and hD5-IL3$_{D1}$ in the absence and presence of PMA and Gö6983.** Schematic diagrams of hD1R, hD5R, hD1-IL3$_{D5}$ and hD5-IL3$_{D1}$ are represented. White circles depict identical residues between hD1R and hD5R while black circles and shaded circles represent unique residues found in hD1R and hD5R, respectively. HEK293 cells transfected with hD1R (**A**), hD5R (**B**) hD1-IL3$_{D5}$ (**C**) and hD5-IL3$_{D1}$ (**D**) were seeded in 12-well plates and labeled with 1 μCi/ml of [$^3$H]-adenine overnight. Cells expressing similar levels of wild type (solid line) and chimeric (dashed line) receptors were pretreated with 300 nM Gö6983 or with the vehicle (0.006% (v/v) DMSO) for 15 min at 37°C. Cells were then incubated in the absence (0.1 mM ascorbic acid) or presence of increasing concentrations of DA with 0.02% (v/v) DMSO (Control, filled symbols) or 1 μM PMA (open symbols) for 30 min. Each point is expressed as the arithmetic mean ± S.E. of 3-5 experiments done in triplicate and plotted as a function of log of DA concentrations. Each point for control and PMA treatments are expressed as percent of the best-fitted $E_{max}$ determined from the corresponding averaged control curve using raw data (CA/TU × 1000) and simultaneous curve fitting with GraphPad Prism. DA-mediated maximal activation ($E_{max}$) and $EC_{50}$ were obtained from best-fitted averaged curves (expressed as percent of control $E_{max}$) using a shared value of Hill slope. Pretreatment with Gö6983 leads to a significant reduction of PMA-induced increase of $E_{max}$ in cells expressing hD1R and hD5R-IL3$_{D1}$ ($p < 0.05$). Likewise, Gö6983 significantly inhibits PMA-induced $EC_{50}$ shift in cells expressing hD5R and hD1R-IL3$_{D5}$ ($p < 0.05$). $B_{max}$ values for N-[methyl-$^3$H]-SCH23390 in pmol/mg membrane proteins (expressed as arithmetic means ± S.E.) were 1.3 ± 0.27 (hD1R), 1.4 ± 0.24 (hD1-IL3$_{D5}$), 2.2 ± 0.43 (hD5R) and 1.9 ± 0.49 (hD5-IL3$_{D1}$). $B_{max}$ values were not statistically different as assessed using one-way ANOVA (with Newman-Keuls post test).

As discussed above, data obtained with chimeras harboring CT and TR suggest a potential role of the terminal region (EL3 and CT) in modulating PMA-induced hD1R sensitization and hD5R desensitization in receptor conformation-dependent manner (**Table 1** and **2**). We then ask if PMA-induced regulation of IL3 chimeras would be modulated following the exchange of both IL3 and TR using two new sets of chimeras: hD1-IL3TR$_{D5}$ and hD5-IL3TR$_{D1}$ (**Fig. 7C** and **D**). In contrast to hD1-IL3$_{D5}$ and hD5-IL3$_{D1}$, DA affinity of hD1-IL3TR$_{D5}$ (~0.2 μM) and hD5-IL3TR$_{D1}$ (~7 μM) is fully switched when compared to hD1R (~9 μM) and hD5R (~0.7 μM), respectively (Iwasiow et al., in preparation). PMA-induced hD1-IL3TR$_{D5}$ desensitization and hD5-IL3TR$_{D1}$ sensitization is highly reminiscent of PMA effects observed in cells expressing hD5R and hD1R, respectively (**Fig. 7**). In fact, as indexed using $EC_{50}$ shifts, PMA-induced desensitization of hD1-IL3TR$_{D5}$ was closer to that observed in cells transfected with hD5R (an $EC_{50}$ shift ratio of ~1.7 fold) than differences between $EC_{50}$ shifts of hD1-IL3$_{D5}$ versus hD5R (~3 fold; **Table 3** and **4**). Nevertheless, the exchange of IL3 and TR together leads to sensitization and desensitization patterns that are still not equivalent to those obtained with corresponding wild type parent receptors and IL3 chimeras (**Table 3** and **4**). Indeed, PMA-induced augmentation of $E_{max}$ in cells expressing hD5-IL3TR$_{D1}$ was lower than the increased $E_{max}$ in cells transfected with hD1R and hD5-IL3$_{D1}$ following PMA treatment (**Table 3** and **4**). Altogether, these results show a crucial role of IL3 in dictating the opposite regulation of DA-dependent hD1R and hD5R responsiveness by PKC. Additionally, our data indicate a potential role for a molecular interplay between IL3 and TR (EL3 and CT) in modulating PKC-induced regulation of DA-mediated responsiveness of human D1-like subtypes. Therefore, hD1R sensitization and hD5R

desensitization by PKC is possibly mediated in an agonist-promoted receptor conformation-dependent fashion. We cannot also rule out that other intracellular regions regulate agonist-promoted receptor conformation for PKC-induced regulation of D1-like subtype responsiveness. Another important unresolved issue of the PMA-induced regulation of hD1R and hD5R is whether similar receptor determinants orchestrate the PMA-induced regulation of agonist-independent (constitutive) activity of D1-like subtypes. In fact, we previously demonstrated that PMA treatment leads to an augmentation and reduction of the constitutive activity of hD1R and hD5R, respectively (Jackson et al. 2005). We then investigated the potential role of IL3 and CT in mediating PMA effects on constitutive activity of hD1R and hD5R.

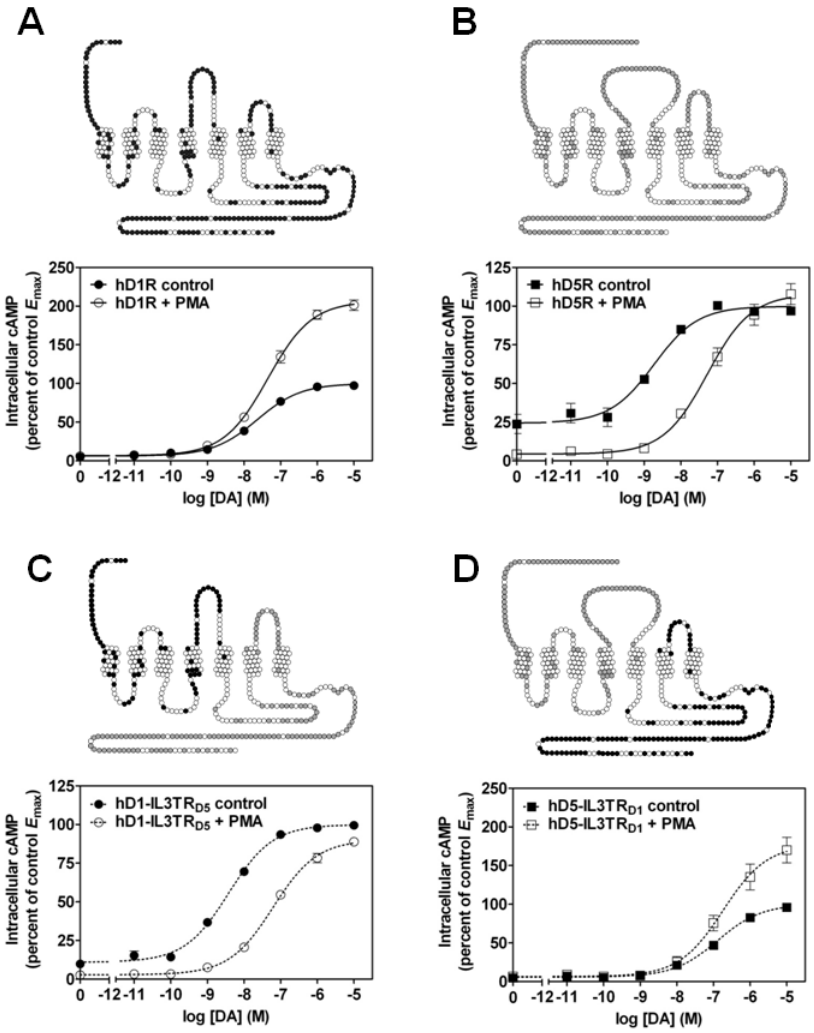


**Figure 7. Dose-response curves of DA for AC stimulation in HEK293 cells transfected with hD1R, hD5R, hD1-IL3TR$_{D5}$ and hD5-IL3TR$_{D1}$ in the absence and presence of PMA.**

Schematic diagrams of hD1R, hD5R, hD1-IL3TR$_{D5}$ and hD5-IL3TR$_{D1}$ are represented. White circles depict identical residues between hD1R and hD5R while black circles and shaded circles represent unique residues found in hD1R and hD5R, respectively. HEK293 cells transfected with hD1R (**A**), hD5R (**B**) hD1-IL3TR$_{D5}$ (**C**) and hD5-IL3TR$_{D1}$ (**D**) were seeded in 12-well plates and labeled with 1 μCi/ml of [$^3$H]-adenine overnight. Cells expressing similar levels of wild type (solid line) and chimeric (dashed line) receptors were incubated in the absence (0.1 mM ascorbic acid) or presence of increasing concentrations of DA with 0.02% (v/v) DMSO (Control, filled symbols) or 1 μM PMA (open symbols) for 30 min at 37°C. Each point is expressed as the arithmetic mean ± S.E. of 5 experiments done in triplicate and plotted as a function of log of DA concentrations. Each point for control and PMA treatments are expressed as percent of the best-fitted $E_{max}$ determined from the corresponding averaged control curve using raw data (CA/TU × 1000) and simultaneous curve fitting with GraphPad Prism. DA-mediated maximal activation ($E_{max}$) and $EC_{50}$ obtained from best-fitted averaged curves (expressed as percent of control $E_{max}$) using a shared value of Hill slope and $B_{max}$ values are reported in **Table 4**.

**Table 4. Best-fitted parameters for dose-response curves of DA in HEK293 cells transfected with hD1R, hD1-IL3TR$_{D5}$, hD5R and hD5-IL3TR$_{D1}$ in the absence and presence of PMA.**

| Receptor | Conditions | $E_{max}$ (% of Control) | $EC_{50}$ (nM) | $EC_{50}$ shift (PMA/Control) |
|---|---|---|---|---|
| hD1R | Control | 100 ± 4 | 23 (13 – 40) | |
| | PMA | 206 ± 4[a,c] | 45 (34 – 59)[a] | 2.0 (1.5 – 2.7)[c] |
| hD1-IL3TR$_{D5}$ | Control | 100 ± 3 | 3.8 (2.2 – 6.7) | |
| | PMA | 90 ± 4[a,b,c] | 62 (34 – 116)[a] | 17 (11 – 26)[b,c] |
| hD5R | Control | 100 ± 3 | 1.9 (1.0 – 3.6) | |
| | PMA | 108 ± 4[b] | 53 (32 – 89)[a] | 29 (17 – 49)[b] |
| hD5-IL3TR$_{D1}$ | Control | 100 ± 5 | 130 (71 – 237) | |
| | PMA | 177 ± 5[a,b,c] | 182 (128 – 259) | 1.5 (1.0 – 2.2)[c] |

Averaged dose-response curves (shown in **Fig. 7**) were analyzed by simultaneous curve fitting with a four-parameter logistic equation using GraphPad Prism. Best-fitted parameters for DA-mediated maximal activation of AC ($E_{max}$) are expressed as percentage of control ± S.E. while best-fitted values for $EC_{50}$ are reported with 95% confidence intervals obtained using simultaneous fitting of averaged curves from 5 experiments. $EC_{50}$ shift (PMA/control) values (expressed as the geometric mean with 95% confidence intervals) were computed from individual best-fitted dose-response curves. Statistical analysis of log transform of $EC_{50}$ shift values was performed using one-way ANOVA with Newman-Keuls post test. $B_{max}$ values for N-[methyl-$^3$H]-SCH23390 in pmol/mg membrane proteins (expressed as arithmetic means ± S.E.) were 1.0 ± 0.27 (hD1R), 1.2 ± 0.12 (hD1-IL3TR$_{D5}$), 1.0 ± 0.28 (hD5R) and 0.91 ± 0.26 (hD5-IL3TR$_{D1}$). $B_{max}$ values were not statistically different as assessed using one-way ANOVA (with Newman-Keuls post test).

[a]$p < 0.05$, control vs. PMA; [b]$p < 0.05$, PMA vs. PMA (hD1R); [c]$p < 0.05$, PMA vs. PMA (hD5R).

**Opposite PKC-induced regulation of DA-independent activity of hD1R and hD5R responsiveness is mediated through IL3 region**

We employed a transfection paradigm (5 μg receptor construct DNA/dish) to reach maximal achievable expression for wild type and chimeric receptors in HEK293 cells (Plouffe et al. 2010). This experimental approach allows detecting a more robust constitutive activity for wild type and chimeric receptors relative to mock-transfected cells as depicted in studies using raw data (**Fig. 8A**). Meanwhile, chimeric receptors display different $B_{max}$ levels. Indeed, hD1-$CT_{D5}$ and hD1-$IL3TR_{D5}$ display lower receptor expression in HEK293 cells (**Fig. 8B**). To address this issue, constitutive activity of wild type and chimeric receptors was normalized relative to $B_{max}$ as there is a linear relationship between $B_{max}$ and the extent of constitutive activity of GPCRs (Samama *et al.* 1993, Tiberi & Caron 1994, Jackson et al. 2000, Plouffe et al. 2010). Using this approach, we show that hD5R, hD1-$CT_{D5}$, hD1-$TR_{D5}$, hD1-$IL3_{D5}$ and hD1-$IL3TR_{D5}$ exhibit higher constitutive activity relative to hD1R (**Fig. 8C**) as previously reported (Iwasiow et al. 1999, Jackson et al. 2000, Tumova et al. 2003). On the other hand, agonist-independent activity of hD5-$CT_{D1}$, hD5-$TR_{D1}$, and hD5-$IL3TR_{D1}$ is significantly reduced relative to that of hD5R (**Fig. 8C**). In fact, the constitutive activity displayed by the three hD5R chimeras is either similar to or lower than hD1R basal activity (**Fig. 8C**). In contrast, the constitutive activity of hD5-$IL3_{D1}$ remains essentially unchanged as compared to cells expressing hD5R (**Fig. 8C**). Therefore, these chimeric receptors are critical not only in delineating the underlying structural determinants dictating the opposite regulation of agonist-independent activity of hD1R and hD5R by PMA but also

in assessing whether PMA effects are controlled by the extent of intrinsic receptor constitutive activity.

In this study, we recapitulated previous published findings showing that PMA treatment regulates in an opposing manner the basal activity of hD1R (increase) and hD5R (decrease) (**Fig. 8D**) (Jackson et al. 2005). Importantly, experiments using hD1-$IL3_{D5}$ and hD5-$IL3_{D1}$ clearly indicate that IL3 is the regulatory region underlying the opposite regulation of basal activity of hD1R and hD5R by PKC (**Fig. 8D**). Additionally, as demonstrated using dopamine dose-response curves, CT and TR structural determinants modulate the extent of PKC effects on agonist-independent activity of human D1-like receptors. However, it seems that CT and TR differentially regulate DA-independent and dependent activity of human wild type D1-like receptors (**Fig. 8D**). This view is supported by different experimental evidence as follows. First, the basal activity of hD1-$TR_{D5}$ is significantly increased in PMA-treated cells whereas constitutive activity of hD1-$CT_{D5}$ is not altered following PMA treatment (**Fig. 8D**). This is in contrast to data showing that PMA-induced sensitization of DA-dependent receptor responsiveness is retained in cells harboring hD1-$TR_{D5}$ and hD1-$CT_{D5}$ (**Fig. 3** and **4**). Second, constitutive activity of hD5-$IL3_{D1}$ is robustly enhanced following PMA treatment, which is in agreement with a role of IL3 in mediating enhanced basal activity by PMA (**Fig. 8D**). However, while hD5-$IL3TR_{D1}$ displays similar constitutive activity relative to hD1R in the absence of PMA; the PMA effect on basal activity of hD5-$IL3TR_{D1}$ is completely abrogated (**Fig. 8D**). In marked contrast, cells expressing hD5-$IL3TR_{D1}$ exhibit a significant augmentation of DA-mediated $E_{max}$ following PMA treatment (**Table 4**).

Collectively, our data strongly imply that IL3 dictates the D1-like subtype-specific regulation of constitutive activity by PKC in a receptor conformation-dependent manner through potential intramolecular interactions between EL3 and CT. In agreement with this notion, we previously demonstrated that distinct molecular interplays between EL3 and CT can impart multiple active conformations to D1-like receptors (Tumova et al. 2003). The exact molecular underpinnings involved in IL3-mediated PKC regulation require further investigation. In the meantime, our studies uncover a new GPCR paradigm whereby key PKC regulatory determinants of IL3 implicated in receptor sensitization process can be "turned off" by different agonist-independent receptor conformations. This indicates that the sensitization of GPCR responsiveness by PKC is more tightly controlled in the absence than in the presence of agonists.

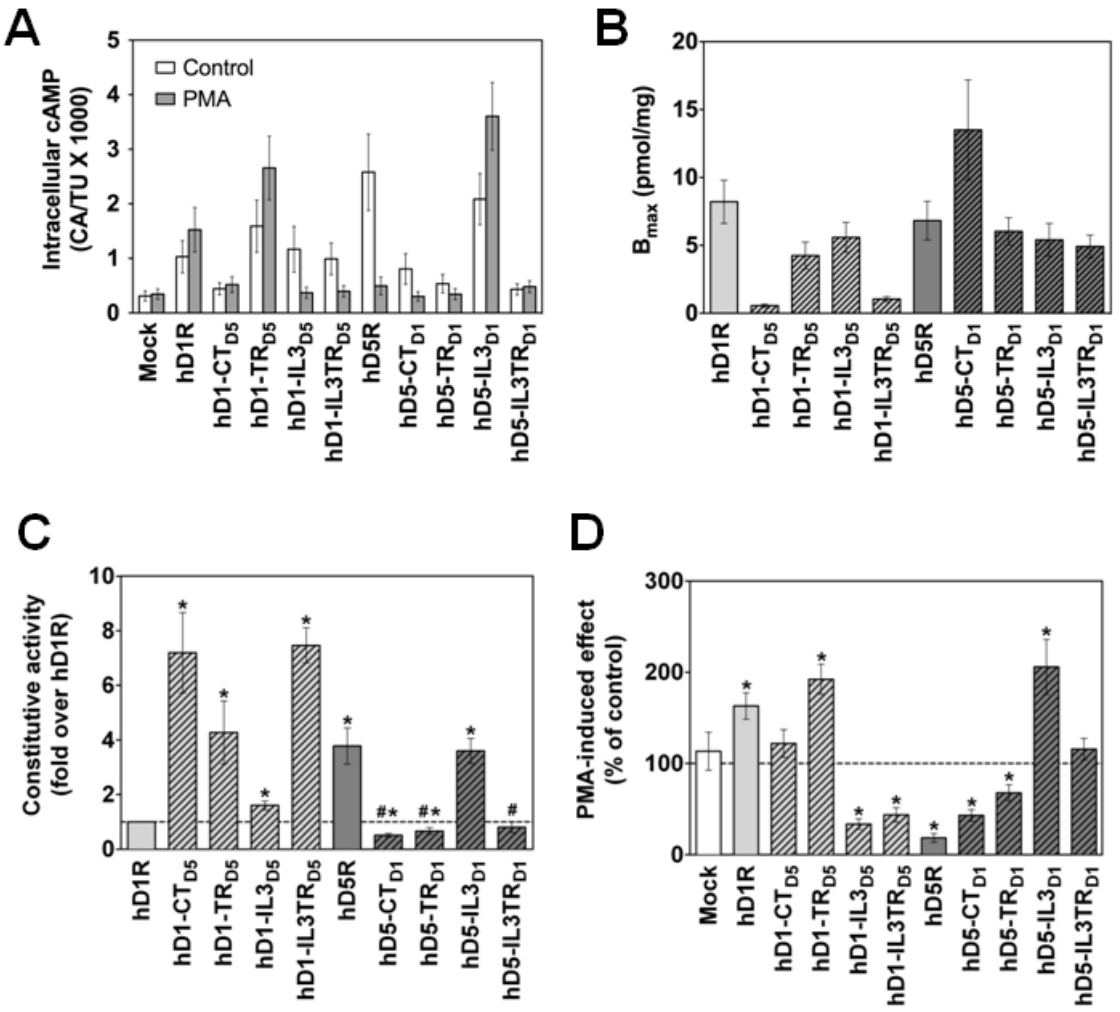


**Figure 8. Effect of PMA treatment on the constitutive activity of hD1R, hD5R and chimeric D1-like receptors expressed in HEK293 cells.**

HEK293 cells transfected with 5 μg /dish of empty pCMV5 expression vector (Mock) and receptor construct DNAs were seeded in 6-well plates and labeled with 2 μCi/ml of [$^3$H]-adenine overnight. Cells were incubated without DA in the presence of 0.02% (v/v) DMSO (Control) or 1 μM PMA for 30 min at 37°C. Results are expressed as the arithmetic mean ± S.E of 8 experiments done in triplicate. (**A**) Basal intracellular cAMP levels expressed as [$^3$H]-cAMP formed (CA) over the total uptake of [$^3$H]-adenine (TU) × 1000 in Mock- and receptor-transfected cells using raw data are reported. (**B**) $B_{max}$ values in pmol/mg membrane proteins for N-[methyl-$^3$H]-SCH23390 in cells transfected with wild type and chimeric receptors are shown. (**C**) Constitutive activity measured in control cells reported in *A* were corrected for $B_{max}$ values and expressed relative to hD1R. Dashed line represents the relative constitutive activity of hD1R set to 1. (**D**) PMA-induced effect on constitutive activity of wild type and chimeric receptors were expressed as percent of control. Dashed line depicts the control level set at 100 for PMA-induced effect.

*$p < 0.05$ when compared to values set to 1(C) or 100 (D) using one-sample *t* tests.

#$p < 0.05$ when compared to hD5R using one-way ANOVA with Newman-Keuls post test.

## Conclusions

In the current study, we highlight a critical role of IL3 in dictating the opposite regulation of the DA-independent and dependent activity of human D1-like receptors by PKC. Our studies also underscore the role of a potential molecular interplay between EL3 and CT in shaping IL3-induced modulation of hD1R and hD5R responsiveness by PKC. Additionally, our data clearly demonstrate that PKC-induced regulation of human D1-like receptor responsiveness is not linked to intrinsic agonist-independent and dependent G protein-coupling properties of hD1R and hD5R but is rather modulated in a GPCR conformation-dependent manner. Our study also challenges the prevailing view suggesting that the actions of PKCs rely exclusively on the presence of consensus PKC motifs whereas our data suggest that the substrate (GPCR) conformation may be important. Therefore, in a similar fashion to the modulation of GPCR activity by G protein-coupled receptor kinases, regulation of hD1R and hD5R responsiveness by PKC may also depend on receptor conformations. Overall, we believe that results reported in this study will be of broad relevance for PKC-mediated regulation of GPCRs.

**Research highlights of chapter 2-manuscript 1**

In this first manuscript, I demonstrated that:

- PMA-mediated hD1R sensitization and hD5R desensitization are not mediated by direct modulation of AC activity but through a regulation at the receptor level.
- IL3 is the key structural determinant that dictates the PMA-mediated opposite regulation of hD1R and hD5R responsiveness.
- Interplay between EL3 and CT potentially modulates IL3-dependent mechanisms of hD1R sensitization and hD5R desensitization upon PMA treatment.

These results suggest that PKC phosphorylation sites are located in IL3 region of hD1R and hD5R. In the next study, I performed single-point mutations of the serine and threonine located in IL3 of hD1R and hD5R using alanine and valine substitutions, respectively. Functional expression, ligand binding and G protein coupling properties properties of single-point mutants (25) were assessed in the next manuscript (manuscript 2) prior to testing them using the PMA paradigm.

# Chapter 3

# Manuscript 2

# Serine and Threonine Clusters in the Third Intracellular Loop of Dopamine D1-like Receptors Modulate Agonist Binding and Activation Properties

(Submitted to *PLoS One*)

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**Short title:** IL3 Mutagenesis of Human D1 and D5 Receptors

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The abbreviations used are: AC, adenylyl cyclase; AR, adrenergic receptors; $B_{max}$, maximal binding capacity; CAM, constitutively active mutant; CA; [$^3$H]-cAMP formed; CT, cytoplasmic tail; DA, dopamine; DHX, dihydrexidine; $EC_{50}$, half maximal effective concentration; $E_{max}$, maximal activation of AC; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; hD1R, human D1R; hD5R, human D5R; IL3, third intracellular loop; HEK, human embryonic kidney; $K_d$, equilibrium dissociation constant; $K_i$, equilibrium dissociation constant of cold drugs; MEM, minimum essential medium; PBS, phosphate-buffered saline; PD3, phosphorylation deficient form of IL3; TM, transmembrane; TU, total uptake.

**Statement of author contributions:**

BP conceived and designed research. BP performed research with the exception of the construction and the corresponding experiments with hD1-T213V, hD1-T215V, hD1-T273V, hD1-S275A, hD5-T244V, hD5-T246V, hD5-T297V and hD5-S299A which was done by BL. BP and MT analyzed data, wrote and edited the manuscript.

## Abstract

Dopamine produces its physiological effects through seven-transmembrane G protein-coupled receptors (GPCRs) grouped in D1-like (D1R and D5R) and D2-like (D2R, D3R and D4R) subtypes. D1-like and D2-like receptors are druggable targets regulating adenylyl cyclase activity by coupling to stimulatory (Gs) and inhibitory (Gi/o) GTP-binding proteins, respectively. Previous observations made by our lab and others raise the question of whether, in addition to their potential role in promoting receptor phosphorylation and desensitization, Ser/Thr residues of the third intracellular loop (IL3) of D1R and D5R can also serve as intrinsic structural elements for the basic ligand binding and G protein coupling properties of these two highly homologous GPCRs. In the present study, we performed single-point mutations of each Ser/Thr of IL3 (12 in D1R and 13 in D5R) and disclosed 3 functionally important conserved Ser/Thr clusters among human D1-like receptors. Notably, these conserved clusters are located in close proximity to key residues encompassing well-established structural microswitches for GPCR activation. Clusters located in the IL3/TM5 junction and cytoplasmic extension of IL3/TM6 junction positively regulate agonist affinity and agonist-independent and -dependent coupling to Gs. In contrast, the cluster found in the IL3/TM6 junction negatively modulates agonist affinity and constitutive activation of D1R and D5R. Interestingly, this cluster also plays a major role in restricting agonist-induced activation of D1R but not D5R. We also delineated a unique Ser cluster in D5R that potentially hinders a higher receptor constitutive activation. Lastly, results obtained with D1R and D5R harboring mutations of all Ser/Thr of IL3 suggest a complex interplay between these Ser/Thr clusters. In conclusion, our results show that IL3 harbors critical structural and

functional Ser/Thr implicated in the basic and subtype-specific mechanisms controlling agonist binding and activation of D1R and D5R. We believe our findings will be of broad relevance for the GPCR field.

## Introduction

Dopamine (DA) is a neurotransmitter playing a major a role in motor, cognition and emotional functions of the brain (Missale et al. 1998, Iversen & Iversen 2007). Neuropsychiatric pathologies such as schizophrenia, Parkinson's disease and drug addiction have been linked to dysfunction of cell-surface receptors controlling the activity of dopaminergic systems (Verhoeff 1999, Goldman-Rakic *et al.* 2004, Le Foll et al. 2009). Dopaminergic receptors are G protein-coupled receptors (GPCRs) and are divided into two subtype classes (Missale et al. 1998). D1-like receptors (D1R and D5R) couple to G protein αs subunit (Gαs) and stimulate adenylyl cyclase (AC) activity. Receptors belonging to the D2-like subtypes ($D2R_{short}$, $D2R_{long}$, D3R and D4R) bind to G protein αi/o subunits (Gαi/o) to inhibit AC activity (Missale et al. 1998). Interestingly, while the seven transmembrane helices (TM1-TM7) of D1R and D5R are nearly identical, these two D1-like subtypes exhibit distinct ligand binding and G protein coupling properties (Jarvie & Caron 1993, Tiberi & Caron 1994). The molecular basis underlying differences in these specific aspects of D1R and D5R functionality remains to be fully appreciated. This is an important issue as studies suggest that D1R and D5R may represent important therapeutic targets (Zhang *et al.* 2007a, Giorgioni *et al.* 2008, Zhang *et al.* 2009).

Studies with constitutively active mutant (CAM) forms of adrenergic receptors (ARs) have led to the formulation of the extended ternary GPCR model, which postulates that receptors in the absence of ligands oscillate between two conformations: an inactive state (R) and an active state (R*) (Samama et al. 1993, Costa & Cotecchia 2005, Cotecchia 2007). In its R state, coupling of the unliganded GPCR to G proteins is hindered and therefore the receptor cannot evoke effector activity. In contrast, following

an allosteric transition from R to R*, the GPCR acquires the conformational competence to couple to and activate G proteins in the absence of ligands culminating in unliganded receptor-induced effector activity in cells. This agonist-independent activity of GPCRs is also referred to as constitutive or basal activity. Importantly, the thermodynamic equilibrium between R and R* states can be pharmacologically regulated in a distinct manner by agonists and inverse agonists. Agonists exhibit higher affinity and stabilize R* state, which leads to enhanced GPCR-induced G protein stimulation. In an opposing manner, inverse agonists preferentially bind with high affinity to R state. Consequently, inverse agonists promote stabilization of R state and silencing of GPCR constitutive activity. Biophysical, biochemical and structural studies suggest that the allosteric transition between R and R* of most GPCRs is not behaving as bimodal switch but is controlled by a wide range of conformational intermediates or substates, which in turn can be stabilized by specific ligands in a multistep process (Kobilka & Deupi 2007, Rasmussen *et al.* 2007, Dror *et al.* 2009, Moukhametzianov *et al.* 2011).

Research using CAMs suggests that there is a wide range of constitutive activity among GPCRs of the same subfamily. Based on their pharmacological and signaling properties, CAM GPCRs can be considered as "improved" versions of their wild type counterparts. In fact, CAM GPCRs displays higher constitutive activity (ligand-independent activity), increased agonist affinity, intrinsic activity and potency (Samama et al. 1993, Costa & Cotecchia 2005, Cotecchia 2007). Natural mutations increasing GPCR constitutive activity have been associated with several pathological conditions such as congenital night blindness, retinitis pigmentosa, tumor formation, hyperthyroidism, dwarfism and male precocious puberty (Seifert & Wenzel-Seifert

2002). Interestingly, heterologous expression studies show that D5R behaves naturally like CAMs when compared to D1R (Tiberi & Caron 1994, Plouffe et al. 2010). Constitutive activity of D5R has also been implicated in the regulation of neuronal functions (Lee *et al.* 1999, Baufreton *et al.* 2003, Baufreton *et al.* 2005, Hersi et al. 2000, Laplante et al. 2004). Furthermore, a study has demonstrated that in a transgenic mouse model of Huntington disease (HD), striatal tissues display a drastic loss of D1R with a concomitant robust increase of D5R expression and elevation of intracellular cAMP levels (Ariano *et al.* 2002). As D5R is normally expressed at significant lower levels than D1R in striatum (Tiberi et al. 1991), results from HD mice thus suggest a potential link between the aberrant striatal cAMP levels in this HD mouse model and the higher constitutive activity and DA affinity of D5R. Meanwhile, the structural elements promoting subtype-specific differences in ligand binding and G protein coupling properties between D1R and D5R remain to be fully appreciated.

Site-directed mutagenesis of α1B-, α2- and β2-ARs showing that residues located in C-terminal portion of the third intracellular loop (IL3) systematically promote CAM phenotype suggest this cytoplasmic region of GPCRs is critical in regulating the allosteric transition between R and R* (Samama et al. 1993, Costa & Cotecchia 2005). This notion is also confirmed by genetic studies demonstrating that natural mutations in IL3 lead to CAM GPCRs linked to diseases (Seifert & Wenzel-Seifert 2002). Interestingly, the swap between two divergent residues in the C-terminal portion of IL3 of D1R (Phe264) and D5R (Iso288) led to a reciprocal partial exchange of constitutive activity and agonist affinity (Charpentier et al. 1996). While reinforcing the functional importance of the C-terminal portion of IL3 in shaping the transition from R to R* of GPCRs, this study also

underscored that mutations within this receptor region could not only increase but decrease intrinsic constitutive activity of GPCRs. Furthermore, the partial exchange effect following the swapping of D1R (Phe264) and D5R (Iso288) residues in IL3 suggests other structural elements are present within and/or outside IL3 to regulate the functional properties of D1R and D5R. In fact, studies using chimeric and truncated D1R and D5R point to the cytoplasmic tail (CT) as one of the intracellular regions besides IL3 that is critical in shaping subtype-specific agonist affinity and constitutive activity of D1-like receptors (Iwasiow et al. 1999, Jackson et al. 2000, Chaar *et al.* 2001, Tumova et al. 2004, Demchyshyn et al. 2000). We previously reported that D1R lacking its CT still undergoes desensitization in the absence of detectable receptor phosphorylation (Jackson et al. 2002). These findings suggest the absence of Ser/Thr phosphorylation process in IL3 of D1R. In fact, the precise role of IL3 phosphorylation in desensitization and intracellular sorting of D1-like receptors remains unclear (Jackson et al. 2002, Jiang & Sibley 1999, Mason *et al.* 2002, Thompson & Whistler 2011). These observations led us to wonder if beyond their potential role in mediating phosphorylation of D1R and D5R, Ser and Thr residues in IL3 could also play a more basic role in regulating D1-like subtype-specific ligand binding and G protein coupling properties. Interestingly, in silico studies of the chemokine receptors suggest that a Thr located at the cytoplasmic end of TM2 may participate in the formation of an ionic lock with a conserved Arg in TM3 to stabilize R state (Smith 2010).

To pinpoint further the role of individual Ser and Thr residues in IL3 of D1R and D5R we employed a single-point mutation strategy. Specifically, we have identified three main conserved Ser/Thr clusters in IL3 acting as positive and negative structural elements

for agonist affinity, constitutive activation and DA-dependent coupling to Gαs. Additionally, our results obtained with mutations of all Ser and Thr in IL3 of human D1R and D5R imply a potential intricate interplay between Ser/Thr clusters. Overall, our study suggests that conserved Ser/Thr clusters of IL3 have a permissive and restrictive role in shaping D1-like ligand binding properties, constitutive activation and agonist-induced responsiveness that are dependent on their location on IL3.

## Materials and Methods

### Reagents and Drugs

Minimal essential media (MEM), phosphate-buffered saline (PBS), gentamicin, trypsin-EDTA and sterile HEPES buffer (1M, pH 7.4) were from Invitrogen (Burlington, Ontario, Canada). Fetal bovine serum (FBS) was obtained from PAA Laboratories Inc. (Etobicoke, Ontario, Canada). Ascorbic acid, (+)-butaclamol, DA, *cis*-flupenthixol, cAMP, 1-methyl-3-isobutylxanthine (IBMX), thioridazine were bought from Sigma-Aldrich (Oakville, Ontario, Canada). Dihydrexidine (DHX) was from Tocris Bioscience (Ellisville, MO, USA). [$^{3}$H]-SCH23390 (~65-91 Ci/mmol) and [$^{3}$H]-adenine (~20 Ci/mmol) was acquired from PerkinElmer (Boston, MA, USA). [$^{14}$C]-cAMP (252 mCi/mmol) was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA, USA). Scintillation counting fluid was from RPI Corp. (Mount Prospect, IL, USA). Dowex AG 50W-4X resin was bought from Bio-Rad Laboratories Inc. (Mississauga, Ontario, Canada). Alumina N Super I was obtained from MP Biomedicals Canada (Montréal, Québec, Canada).

### Expression Constructs

Human D1-like receptors (hD1R and hD5R) were cloned in the pCMV5 expression vector. The Ser and Thr phosphorylation-deficient forms of IL3 of hD1R and hD5R (PD3-hD1R and PD3-hD5R) in pCMV5 were generated using restriction endonuclease digestions of wild type receptors and the full phosphorylation-deficient hD1R and hD5R constructs in pUC57 vector, which were custom made by Genscript Corp. (Piscataway, NJ, USA). Single-point mutations of Ser (Ser-to-Ala) and Thr (Thr-

to-Val) in IL3 were engineered using a polymerase chain reaction-based overlap extension approach with hD1R- and hD5R-pCMV5 expression constructs as DNA templates (*Supplementary Methods*). Custom DNA oligonucleotides were from Sigma Genosys (Burlington, Ontario, Canada). PCR primer sequences are listed in *Supplementary information* (*Tables S1-S2*). Coding sequences were verified by automated fluorescent DNA sequencing (Applied Biosystems 3730 DNA Analyzer) performed at the StemCore Laboratories of the Ontario Genomics Innovation Centre (Ottawa, Ontario, Canada).

**Supplementary Methods**

***Construction of single-point mutation of hD1R and hD5R.***

The human D1R and D5R subcloned in the expression vector pCMV5 were used as DNA templates. Two PCR products, A and B, were amplified separately using P1-P2 and P3-P4 primer pairs, respectively (Table S1-S2). An overlapping region between A and B allowed the amplification of the final PCR product using primers P5-P6 (Table S1-S2). PCR reactions using P1-P2 and P3-P4 primer pairs were carried out with Expand High Fidelity DNA polymerase (Roche Diagnostics, Laval, Canada) as follows: 1 cycle (94°C for 3 min, 50°C for 1 min, 72°C for 3 min), 25 cycles (94°C for 45 s, 50°C for 1 min, 72°C for 1 min) completed by an anneal extension step at 72°C for 8 min. Overlap PCR reactions performed with P5 and P6 primers were done using the following conditions: 1 cycle (94°C for 3 min, 50°C for 1 min, 72°C for 10 min), 25 cycles (94°C for 45 s, 50°C for 1 min, 72°C for 1 min) completed by an anneal extension step at 72°C for 8 min. PCR products were separated on 1-1.8% (w/v) agarose gel, excised and purified on QIAEX

resin (Qiagen). Purified DNA bands obtained from overlap PCR reactions were digested with appropriate restriction enzymes and ligated in-frame with the expression vector pCMV5 containing wild type human D1R or D5R DNA sequences. Mutant hD1R cassettes were subcloned in linearized hD1R-pCMV5 (*Hind*III-*Xba*I) to generate expression constructs for single-point hD1R mutants. Mutant hD5R cassettes were subcloned in linearized hD5R-pCMV5 (*Bsm*I-*Eag*I) to produce expression constructs for single-point hD5R mutants.

**Transfection and Cell Culture**

HEK293 cells (CRL-1573; ATCC, Manassas, VA, USA) seeded in 100-mm dishes ($2.5 \times 10^6$ cells/dish) were grown in complete MEM (supplemented with 10% (v/v) heat-inactivated FBS and 40 μg/ml of gentamicin) at 37°C in a humidified 5% $CO_2$ incubator and transfected using a modified calcium phosphate precipitation method as described (Plouffe et al. 2010). For radioligand binding and constitutive activity studies, cells were transfected using 5 μg of plasmid DNA per dish. For dose-response curve experiments, cells were transfected with 0.008-0.3 μg of plasmid DNA per dish except for hD1-T273V, which required 5 μg of plasmid DNA. Empty pCMV5 vector was used to normalize the total amount of DNA to 5 μg per 100-mm dish. After an overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells were washed with PBS, trypsinized and reseeded in 150-mm dishes (radioligand binding), 6-well plates (constitutive activity studies) or 12-well plates (dose-response curves) as described (Plouffe et al. 2010). In addition, for each expression constructs tested in constitutive activity and dose-response curve studies, one 100-mm dish was seeded with transfected

cells and utilized to assess receptor expression using a saturating concentration of [$^3$H]-SCH23390 (~7-12 nM). Cells used in radioligand binding studies were grown for an additional 48 h prior to membrane preparation. For cAMP assay studies, cells seeded in 6- and 12-well plates were grown in complete MEM until next day. Culture medium was then aspirated and cells incubated overnight in MEM (5% (v/v) FBS and 40 μg/ml gentamicin) containing [$^3$H]-adenine at 2 μCi/ml (constitutive activity studies) or 1 μCi/ml (dose-response curves).

**Membrane Preparation**

Transfected HEK293 cells were washed with cold PBS, harvested into ice-cold lysis buffer (10mM Tris-HCl, pH 7.4 and 5 mM EDTA), and centrifuged at 40,000 *g* for 20 min at 4°C. Crude membrane pellets were resuspended in lysis buffer using a Brinkmann Polytron (at a velocity of 17,000 rpm for 15 sec) and centrifuged at 40,000 *g* for 20 min at 4°C as described (Plouffe et al. 2010). The final pellet was resuspended in lysis buffer, and membranes were either used immediately (saturation studies) or frozen in liquid nitrogen and stored at -80°C until used (competition studies).

**Radioligand Binding**

Fresh or frozen membranes were diluted in resuspension buffer (62.5 mM Tris-HCl, pH 7.4 and 1.25 mM EDTA) and mixed briefly using a Brinkmann Polytron. All drugs were dissolved in milli-Q-water except DA, which was prepared in ascorbic acid (final concentration in assays: 0.1 mM). Binding reactions were carried out using 100 μl of membranes and 50 μl [$^3$H]-SCH23390 in the presence of 50 μl milli-Q-water or 50 μl

of cold drugs in a final volume of 500 µl (final in assays: 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM EDTA). Saturation studies were done using increasing concentrations of [$^3$H]-SCH23390 diluted in milli-Q-water (~0.01-12 nM). Total and nonspecific binding were assessed in the absence and presence of *cis*-flupenthixol at final concentration of 10 µM. For competition studies, frozen membranes were thawed on ice and incubated with a constant concentration of [$^3$H]-SCH23390 (~0.6-1.2 nM) and increasing concentrations of DA, dihydrexidine, (+)-butaclamol and thioridazine. Membranes were incubated for 90 min at room temperature and binding reactions terminated using rapid filtration through glass fiber filters (GF/C, Whatman). The filters were washed three times with 5 ml of cold washing buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl), and radioactivity bound to filters was quantified by liquid scintillation counting (Beckman, LS 6500). Protein concentrations were measured using Bio-Rad assay kit with bovine serum albumin as standard.

**Whole Cell cAMP Assay**

DA-independent and dependent regulation of AC activity by human wild type and mutant D1-like receptors were assessed in the presence of IBMX (final concentration in assays: 1 mM) for 30 min at 37ºC using whole cell cAMP assays performed with 6- and 12-well plates as detailed elsewhere (Plouffe et al. 2010). The amount of intracellular [$^3$H]-cAMP in each well of 6-well (constitutive activity) and 12-well plates (DA doses-response curves) was assessed from KOH-neutralized perchloric acid samples (containing 0.1 mM cAMP and ~3.3 nCi of [$^{14}$C]-cAMP as internal standard for column recovery), which were purified by a sequential chromatography over Dowex and alumina

columns (Plouffe et al. 2010). The amount of intracellular [$^3$H]-cAMP formed (CA) over the total amount of intracellular [$^3$H]-adenine (TU) was determined to calculate the relative AC activity and expressed as CA/TU × 1000. $B_{max}$ values were obtained using fresh membranes prepared in resuspension buffer and incubated with a saturating concentration (6-12 nM) of [$^3$H]-SCH23390 in the absence or presence 10 µM *cis*-flupenthixol (to delineate nonspecific binding).

**Data Treatment and Statistical Analysis**

Binding isotherms were analyzed using nonlinear curve fitting program from GraphPad Prism 5.03 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com) to determine the equilibrium dissociation constant ($K_d$, nM) and maximal binding capacity ($B_{max}$, pmol/mg membrane proteins) of [$^3$H]SCH23390 (saturation studies), and equilibrium dissociation constant of cold drugs ($K_i$, nM; competition studies). Geometric means of $K_d$ and $K_i$, and arithmetic means of $B_{max}$ are expressed with the lower and upper 95% confidence intervals. Constitutive activation values as well as affinity and $B_{max}$ ratios were computed as arithmetic means ± S.E. Raw data (CA/TU × 1000) measured for individual dose-response curves of DA in cells expressing similar levels of wild type and mutant receptors were analyzed using a four-parameter logistic equation from GraphPad Prism. Raw data were then normalized as percent of the best-fitted maximal activation of AC value ($E_{max}$) obtained with wild type receptor. Normalized data were averaged and curves analyzed again using simultaneous fitting with a shared slope parameter. Best-fitted parameters of $E_{max}$ with approximate S.E. and half maximal effective concentration of DA ($EC_{50}$) with 95% confidence

intervals are reported as calculated by GraphPad Prism. Curve fitting using unconstrained and constrained parameters were also employed to establish the statistical significance of differences between best-fitted values of wild type and mutant receptors. One-sample *t* test, unpaired *t* test and one-way ANOVA with Dunnett's post test were performed using GraphPad Prism with a critical level of probability of $\alpha = 0.05$.

**Table S1. Sequences of oligonucleotide primers for the construction of hD1R single-point mutations.** Mutated nucleotides leading to Ser-to-Ala or Thr-to-Val are underlined. Nucleotides introducing a silent mutation are in bold if applicable. Engineered restriction sites are in brackets.

| Construct | Oligonucleotide primers (5'→3') |
|---|---|
| hD1-T213V (*Acc*I) | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**: CCTGGTGTAGACGACAATCATGATGGCCACAGGGAT |
| | **P3**: ATCATGATTGTCGTCTACACCAGGATCTACAGGATT |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |
| hD1-T215V (***Box*I**) | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**: GATCCTGACGTAGGT**C**ACAATCATGATGGCCACAGG |
| | **P3**: ATGATTGT**G**ACCTACGTCAGGATCTACAGGATTGCT |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |
| hD1-T243V (*BstE*II) | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**: ACCTGTGGTGACCTGGCAATTCTTGGCGTGGACTGC |
| | **P3**: TGCCAGGTCACCACAGGTAATGGAAAGCCTGTCGAA |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |
| hD1-T244V (***Pst*I**) | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**: TGTGACGGTCTGGCAATTCTTGGCGTGGACTGC**A**GCCCTCTCCAAGGC |
| | **P3**: AGGGC**T**GCAGTCCACGCCAAGAATTGCCAGACCGTCACAGGTAATGGA |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |
| hD1-T245V (*Box*I) | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**: ATTACCGACGGTGGTCTGGCAATTCTTGGCGTGGAC |
| | **P3**: CAGACCACCGTCGGTAATGGAAAGCCTGTCGAATGT |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |
| hD1-S254A (*Fsp*I) | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**: CGGTTGCGCACATTCGACAGGCTTTCCATTACCTGT |
| | **P3**: GTCGAATGTGCGCAACCGGAAAGTTCTTTTAAGATG |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |

**Table S1.** *Continued*.

| Construct | Oligonucleotide primers (5'→3') |
|---|---|
| hD1-S258A (***Stu*I**) | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**: CTTAAAAGAGGC**C**TCCGGTTGAGAACATTCGACAGG |
| | **P3**: CAACCGGA**G**GCCTCTTTTAAGATGTCCTTCAAAAGA |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |
| hD1-S259A (***Eco47*III**) | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**: GGACATCTTAAAAGC**G**CTTTCCGGTTGAGAACATTC |
| | **P3**: GAAAG**C**GCTTTTAAGATGTCCTTCAAAAGAGAAACT |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |
| hD1-S263A (*Bsm*I) | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**: TTCTCTTTTGAATGCCATCTTAAAAGAACTTTCCGG |
| | **P3**: TTTAAGATGGCATTCAAAAGAGAAACTAAAGTCCTG |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |
| hD1-T268V (***Afl*II**) | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**: CTT**A**AGGACTTTAACTTCTCTTTTGAAGGACATCTT |
| | **P3**: AGAGAAGTTAAAGTCCT**T**AAGACTCTGTCGGTGATC |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |
| hD1-T273V (***Afl*II**) | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**: CACCGACAGAACCTT**A**AGGACTTTAGTTTCTCTTTT |
| | **P3**: ACTAAAGTCCT**T**AAGGTTCTGTCGGTGATCATGGGTGTG |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |
| hD1-S275A (***Afl*II**)) | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**: CATGATCACCGCCAGAGTCTT**A**AGGACTTTAGTTTCTCTTTTGAA |
| | **P3**: ACTAAAGTCCT**T**AAGACTCTGGCGGTGATCATGGGTGTGTTTGTG |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |

**Table S2. Sequences of oligonucleotide primers for the construction of hD5R single-point mutations.** Mutated nucleotides leading to Ser-to-Ala or Thr-to-Val are underlined. Nucleotides introducing a silent mutation are in bold if applicable. Engineered restriction sites are in brackets.

| Construct | Oligonucleotide primers (5'→3') |
|---|---|
| hD5-T244V (***Mlu*I**) | **P1**: TACGGTGGGAGG |
| | **P2**: GATGCGGTAGAT**A**CGCGTGTAGACCACGATCATGATGGCAAC |
| | **P3**: ATGATCGTGGTCTACACGCG**T**ATCTACCGCATCGCCCAGGTG |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |
| hD5-T246V (*Fsp*I) | **P1**: TACGGTGGGAGG |
| | **P2**: GTAGATGCGCACGTAGGTCACGATCATGATGGCAAC |
| | **P3**: ATCGTGACCTACGTGCGCATCTACCGCATCGCCCAG |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |
| hD5-S260A (***Xho*I**) | **P1**: TACGGTGGGAGG |
| | **P2**: CCTCTC**G**AGGGAGGCAATCCTGCGGATCTGCACCTG |
| | **P3**: AGGATTGCCTCCCT**C**GAGAGGGCCGCAGAGCACGCG |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |
| hD5-S261A (***Xho*I**) | **P1**: TACGGTGGGAGG |
| | **P2**: CTC**G**AGGGCGGAAATCCTGCGGATCTGCACCTGGGC |
| | **P3**: TCCGCCCT**C**GAGAGGGCCGCAGAGCACGCGCAGAGC |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |
| hD5-S271A (*Stu*I) | **P1**: TACGGTGGGAGG |
| | **P2**: CCGGCAGGCCTGCGCGTGCTCTGCGGCCCTCTCCAG |
| | **P3**: GCGCAGGCCTGCCGGAGCAGCGCAGCCTGCGCGCCC |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |

**Table S2.** *Continued*.

| Construct | Oligonucleotide primers (5'→3') |
|---|---|
| hD5-S274A (*Nhe*I) | **P1:** TACGGTGGGAGG |
| | **P2:** GGCTGCGCTAGCCCGGCAGCTCTGCGCGTGCTCTGC |
| | **P3:** AGCTGCCGGGCTAGCGCAGCCTGCGCGCCCGACACC |
| | **P4:** TCATGTGGATGTAGGCAG |
| | **P5:** ACCTGGCCAACTGGA |
| | **P6:** TGTTCACCGTCTCCA |
| hD5-S275A (*Eco47*III) | **P1:** TACGGTGGGAGG |
| | **P2:** GCAGGCTGCAGCGCTCCGGCAGCTCTGCGCGTGCTC |
| | **P3:** TGCCGGAGCGCTGCAGCCTGCGCGCCCGACACCAGC |
| | **P4:** TCATGTGGATGTAGGCAG |
| | **P5:** ACCTGGCCAACTGGA |
| | **P6:** TGTTCACCGTCTCCA |
| hD5-T282V (***Box*I**) | **P1:** TACGGTGGGAGG |
| | **P2:** CAG**A**CTGACGTCGGGCGCGCAGGCTGCGCTGCTCCG |
| | **P3:** CCCGACGTCAG**T**CTGCGCGCTTCCATCAAGAAGGAG |
| | **P4:** TCATGTGGATGTAGGCAG |
| | **P5:** ACCTGGCCAACTGGA |
| | **P6:** TGTTCACCGTCTCCA |
| hD5-S283A (***Fsp*I)** | **P1:** TACGGTGGGAGG |
| | **P2:** GCGCAGGGCGGTGTCGGG**T**GCGCAGGCTGCGCTGCTCCGGCAGCTCTG |
| | **P3:** GCCTGCGC**A**CCCGACACCGCCCTGCGCGCTTCCATCAAGAAGGAGACC |
| | **P4:** TCATGTGGATGTAGGCAG |
| | **P5:** ACCTGGCCAACTGGA |
| | **P6:** TGTTCACCGTCTCCA |
| hD5-S287A (***Box*I**) | **P1:** TACGGTGGGAGG |
| | **P2:** GATGGCAGCGCGCAG**A**CTGGTGTCGGGCGCGCAGGC |
| | **P3:** GCGCCCGACACCAG**T**CTGCGCGCTGCCATCAAGAAG |
| | **P4:** TCATGTGGATGTAGGCAG |
| | **P5:** ACCTGGCCAACTGGA |
| | **P6:** TGTTCACCGTCTCCA |
| hD5-T292V (***Afl*II**) | **P1:** TACGGTGGGAGG |
| | **P2:** GGTCTT**A**AGAACCTTGACCTCCTTCTTGATGGAAGC |
| | **P3:** AAGGAGGTCAAGGTTCT**T**AAGACCCTGTCGGTGATC |
| | **P4:** TCATGTGGATGTAGGCAG |
| | **P5:** ACCTGGCCAACTGGA |
| | **P6:** TGTTCACCGTCTCCA |

**Table S2.** *Continued*.

| Construct | Oligonucleotide primers (5'-3') |
|---|---|
| hD5-T297V | **P1**: TACGGTGGGAGG |
| | **P2**: CACCGACAGGACCTTAAGAACCTTGGTCTCCTTCTT |
| | **P3**: ACCAAGGTTCTTAAGGTCCTGTCGGTGATCATGGGG |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |
| hD5-S299A | **P1**: TACGGTGGGAGG |
| | **P2**: CATGATCACCGCCAGGGTCTTAAGAACCTTGGTCTCCTTCTT |
| | **P3**: ACCAAGGTTCTTAAGACCCTGGCGGTGATCATGGGGGTCTTC |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |

## RESULTS

The IL3 region of hD1R and hD5R contains 12 and 13 Ser/Thr residues, respectively (**Fig. 1**). The examination of IL3 sequences reveals that 7 Ser/Thr residues are fully conserved between hD1R and hD5R (**Fig. 1**). These residues are found in IL3/TM5 and IL3/TM6 junctions as well as in the cytoplasmic extension of IL3/TM6 (**Fig. 1**). Notably, these regions are critical for the structure and activation of family A GPCRs (Ahuja & Smith 2009, Rosenbaum *et al.* 2009, Hofmann *et al.* 2009, Smith 2010). Herein, we performed single-point mutations (Ser-to-Ala- and Thr-to-Val) on each Ser and Thr of IL3 of human D1-like receptors to assess their role (and more specifically the importance of hydroxyl group on the side chain) in modulating ligand binding and G protein coupling functions. We also generated two receptor constructs referred to as PD3-hD1R and PD3-hD5R, in which all Ser and Thr of IL3 were mutated to determine the overall contribution of these residues in regulating ligand binding and G protein-coupling properties of human D1-like receptors.

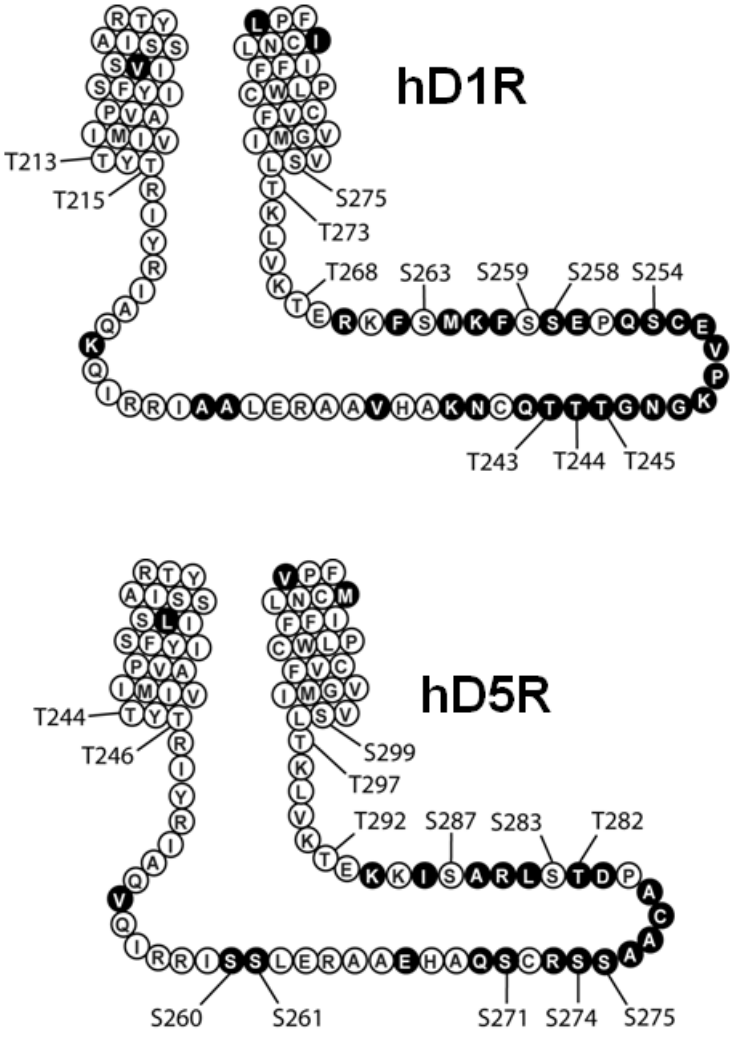


**Figure 1. Schematic diagram of receptor regions encompassing TM5, IL3 and TM6 of hD1R and hD5R.**
Amino acid sequence of hD1R (**top**) and hD5R (**bottom**) are shown. Identical amino acids between hD1R and hD5R are illustrated using white circles. Position of mutated Ser and Thr residues are indicated for each receptor.

**High affinity binding of the classical D1-like radioligand [$^3$H]-SCH23390 and functional expression of hD1R and hD5R mutants**

Saturation studies show that IL3 mutations have no major impact on the affinity of hD1R and hD5R for [$^3$H]-SCH23390 (**Table 1** and **Fig. 2A**). Additionally, the vast majority of $B_{max}$ measured with IL3 mutants were either unchanged or higher in comparison with values obtained in HEK293 cells expressing wild type receptors (**Table 1** and **Fig. 2B**). However, mutations of Ser and Thr residues located at the junction of IL3 and TM6 of hD1R (hD1-T273V and hD1-S275A) lead to a significant reduction of $B_{max}$ relative to the wild type receptor (**Table 1** and **Fig. 2B**). Similar mutations done in this region of hD5R promoted a lesser reduction (hD5-T297V) or no effect (hD5-S299A) on $B_{max}$ (**Table 1** and **Fig. 2B**). Interestingly, PD3-hD1R and PD3-hD5R express at a slightly higher $B_{max}$ than their respective wild type parent receptors (**Table 1** and **Fig. 2B)**. These results suggest mutations of all Ser and Thr residues of IL3 may counteract the negative regulation of $B_{max}$ observed in cells expressing hD1-T273V, hD1-S275A and hD5-T297V. This counteracting effect is potentially explained by the positive regulation of $B_{max}$ seen with other mutations, particularly Thr245Val and Thr292Val in hD1R and hD5R, respectively. Altogether, these results suggest that specific Ser and Thr in IL3 play distinct roles (positive and negative) in the regulation of hD1R and hD5R expression.

**Table 1. Equilibrium dissociation constants ($K_d$) and maximal binding capacity ($B_{max}$) values of [$^3$H]-SCH23390 for human wild type and mutant D1-like receptors**

| Receptors | $K_d$ (nM) | $B_{max}$ (pmol/mg) |
|---|---|---|
| **hD1R** | **0.52 [0.45-0.61]** | **12.7 [10.5-15.0]** |
| hD1-T213V | 0.61 [0.49-0.76] | 16.0 [12.4-19.5] |
| hD1-T215V | 0.70 [0.57-0.86] | 15.4 [11.1-19.6] |
| hD1-T243V | 0.42 [0.32-0.55] | 17.8 [10.7-25.0] |
| hD1-T244V | 0.40 [0.31-0.53] | 18.6 [12.9-24.2] |
| hD1-T245V | 0.43 [0.34-0.53] | 26.2 [17.9-34.6] |
| hD1-S254A | 0.35 [0.31-0.41] | 16.6 [11.2-22.0] |
| hD1-S258A | 0.31 [0.24-0.40] | 11.0 [7.4-14.6] |
| hD1-S259A | 0.39 [0.34-0.44] | 18.3 [14.8-21.8] |
| hD1-S263A | 0.36 [0.29-0.45] | 18.2 [13.7-22.8] |
| hD1-T268V | 0.35 [0.29-0.42] | 13.9 [7.3-20.6] |
| hD1-T273V | 0.49 [0.41-0.60] | 2.0 [1.4-2.5] |
| hD1-S275A | 0.50 [0.42-0.59] | 7.2 [5.5-8.9] |
| PD3-hD1R | 0.44 [0.34-0.57] | 17.3 [14.4-20.3] |
| **hD5R** | **0.78 [0.53-1.14]** | **10.9 [8.8-13.0]** |
| hD5-T244V | 1.04 [0.83-1.30] | 13.2 [8.5-17.8] |
| hD5-T246V | 1.33 [0.92-1.93] | 12.7 [8.4-17.0] |
| hD5-S260A | 0.42 [0.33-0.52] | 11.7 [9.4-14.0] |
| hD5-S261A | 0.44 [0.30-0.63] | 13.3 [8.6-18.0] |
| hD5-S271A | 0.45 [0.29-0.70] | 15.3 [11.2-19.3] |
| hD5-S274A | 0.49 [0.29-0.84] | 14.3 [6.9-21.6] |
| hD5-S275A | 0.42 [0.31-0.57] | 14.4 [9.1-19.6] |
| hD5-T282V | 0.43 [0.30-0.64] | 13.5 [8.8-18.1] |
| hD5-S283A | 0.42 [0.28-0.62] | 13.0 [9.3-16.6] |
| hD5-S287A | 0.37 [0.18-0.76] | 12.6 [9.4-15.8] |
| hD5-T292V | 0.46 [0.33-0.64] | 16.9 [13.6-20.1] |
| hD5-T297V | 0.87 [0.59-1.29] | 4.9 [3.3-6.5] |
| hD5-S299A | 1.05 [0.77-1.43] | 10.5 [7.7-13.2] |
| PD3-hD5R | 0.50 [0.33-0.76] | 13.4 [10.0-16.7] |

$K_d$ (nM) and $B_{max}$ values (pmol/mg of membrane proteins) are expressed as geometric and arithmetic means, respectively. Means are from four to eighteen experiments done in duplicate determinations with the lower and upper 95% confidence intervals in brackets.

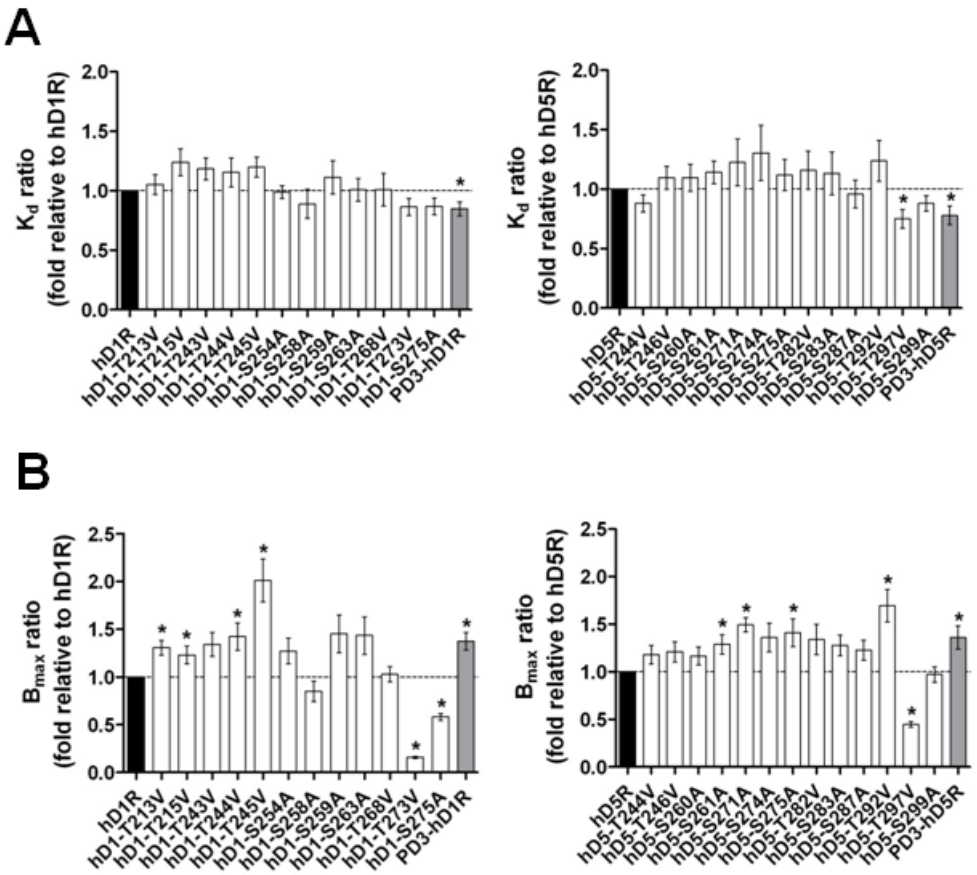


**Figure 2. Ratio values of equilibrium dissociation constant ($K_d$) and maximal binding capacity ($B_{max}$) of [$^3$H]-SCH23390 at hD1R and hD5R mutants.**
Individual raw $K_d$ (**A**) and $B_{max}$ (**B**) values of hD1R (**left**) and hD5R (**right**) mutants obtained with saturation curves of [$^3$H]-SCH23390 (averages are shown in **Table 1**) were expressed as fold change relative to their respective wild type receptor value. Ratios are reported as arithmetic means ± S.E. from four to eighteen experiments performed in duplicate determinations. $K_d$ and $B_{max}$ ratios of each mutant receptor were compared with wild type value set to 1 using one-sample *t* test. *, $p<0.05$.

**Ser and Thr of IL3 regulate agonist but not inverse agonist binding**

Affinity values of agonists (DA and DHX) and inverse agonists (thioridazine and (+)-butaclamol) for wild type and mutant forms of hD1R and hD5R are reported in **Table 2**. Mutations of Ser and Thr in IL3 mediated changes to agonist affinities while having no effect on inverse agonist binding (**Fig. 3**). Specifically, in both hD1R and hD5R, mutations of Ser and Thr residues in IL3/TM5 and IL3/TM6 junctions promoted opposite effects on agonist binding (**Fig. 3A**). Indeed, mutations of conserved Ser/Thr in IL3/TM6 junctions produced an increase in agonist affinity while mutations of the two conserved Thr in IL3/TM5 junctions led to a reduction of agonist affinity (**Fig. 3A**). Likewise, mutations of a conserved Thr in the cytoplasmic extension of IL3/TM6 (Thr268 in hD1R and Thr292 in hD5R) also lead to a loss of agonist affinity. Interestingly, the extent of agonist affinity reduction was stronger in hD5R than hD1R. Moreover, the overall contribution of Ser and Thr of IL3 in the regulation of agonist affinity is different between hD1R and hD5R. Losses and gains in agonist affinities mediated by individual mutations in hD1R likely neutralize each other in PD3-hD1R, which display no change in agonist affinity relative to hD1R (**Table 2** and **Fig. 3A**). In striking contrast, PD3-hD5R affinity for DA and DHX was significantly decreased in comparison with wild type parent receptor (**Table 2** and **Fig. 3A**). Results obtained with PD3-hD5R are in agreement with a greater reduction of agonist affinity following the mutations of Thr246 and Thr292 in hD5R (**Fig. 3A**). Altogether, these results suggest that Ser and Thr of IL3 play a more important role in the regulation of agonist affinity of hD5R than hD1R.

Constitutively active mutants (CAMs) of GPCRs have typically higher agonist affinity and produce a greater agonist-independent stimulation of downstream effectors

by virtue of their elevated basal activity (Samama et al. 1993, Costa & Cotecchia 2005). As stated previously, these CAM features are also recapitulated in cells expressing hD5R when compared with those harboring hD1R. We then asked whether changes observed in agonist affinity of Ser and Thr single-point mutants are also associated with changes in basal activity relative to their wild type parent receptor.

**Table 2. Equilibrium dissociation constant values ($K_i$) of unlabeled dopaminergic ligands for human wild type and mutant D1-like receptors.**

| Receptors | $K_i$ (nM) | | | |
|---|---|---|---|---|
| | **Dopamine** | **Dihydrexidine** | **Thioridazine** | **(+)-Butaclamol** |
| **hD1R** | **5606 [3465-9071]** | **402 [321-504]** | **61 [53-70]** | **4.3 [3.1-5.8]** |
| hD1-T213V | 13772 [8608-22035] | 632 [498-804] | 70 [46-106] | 3.8 [2.6-5.5] |
| hD1-T215V | 17549 [10241-30070] | 690 [578-824] | 76 [55-105] | 4.1 [2.8-6.1] |
| hD1-T243V | 2695 [1733-4192] | 212 [141-318] | 71 [47-109] | 7.7 [4.4-13] |
| hD1-T244V | 2842 [2301-3511] | 176 [118-260] | 69 [59-81] | 7.0 [4.4-11] |
| hD1-T245V | 3187 [2799-3629] | 285 [183-445] | 73 [59-90] | 7.4 [4.6-12] |
| hD1-S254A | 2385 [1678-3389] | 250 [202-310] | 57 [45-73] | 7.1 [3.4-15] |
| hD1-S258A | 1783 [1278-2488] | 193 [135-277] | 53 [39-73] | 4.9 [2.2-11] |
| hD1-S259A | 3333 [2141-5188] | 245 [182-331] | 54 [41-71] | 6.1 [4.1-9.3] |
| hD1-S263A | 3035 [2133-4319] | 248 [164-374] | 63 [46-87] | 6.3 [3.6-11] |
| hD1-T268V | 7049 [4821-10307] | 356 [263-484] | 49 [34-72] | 6.2 [4.1-9.5] |
| hD1-T273V | 3301 [2229-4890] | 236 [196-284] | 36 [25-53] | 2.5 [1.7-3.7] |
| hD1-S275A | 5673 [3568-9019] | 312 [221-441] | 62 [47-83] | 3.8 [2.0-7.3] |
| PD3-hD1R | 6493 [4073-10353] | 418 [308-569] | 44 [36-53] | 4.7 [2.0-11] |
| **hD5R** | **755 [503-1132]** | **39.5 [30-52]** | **200 [163-246]** | **24.8 [20.8-29.6]** |
| hD5-T244V | 1424 [996-2035] | 76.1 [48.5-120] | 209 [139-313] | 24.7 [18.2-33.7] |
| hD5-T246V | 5438 [3584-8251] | 199 [140-282] | 205 [160-262] | 30.6 [20.2-46.3] |
| hD5-S260A | 247 [212-288] | 22.9 [21.4-24.3] | 171 [117-251] | 25.1 [16.7-37.6] |
| hD5-S261A | 322 [216-482] | 28.0 [15.1-52.0] | 208 [146-295] | 27.6 [21.2-35.8] |
| hD5-S271A | 358 [213-604] | 27.3 [14.3-52.3] | 202 [164-250] | 25.0 [19.5-31.9] |
| hD5-S274A | 342 [188-622] | 31.0 [22.8-42.2] | 187 [136-257] | 25.5 [21.0-31.1] |
| hD5-S275A | 336 [195-577] | 28.5 [19.0-42.8] | 174 [117-258] | 24.3 [18.6-31.6] |
| hD5-T282V | 358 [216-594] | 26.0 [16.0-42.3] | 192 [111-331] | 28.2 [21.7-36.8] |
| hD5-S283A | 310 [183-526] | 25.2 [18.2-34.8] | 190 [139-259] | 24.8 [17.3-35.5] |
| hD5-S287A | 365 [196-680] | 30.4 [19.7-47.0] | 202 [131-310] | 22.9 [16.0-32.7] |
| hD5-T292V | 1214 [697-2113] | 92.7 [74.2-116] | 197 [129-300] | 26.4 [17.9-38.8] |
| hD5-T297V | 412 [271-627] | 24.1 [16.2-35.8] | 125 [90-172] | 19.1 [13.8-26.4] |
| hD5-S299A | 742 [567-972] | 44.4 [27.0-73.1] | 150 [108-210] | 25.0 [14.4-43.3] |
| PD3-hD5R | 1883 [928-3819] | 114 [86.4-150] | 153 [105-222] | 20.2 [15.6-26.1] |

$K_i$ (nM) are expressed as geometric means from four to sixteen experiments done in duplicate determinations with the lower and upper 95% confidence intervals in brackets.

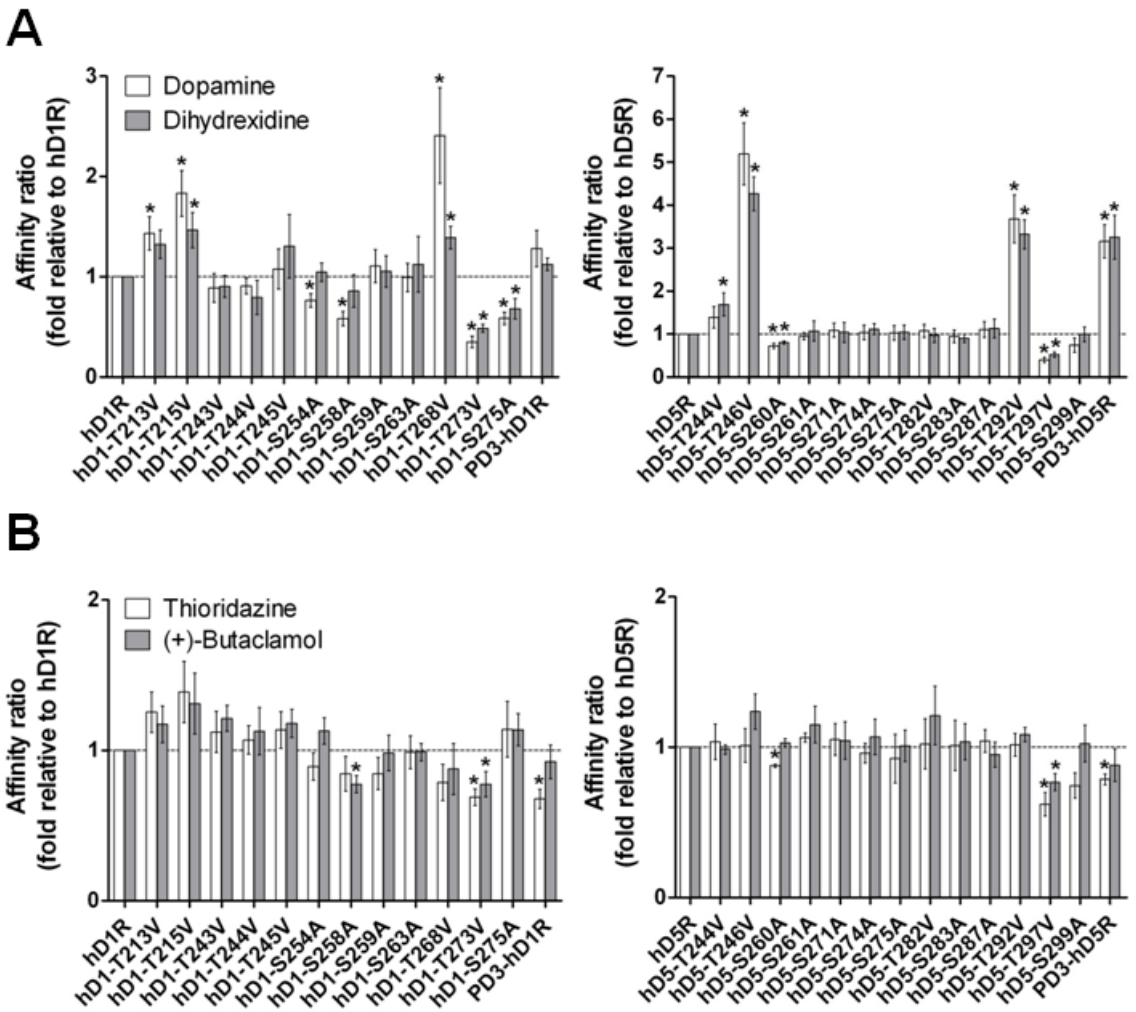


**Figure 3. Affinity ratios of dopaminergic agonists and inverse agonists at hD1R and hD5R mutants.**

Individual raw $K_i$ values of agonists (**A**) and inverse agonists (**B**) for hD1R (**left**) and hD5R (**right**) mutants obtained with competition curves (averages are shown in **Table 2**) were expressed as affinity ratio relative to their respective wild type receptor value. Affinity ratios of agonists (dopamine, dihydrexidine) and inverse agonists (thioridazine, (+)-butaclamol) are reported as arithmetic means ± S.E. from four to sixteen experiments performed in duplicate determinations. The affinity ratio of each mutant receptor was compared with wild type value set to 1 using one-sample *t* test. *, $p<0.05$.

**Constitutive activity of hD1R and hD5R is regulated by Ser and Thr of IL3 in a cluster localization-dependent manner.**

The impact of Ser and Thr mutations in IL3 on receptor constitutive activity was tested in cells expressing the highest achievable $B_{max}$ for wild type and mutant constructs as determined using saturation studies (**Table 1**). **Figure 4A** shows the results obtained from studies using averaged raw data. In a similar fashion to agonist binding data, we observe opposite effects of mutations on constitutive activity of human D1-like receptors that are dependent on the Ser/Thr localization in IL3 (**Fig. 4A**). To assess more accurately the impact of these changes, raw data for mock and mutants were expressed as fold values of their respective wild type receptor (**Fig. 4B**). Moreover, there is a well established linear relationship between the extent of GPCR basal activity and receptor expression levels (Samama et al. 1993, Tiberi & Caron 1994, Jackson et al. 2000, Ballesteros *et al.* 2001). Therefore, because several mutants exhibit different $B_{max}$ relative to their wild type parent receptor, the basal activity was also corrected for expression levels and then computed as fold of wild type value (**Fig. 4C**). Our normalized data suggest that Ser and Thr mutations leading to changes in constitutive activity can be grouped into activating and inactivating mutations of the basal activity of hD1R and hD5R (**Fig. 4C**). These mutations can be further refined according to the effect or lack thereof on agonist affinity (**Fig. 3A**) as discussed below. Altogether, our results imply that distinct Ser/Thr clusters of IL3 regulate constitutive activity of human D1-like receptors in a localization-specific fashion.

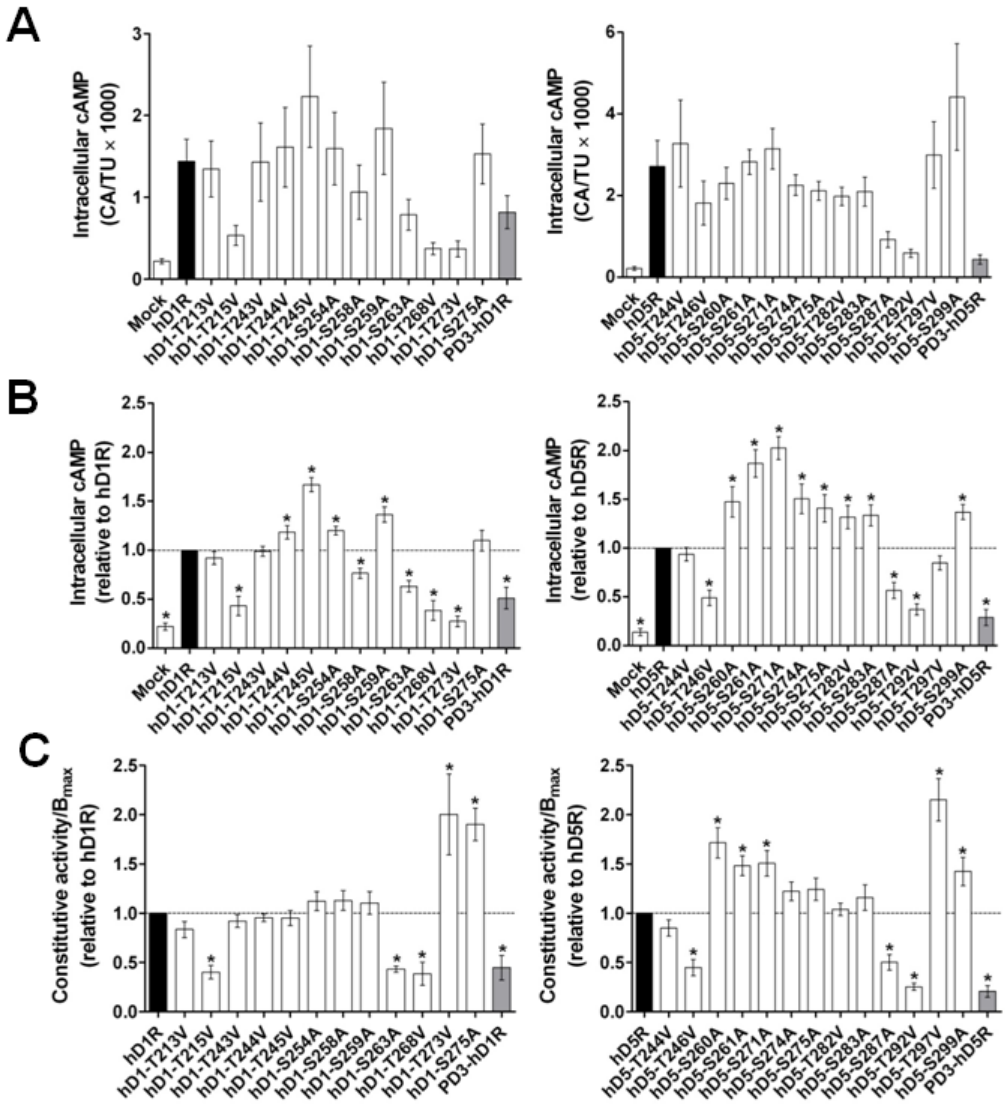


**Figure 4. Constitutive activity of human wild type and mutant D1-like receptors.**
HEK293 cells transfected with empty vector (mock) and receptor constructs were metabolically labeled with 2 μCi/ml of [$^3$H]-adenine overnight. Intracellular cAMP levels were measured in 6-well dishes in the absence of dopamine and expressed as [$^3$H]-cAMP formed (CA) over the total amount of [$^3$H]-adenine uptake (TU) × 1000. All values are reported as arithmetic means ± S.E. of four to eighteen experiments done in triplicate determinations. **A**, Raw data for basal intracellular cAMP levels in cells expressing wild type and mutant hD1R (**left**) and hD5R (**right**). **B**, Constitutive activity (raw data) of mutant hD1R and hD5R is expressed relative to their respective wild type receptor. **C**, Mutant receptor constitutive activity data shown in **B** were corrected for $B_{max}$ values. $B_{max}$ values in pmol/mg of membrane proteins (expressed as arithmetic mean ± S.E.) are as follows: 13.6 ± 0.8 (hD1R); 14.5 ± 1.4 (hD1-T213V); 13.4 ± 1.3 (hD1-T215V); 15.6 ± 0.8 (hD1-T243V); 17.7 ± 1.0$^{\#}$ (hD1-T244V); 25.7 ± 1.6$^{\#}$ (hD1-T245V); 15.7 ± 0.7 (hD1-S254A); 10.0 ± 1.0 (hD1-S258A); 18.3 ± 1.3$^{\#}$ (hD1-S259A); 20.8 ± 1.1$^{\#}$ (hD1-S263A); 14.8 ± 1.7 (hD1-T268V); 1.8 ± 0.2$^{\#}$ (hD1-T273V); 7.4 ± 0.6$^{\#}$ (hD1-S275A); 20.0 ± 1.0$^{\#}$ (PD3-hD1R); 8.9 ± 0.9 (hD5R); 10.3 ± 1.1 (hD4-T244V); 10.5 ± 1.3 (hD5-T246V); 6.8 ± 1.3 (hD5-S260A); 9.7 ± 1.5 (hD5-S261A); 10.5 ± 1.7 (hD5-S271A); 9.4 ± 1.5 (hD5-S274A); 9.0 ± 1.4 (hD5-S275A); 9.7 ± 1.6 (hD5-T282V); 9.2 ± 1.6 (hD5-S283A); 9.0 ± 1.6 (hD5-S287A); 11.4 ± 1.9 (hD5-T292V); 3.7 ± 0.4$^{\#}$ (hD5-T297V); 8.5 ± 0.8 (hD5-S299A); 10.3 ± 2.0 (PD3-hD5R). Statistical differences between $B_{max}$ values of mutants and their respective wild type receptor were determined using one-way ANOVA with Dunnett's post test. *, $p<0.05$ when compared with the constitutive activity value of wild type receptors set to 1 using one sample *t* tests. $^{\#}$, $p<0.05$ when compared with the $B_{max}$ value of their respective wild type receptor.

**Ser/Thr clusters in IL3/TM5 and IL3/TM6 junctions play an opposite role in the transition from R to R***

Mutations of Thr residues in the IL3/TM5 junction leading to agonist affinity loss mediated also a diminution of the basal activity of hD1R and hD5R (**Fig. 3A** and **4C**). In contrast, mutations of Ser/Thr in IL3/TM6 junction of hD1R and hD5R leading to increased agonist affinity promoted also a significant augmentation of the receptor basal activity (**Fig. 3A** and **4C**). Therefore, in the absence of agonists, Thr residues in IL3/TM5 junction (most predominantly Thr215 in hD1R and Thr246 in hD5R) may serve as positive elements facilitating the formation of the active state (R*) of hD1R and hD5R. Meanwhile, the Ser/Thr cluster in IL3/TM6 junction of hD1R (Thr273 and Ser275) and hD5R (Thr297 and Ser299) play a critical role in maintaining unliganded human D1-like dopaminergic receptors into an inactive state (R). Interestingly, $B_{max}$ of some of these CAMs was generally lower than that measured with their respective wild type receptor. The lower $B_{max}$ may be consistent with the decreased receptor stability that has been previously reported for other CAM GPCRs (Pei *et al.* 1994, Gether *et al.* 1997a, Rasmussen *et al.* 1999).

**Ser/Thr clusters in cytoplasmic extension of IL3/TM6 junction facilitate the transition from R to R***

Mutational and biophysical studies have previously highlighted the importance of the cytoplasmic extension of IL3/TM6 junction in regulating the constitutive activity of GPCRs (Samama et al. 1993, Costa & Cotecchia 2005, Charpentier et al. 1996, Ballesteros et al. 2001, Farrens *et al.* 1996, Gether *et al.* 1997b, Altenbach *et al.* 2008).

These studies have shown that mutations of residues in this cytoplasmic region of GPCRs systematically lead to CAMs. However, we previously showed that the reciprocal exchange of Phe264 (hD1R) and Ile288 (hD5R) located in the cytoplasmic extension of IL3/TM6 junction mediates a partial increase and decrease of the constitutive activity of hD1R and hD5R, respectively (Charpentier et al. 1996). In the present study, we demonstrated that mutations of conserved Ser and Thr in the cytoplasmic extension of IL3/TM6 junction of hD1R (Ser263 and Thr268) and hD5R (Ser287 and Thr292) culminated in a reduction of the constitutive activity, suggesting a positive role of these residues in the regulation of the transition of R (inactive) to R* (active) conformation of human D1-like receptors (**Fig. 4C**).

**The hD5R harbors unique Ser residues prohibiting higher constitutive activity**

So far, we have shown that Ser/Thr residues of IL3/TM5 and IL3/TM6 junctions as well as those found in the cytoplasmic extension of IL3/TM6 mediate changes in constitutive activity and agonist affinity. Given that Ser/Thr in these IL3 regions are fully conserved between hD1R and hD5R, our findings suggest that these residues are involved in a basic mechanism regulating the constitutive activation and agonist affinity of human D1-like receptors. However, these data do not explain subtype-specific differences that exist in agonist affinity and constitutive activation between hD1R and hD5R. Interestingly, mutations of Ser260, Ser261 and Ser271 located outside the TM5/IL3/TM6 junctions and cytoplasmic extension of IL3/TM6 of hD5R promoted an augmentation of the basal activity (**Fig. 4C**). These results suggest that Ser260, Ser261 and Ser271 of hD5R may hinder the stabilization of the unliganded receptor in its active

state. Interestingly, the corresponding residues in hD1R are "naturally mutated" into Ala (Ala229 and Ala230) or replaced with another weakly polar residue (Thr243). Thus, these unique Ser in hD5R may interfere with the expression of a higher constitutive activity for hD5R. Altogether, these findings raise an important question about how positive and negative Ser/Thr elements in IL3 globally shape the constitutive activation of hD1R and hD5R. This question was addressed using PD3-hD1R and PD3-hD5R harboring an IL3 region with all Ser/Thr mutated.

**Distinct functional interplays between Ser/Thr of IL3 promote the formation of agonist-independent hD1R and hD5R active states**

Mutations of all Ser/Thr within IL3 promoted a robust diminution of the constitutive activity of hD1R and hD5R (**Fig. 4C**). The basal activity reduction seen with PD3-hD1R is possibly explained by the additive effect of individual mutations (2 activating and 3 inactivating), which elicit 2-fold gains and 2-fold losses, respectively. However, in spite of having more single-point mutations leading to increase than decrease of agonist-independent activity (5 activating vs. 3 inactivating mutations), the basal activity of PD3-hD5R is drastically decreased by 80% relative to hD5R (**Fig. 4C**). This finding shows that Ser/Thr residues of IL3 largely contribute in a non additive manner to hD5R constitutive activity suggesting that inactivating mutations in IL3 of hD5R mediate a stronger effect over activating mutations. Thus, it implies that the positive Ser/Thr elements must prevail over the negative Ser/Thr elements in controlling the extent of hD5R constitutive activation. These results highlight some of the positive

structural elements of IL3 contributing to the higher basal activity of hD5R when compared to hD1R.

Overall, we have demonstrated that the structural and/or functional role of Ser/Thr of IL3 in the stabilization of R and R* is potentially mediated in a localization-dependent manner. As agonists stabilize R*, we next investigated the impact of IL3 mutations in controlling DA-induced activation of hD1R and hD5R.

**Mutations of Ser and Thr of IL3 reveal striking differences between DA-induced activation of hD1R and hD5R**

We show that the conserved Ser and Thr of IL3 between hD1R and hD5R have distinct functional roles in DA-induced activation properties (**Figs. 5-6**). Interestingly, these residues regulate in a subtype-specific manner DA-induced hD1R and hD5R stimulation while also mediating opposite effects according to their position relative to TM5 and TM6. On one hand, mutations of Thr213 and Thr215 in IL3/TM5 junction of hD1R decreased DA potency as indexed by rightward shifts in $EC_{50}$ (**Fig. 5** and **Table 3**). Likewise, mutations of the corresponding Thr in IL3 of hD5R (Thr244 and Thr246) led also to a loss in DA potency (**Fig. 6** and **Table 4**). These results suggest that Thr residues located in IL3/TM5 junction facilitate DA-induced coupling of human D1-like receptors to Gαs. On the other hand, mutations of Ser and Thr in IL3/TM6 junction and its cytoplasmic extension can be divided into residues either worsening (hD1R and hD5R) or improving (hD1R only) DA-induced receptor activation (**Figs. 5-6**, **Tables 3-4**). For instance, mutations of Ser/Thr residues located in cytoplasmic extension of IL3/TM6 junction (Ser263 and Thr268 in hD1R; Ser287 and Thr292 in hD5R) mediated rightward $EC_{50}$ shifts ranging from 2 to 13 fold relative to wild type parent receptors. Importantly,

the effects on $EC_{50}$ of DA promoted by mutations of these two residues were stronger in hD1R than hD5R (**Tables 3-4**). All mutations causing rightward shift in $EC_{50}$ of DA mediated also a loss of DA affinity. As agonist affinity and $EC_{50}$ (agonist potency) have been classically linked together, the loss of DA potency may be explained by the reduced DA affinity of these single-point mutants. However, differences between the extent of DA affinity shifts and $EC_{50}$ rightward shifts imply that these mutations have a greater impact on DA-induced Gαs coupling than DA binding. Moreover, the lower constitutive activity of these mutants suggests that Gαs coupling is diminished. In light of these findings, we believe that mutations of Ser and Thr reducing DA potency (notably for hD1R) are also associated with an uncoupling mechanism.

In an opposite fashion, mutations of Thr273 and Ser275 in IL3/TM6 junction of hD1R drastically increased the DA-induced receptor activation as documented by the higher $E_{max}$ (**Fig. 5** and **Table 3**). These results are consistent with the increased constitutive activity of hD1-T273V and hD1-S275A (**Fig. 4C**). Thus, Thr273 and Ser275 play a crucial role in restricting DA-independent and dependent activation of hD1R. Interestingly, mutations of these conserved Ser and Thr in hD5R (Thr297 and Ser299) had no effect on receptor ability to produce intracellular cAMP (**Fig. 6** and **Table 4**). Therefore, in striking contrast to hD1R, Ser and Thr in IL3/TM6 junction of hD5R do not play a role in restricting agonist-dependent stimulation of hD5R. Alternatively, these finding may suggest that the constraints imparted by these residues are more easily alleviated in hD5R following DA binding.

Furthermore, our study demonstrates that Ser and Thr located outside of IL3/TM5 and IL3/TM6 junctions, or cytoplasmic extension of IL3/TM6, have no major role in

regulating DA-induced activation of hD1R and hD5R (**Figs. 5-6**). This is intriguing with respect to hD5R as mutations of Ser260, Ser261 and Ser271 suggest that these residues restrain the transition of unliganded hD5R towards a more active conformation (**Fig. 6** and **Table 4**). These findings are similar to those observed with CAM hD5-T297V and hD5-S299A implying that an increase in basal activity does not necessarily lead to enhanced DA-induced coupling to Gαs. In general, these data show that agonist-induced stimulation of hD5R is significantly more resistant to allosteric changes brought by mutations of Ser/Thr of IL3 leading to higher constitutive activation. Taking into account that TM6 sequences of hD1-like receptors are virtually identical (**Fig. 1**), our results potentially highlight marked mechanistic differences in the DA-induced activation of hD1R and hD5R.

**Table 3. Best-fitted $EC_{50}$ and $E_{max}$ values of dopamine in cells expressing human wild type and mutant D1 receptors.**

| Receptors | $EC_{50}$ (nM) | $E_{max}$ (% of hD1R) | $B_{max}$ (pmol/mg) |
|---|---|---|---|
| hD1R | 14 [7.0-26] | 101 ± 4 | 1.7 ± 0.14 |
| hD1-T213V | 33 [16-69] | 96 ± 5 | 1.6 ± 0.26 |
| hD1R | 13 [7.1-23] | 99 ± 4 | 1.6 ± 0.09 |
| hD1-T215V | 53 [30-94]* | 105 ± 5 | 1.5 ± 0.17 |
| hD1R | 18 [6.6-48] | 99 ± 7 | 1.2 ± 0.29 |
| hD1-T243V | 14 [5.2-38] | 99 ± 6 | 1.4 ± 0.46 |
| hD1R | 11 [4.3-28] | 99 ± 6 | 1.9 ± 0.16 |
| hD1-T244V | 7.3 [2.8-19] | 94 ± 5 | 2.2 ± 0.65 |
| hD1R | 15 [9.5-24] | 96 ± 3 | 1.1 ± 0.27 |
| hD1-T245V | 15 [9.3-24] | 95 ± 3 | 1.6 ± 0.72 |
| hD1R | 12 [4.3-32] | 101 ± 6 | 2.1 ± 0.25 |
| hD1-S254A | 7.3 [2.7-20] | 101 ± 5 | 2.2 ± 0.65 |
| hD1R | 21 [12-37] | 98 ± 4 | 0.91 ± 0.32 |
| hD1-S258A | 23 [13-42] | 98 ± 4 | 0.79 ± 0.35 |
| hD1R | 12 [5.2-28] | 99 ± 5 | 1.8 ± 0.41 |
| hD1-S259A | 9.8 [4.2-23] | 101 ± 5 | 2.0 ± 0.63 |
| hD1R | 15 [9.9-22] | 99 ± 3 | 1.2 ± 0.16 |
| hD1-S263A | 43 [28-66]* | 93 ± 3 | 1.2 ± 0.22 |
| hD1R | 10 [4.6-22] | 99 ± 5 | 2.3 ± 0.65 |
| hD1-T268V | 133 [64-276]* | 104 ± 5 | 1.6 ± 0.40 |
| hD1R | 13 [1.7-99] | 101 ± 11 | 1.5 ± 0.13 |
| hD1-T273V | 16 [7.0-35] | 263 ± 14* | 1.3 ± 0.20 |
| hD1R | 14 [7.1-27] | 99 ± 4 | 1.6 ± 0.09 |
| hD1-S275A | 7.2 [4.3-12] | 129 ± 4* | 1.7 ± 0.16 |
| hD1R | 11 [7.1-17] | 97 ± 3 | 1.5 ± 0.22 |
| PD3-hD1R | 42 [25-73]* | 84 ± 3* | 1.3 ± 0.27 |

Best-fitted parameters for $EC_{50}$ and $E_{max}$ from averaged dose-response curves of DA shown in **Figs. 5** and **7A** are reported. $EC_{50}$ values are expressed with 95% confidence intervals in brackets while $E_{max}$ values are reported as percent of hD1R value ± approximate S.E. as obtained with GraphPad Prism software. Statistical significance between matched hD1R and mutant forms was determined by comparing best-fitted curve using unconstrained and constrained parameters for a given parameter. $B_{max}$ values in pmol/mg membrane proteins for wild type and mutant receptors are expressed as arithmetic means ± S.E. Differences between $B_{max}$ are not statistically different as assessed using unpaired *t* test ($p > 0.05$). *, $p < 0.05$.

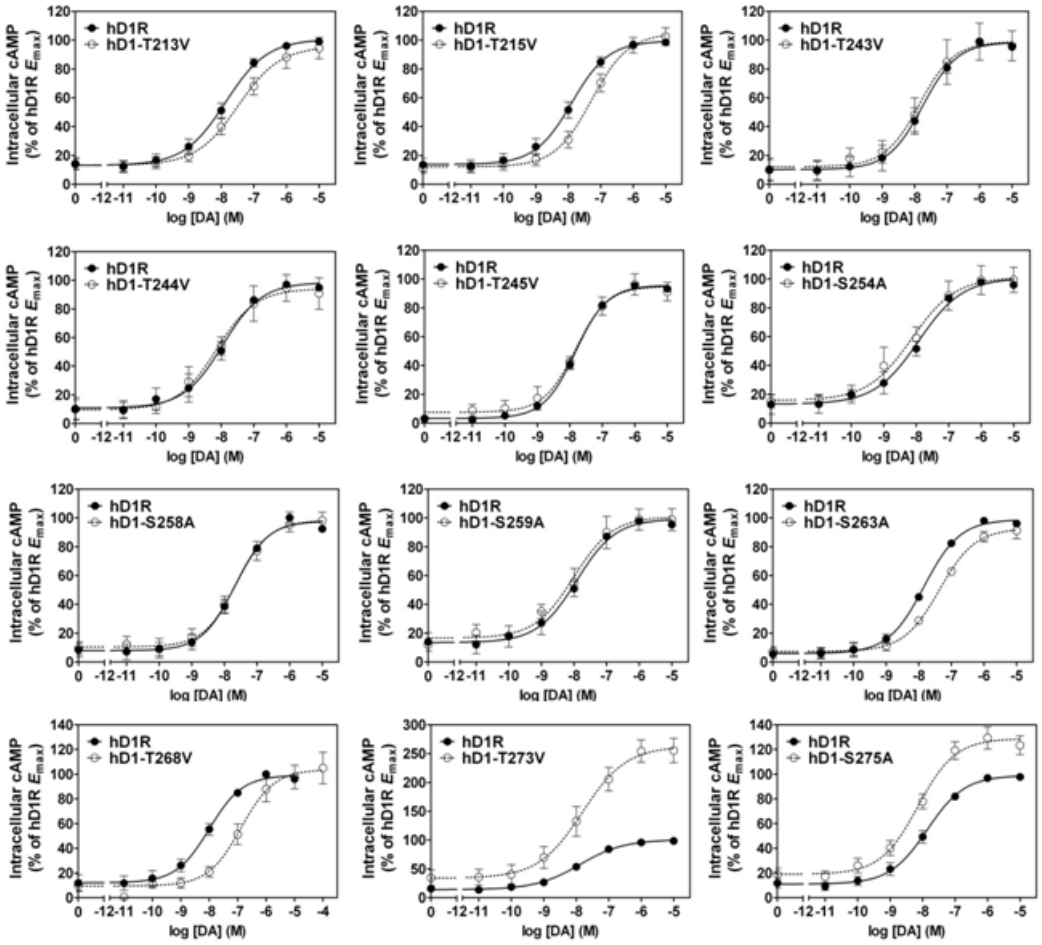


**Figure 5. DA-mediated stimulation of adenylyl cyclase activity by hD1R and single-point mutants.**

HEK293 cells transfected with wild type (solid line, filled circles) and single-point mutant (broken line, open circles) hD1R constructs were metabolically labeled with 1 μCi/ml of [$^3$H]-adenine overnight. Intracellular cAMP levels measured in 12-well dishes in the presence of increasing DA concentrations were computed as [$^3$H]-cAMP formed (CA) over the total amount of [$^3$H]-adenine uptake (TU) × 1000 (CA/TU × 1000). CA/TU × 1000 values of wild type and single-point mutant hD1R were expressed as the percentage of best-fitted $E_{max}$ of the wild type receptor obtained with raw data. Each point represents the arithmetic mean ± S.E. of four to twelve experiments done in triplicate determinations and curves are plotted as a log function of DA concentrations. $B_{max}$ and best-fitted parameters for $EC_{50}$ and $E_{max}$ are reported in **Table 3**. DA, dopamine.

**Table 4. Best-fitted $EC_{50}$ and $E_{max}$ values of dopamine in cells expressing human wild type and mutant D5 receptors.**

| Receptors | $EC_{50}$ (nM) | $E_{max}$ (% of hD5R) | $B_{max}$ (pmol/mg) |
|---|---|---|---|
| hD5R | 0.95 [0.54-1.7] | 99 ± 3 | 2.4 ± 0.25 |
| hD5-T244V | 1.8 [1.1-3.2] | 99 ± 3 | 2.4 ± 0.31 |
| hD5R | 0.91 [0.50-1.7] | 99 ± 3 | 2.3 ± 0.25 |
| hD5-T246V | 4.9 [2.8-8.7]* | 97 ± 3 | 2.5 ± 0.38 |
| hD5R | 0.70 [0.35-1.4] | 99 ± 3 | 2.1 ± 0.26 |
| hD5-S260A | 0.44 [0.22-0.88] | 109 ± 3* | 2.1 ± 0.20 |
| hD5R | 1.0 [0.59-1.8] | 99 ± 3 | 1.8 ± 0.20 |
| hD5-S261A | 0.99 [0.57-1.8] | 97 ± 3 | 1.8 ± 0.22 |
| hD5R | 0.95 [0.56-1.6] | 99 ± 3 | 1.8 ± 0.25 |
| hD5-S271A | 0.88 [0.51-1.6] | 101 ± 3 | 2.0 ± 0.35 |
| hD5R | 0.86 [0.53-1.4] | 98 ± 3 | 1.7 ± 0.20 |
| hD5-S274A | 0.86 [0.53-1.4] | 99 ± 3 | 1.5 ± 0.25 |
| hD5R | 0.62 [0.31-1.2] | 99 ± 3 | 2.0 ± 0.26 |
| hD5-S275A | 0.50 [0.25-1.0] | 99 ± 3 | 1.8 ± 0.22 |
| hD5R | 0.87 [0.47-1.6] | 99 ± 3 | 1.9 ± 0.42 |
| hD5-T282V | 0.84 [0.44-1.6] | 99 ± 3 | 2.0 ± 0.52 |
| hD5R | 0.52 [0.29-0.92] | 99 ± 3 | 1.8 ± 0.30 |
| hD5-S283A | 0.37 [0.20-0.68] | 100 ± 3 | 1.8 ± 0.35 |
| hD5R | 0.96 [0.43-2.1] | 99 ± 4 | 1.6 ± 0.38 |
| hD5-S287A | 1.9 [0.81-4.5] | 94 ± 4 | 1.3 ± 0.25 |
| hD5R | 0.77 [0.41-1.5] | 99 ± 3 | 1.9 ± 0.32 |
| hD5-T292V | 4.3 [2.2-8.4]* | 95 ± 4 | 1.6 ± 0.28 |
| hD5R | 0.92 [0.53-1.6] | 99 ± 3 | 2.3 ± 0.25 |
| hD5-T297V | 0.85 [0.49-1.5] | 104 ± 3 | 2.2 ± 0.33 |
| hD5R | 0.95 [0.52-1.7] | 99 ± 3 | 2.5 ± 0.23 |
| hD5-S299A | 0.95 [0.52-1.7] | 105 ± 3 | 2.7 ± 0.27 |
| hD5R | 0.95 [0.69-1.3] | 98 ± 2 | 1.7 ± 0.19 |
| PD3-hD5R | 5.1 [3.8-6.8]* | 114 ± 2* | 1.7 ± 0.24 |

Best-fitted parameters for $EC_{50}$ and $E_{max}$ from averaged dose-response curves of DA shown in **Fig. 6** and **7B** are reported. $EC_{50}$ values are expressed with 95% confidence intervals in brackets while $E_{max}$ values are reported as percent of hD5R value ± approximate S.E. as obtained with GraphPad Prism software. Statistical significance between matched hD5R and mutant forms was determined by comparing best-fitted curve using unconstrained and constrained parameters for a given parameter. $B_{max}$ values in pmol/mg membrane proteins for wild type and mutant receptors are expressed as arithmetic means ± S.E. Differences between $B_{max}$ are not statistically different as assessed using unpaired *t* test ($p > 0.05$). *, $p < 0.05$.

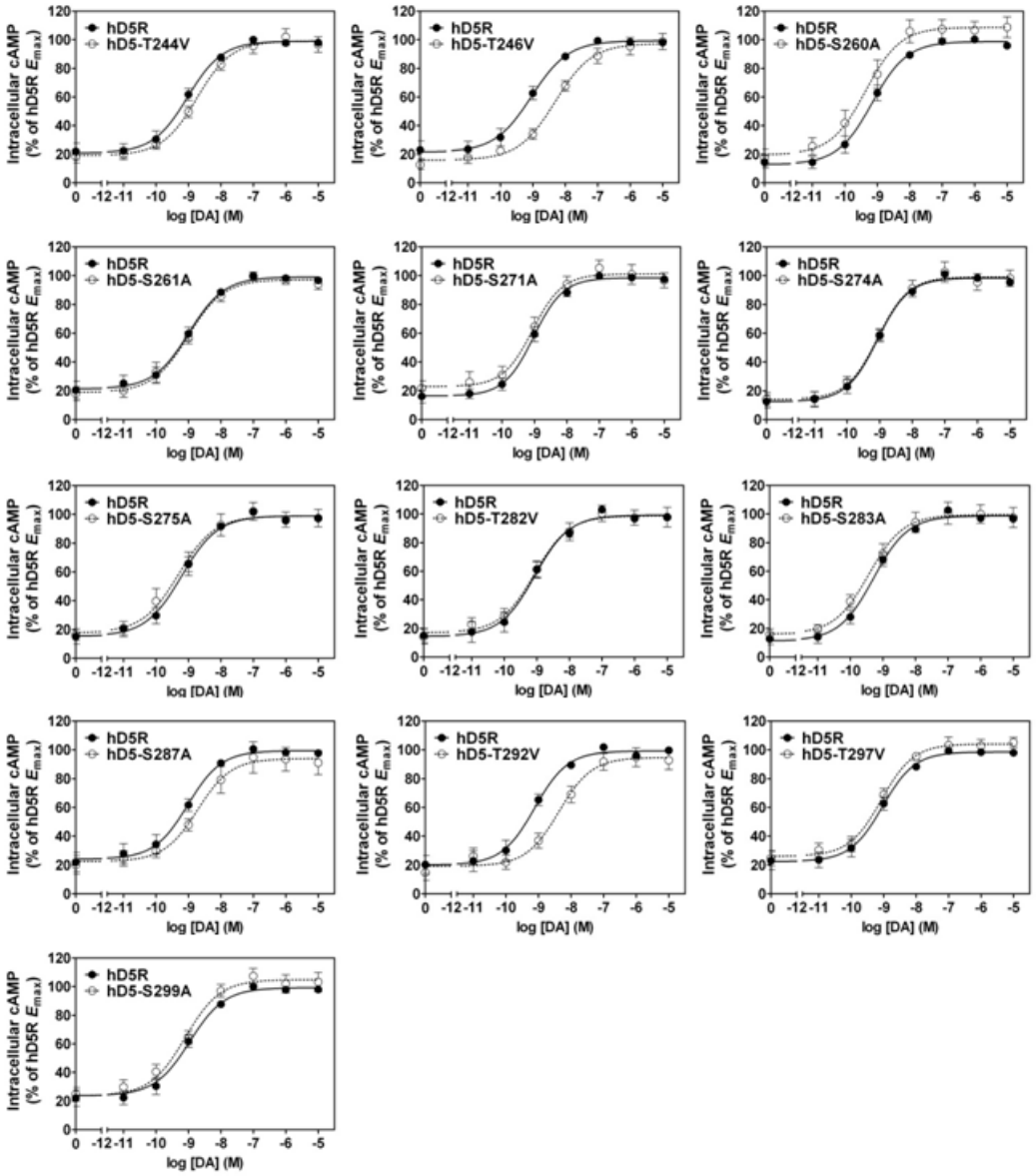


**Figure 6. DA-mediated stimulation of adenylyl cyclase activity by hD5R and single-point mutants.**

HEK293 cells transfected with wild type (solid line, filled circles) and single-point mutant (broken line, open circles) hD5R constructs were metabolically labeled with 1 μCi/ml of [$^3$H]-adenine overnight. Intracellular cAMP levels measured in 12-well dishes in the presence of increasing DA concentrations were computed as [$^3$H]-cAMP formed (CA) over the total amount of [$^3$H]-adenine uptake (TU) × 1000 (CA/TU × 1000). CA/TU × 1000 values of wild type and single-point mutant hD5R were expressed as the percentage of best-fitted $E_{max}$ of the wild type receptor obtained with raw data. Each point represents the arithmetic mean ± S.E. of four to twelve experiments done in triplicate determinations and curves are plotted as a log function of DA concentrations. $B_{max}$ and best-fitted parameters for $EC_{50}$ and $E_{max}$ are reported in **Table 4**. DA, dopamine.

**Distinct functional interplays between Ser/Thr of IL3 regulate DA-induced activation of hD1R and hD5R**

Dose-response curves with single-point mutants raised two important issues regarding the overall contribution of Ser/Thr residues of IL3 in DA-induced activation of hD1R and hD5R. First, our study indicates that different Ser/Thr residues have opposite roles in DA-induced hD1R activation. Second, most of single-point mutations had no major effect on DA-mediated activation of hD5R vis-à-vis the number of mutations altering basal activity. We then wondered whether multiple Ser/Thr residues are required in shaping DA-induced activation of human D1-like receptors using PD3-hD1R and PD3-hD5R. The lower $E_{max}$ and rightward shift in $EC_{50}$ for DA in cells expressing PD3-hD1R suggest a decreased DA-induced PD3-hD1R responsiveness relative to the wild type receptor (**Fig. 7A** and **Table 3**). These effects cannot be easily reconciled with the robust increase in $E_{max}$ seen with single-point mutations of Thr273 and Ser275 in IL3 of hD1R (**Fig. 5** and **Table 3).** Furthermore, cells expressing PD3-hD5R display a rightward shift in $EC_{50}$ and a slight $E_{max}$ increase for DA in comparison to hD5R-transfected cells (**Fig. 7B** and **Table 4**). The increase in $E_{max}$ of PD3-hD5R is potentially explained by the mutation of Ser260 in IL3 (**Fig. 6** and **Table 4**). However, the extent of rightward shift in $EC_{50}$ of DA (~5 fold) in cells transfected with PD3-hD5R is not in line with additive or synergistic effects mediated by individual Ser/Thr mutations. Indeed, the two strongest single-point uncoupling mutations in hD5R (hD5-T246V and hD5-T292V) both promote a 5-fold rightward shift in $EC_{50}$ value. Notwithstanding these unresolved issues, the global change mediated by mutations of all Ser/Thr of IL3 clearly culminates in the inhibition of DA-mediated hD1R and hD5R activation through a complex combinatorial

effect. Meanwhile, how changes measured in DA-induced activation of single-point mutants mechanistically translate into the overall change noted in DA-mediated activation of PD3-hD1R and PD3-hD5R is a challenging question that requires further studies.

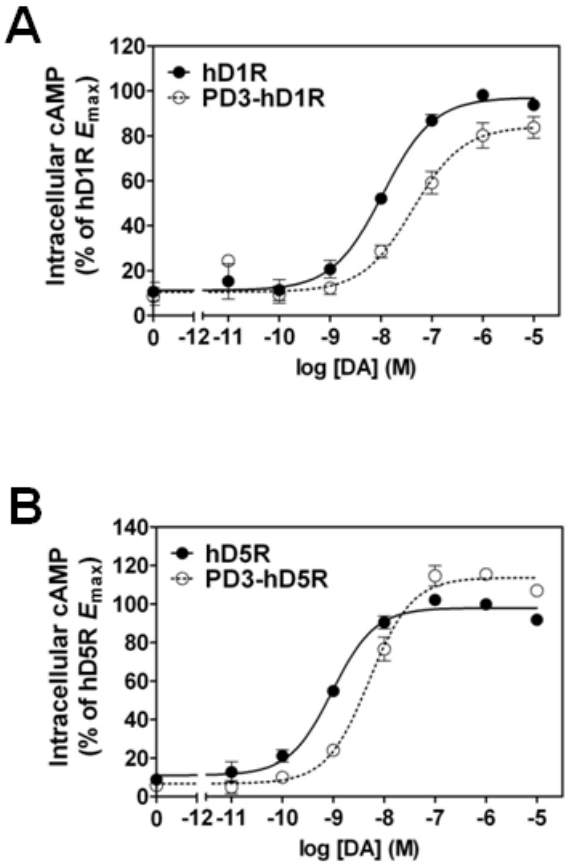


**Figure 7. DA-mediated stimulation of adenylyl cyclase activity by PD3-hD1R and PD3-hD5R.**

HEK293 cells transfected with wild type (solid line, filled circles) and PD3 receptor constructs (broken line, open circles) were metabolically labeled with 1 μCi/ml of [$^3$H]-adenine overnight. Intracellular cAMP levels measured in 12-well dishes in the presence of increasing DA concentrations were computed as [$^3$H]-cAMP formed (CA) over the total amount of [$^3$H]-adenine uptake (TU) × 1000 (CA/TU × 1000). CA/TU × 1000 values were expressed as the percentage of best-fitted $E_{max}$ of the wild type receptor obtained with raw data. Each point represents the arithmetic mean ± S.E. of eight experiments done in triplicate determinations and curves are plotted as a log function of DA concentrations. $B_{max}$ and best-fitted parameters for $EC_{50}$ and $E_{max}$ are reported in **Tables 3-4**. Dose-response curves of DA for hD1R and PD3-hD1R (**A**), and hD5R and PD3-hD5R (**B**) are shown. DA, dopamine.

## Discussion

Our results suggest that Ser and Thr of IL3 play an important role in the regulation of agonist-independent and dependent coupling properties of hD1R and hD5R. Furthermore, PD3-hD5R exhibits a reduction in agonist affinity. As agonist affinity of PD3-hD1R remains unchanged, it implies that Ser and Thr of IL3 play a differential role in shaping the functional properties of human D1-like receptors. IL3 connects TM5 and TM6, two helices playing a critical role in the GPCR activation (Ahuja & Smith 2009, Hofmann et al. 2009, Rosenbaum et al. 2009, Rasmussen *et al.* 2011, Rosenbaum *et al.* 2011). Biophysical and biochemical studies using rhodopsin and β2-AR, two prototypical family A GPCRs, suggest a global toggle switch mechanism in the regulation of receptor activation. This global switch mechanism is characterized by a cytoplasmic outward movement of TM6 and cytoplasmic inward movement of TM3, TM5 and TM7 (Park *et al.* 2008a, Scheerer *et al.* 2008, Ahuja & Smith 2009, Rosenbaum et al. 2009, Hofmann et al. 2009, Choe *et al.* 2011, Standfuss *et al.* 2011). Expression of this global activation mechanism involves the modulation of several local molecular microswitches that participate in the rearrangement and stabilization of intramolecular interactions between cytoplasmic sides of these helices (Ahuja & Smith 2009, Hofmann et al. 2009). Using a single-point mutational approach, we have identified three distinct clusters of Ser/Thr residues that may be involved in the control of local molecular switches of GPCR activation.

Two highly conserved amino acids in family A GPCRs shape a network of polar interactions that ties together the cytoplasmic sides of TM3 and TM6 to stabilize an inactive state and to prevent the outward movement of TM6 (Ahuja & Smith 2009,

Rosenbaum et al. 2009, Hofmann et al. 2009, Choe et al. 2011, Standfuss et al. 2011). In fact, biochemical and biophysical studies suggest an "ionic lock" between two highly conserved residues in family A GPCRs, Arg and Glu, located in the cytoplasmic ends of TM3 and TM6, respectively. Interestingly, these two highly conserved amino acids are also found in the D1-class receptors (Arg121 and Glu267 in hD1R; Arg138 and Glu291 in hD5R). Potentially, these residues also form an "ionic lock" in hD1R and hD5R. It has been postulated that one of the molecular microswitches in controlling GPCR activation encompasses the cytoplasmic "ionic lock" between Arg of TM3 and Glu of TM6. Furthermore, crystallographic studies of activated forms of rhodopsin have clearly demonstrated that these crystal rhodopsin structures display the cytoplasmic features of an active conformation (Park et al. 2008a, Scheerer et al. 2008, Ahuja & Smith 2009, Hofmann et al. 2009, Choe et al. 2011, Standfuss et al. 2011). Specifically, during the transition from an inactive (R) to active (R*) state, a Tyr residue in the cytoplasmic side of TM5 is reoriented to interact with the conserved Arg located in the cytoplasmic end of TM3. This Arg-Tyr interaction releases the "Arg-Glu ionic lock" in opsin (Park et al. 2008a, Scheerer et al. 2008, Hofmann et al. 2009, Ahuja & Smith 2009). Furthermore, studies suggest that this highly conserved Tyr in IL3/TM5 junction of family A GPCRs undergoes distinct structural rearrangements in various crystallized forms of agonist-bound and activated receptors (Rasmussen et al. 2011, Xu *et al.* 2011). This Tyr is also conserved in hD1R (Tyr214) and hD5R (Tyr245) (**Fig. 1**). Interestingly, an analysis of GPCR database (http://www.gpcr.org) indicates that the flanking of this Tyr by two Thr in hD1R and hD5R is unique among family A GPCRs. We hypothesize that flanking Thr strengthen the polar interaction between Tyr of TM5 and Arg of TM3 potentially through

an enriched hydroxyl environment surrounding the Tyr residue. These Thr residues may also modulate hydrogen bond network and packing interactions regulating the rearrangement of this Tyr. Notably, mutations of the two flanking Thr residues (more predominantly Thr215 in hD1R and Thr246 in hD5R) reduce basal activity and DA-induced stimulation of human D1-like receptors (**Figs. 4-6**). Interestingly, these mutations have a subtle impact on agonist affinity of hD1R in spite of being localized at the junction of IL3 and TM5; a TM helix containing critical residues for agonist binding (Pollock *et al.* 1992). This implies that mutations of these Thr do not significantly alter packing interactions of TM residues for agonist binding to hD1R. In an opposing fashion, mutation of Thr246 in hD5R promotes a drastic loss of agonist affinity (**Fig. 3A**). This is a very interesting finding as the TM5 and its cytoplasmic extension in human D1-like receptors are essentially identical (**Fig. 1**). Therefore, it is tempting to speculate that these two residues may contribute to imparting unique and subtype-specific functional and structural properties to D1-like receptors.

An efficient breaking of the "Arg-Glu ionic lock" in human D1-like receptors may be promoted by Ser/Thr of the cytoplasmic extension of IL3/TM6 junction surrounding the Glu residue (Glu267 in hD1R and Glu291 in hD5R). In this study, we showed that mutations of Ser and Thr (Ser263 and Thr268 in hD1R; Ser287 and Thr292 in hD5R) located in the vicinity of this Glu residue strongly reduce constitutive activation of human D1-like subtypes (**Fig. 4C**). These two amino acids likely facilitate and/or maintain the release of "Arg-Glu ionic lock". In addition, loss of DA potency at these mutants supports further this view (**Fig. 5 and 6**). Consequently, these two amino acids may not only assist in the transition of unliganded hD1R and hD5R from R to R* but

may also play an important role in the stabilization of agonist-R* complex and activation of human D1-like receptors. It is worth noting that of the two residues, mutation of the Thr located adjacent to Glu (Thr268 in hD1R and Thr292 in hD5R) mediates the stronger uncoupling effect on DA-induced receptor stimulation and loss of agonist affinity (**Fig. 3A, 5 and 6**). With respect to the loss of agonist affinity, it is unlikely that this Thr participates directly in agonist binding because of the Thr localization in the cytoplasmic extension of IL3/TM6 junction. It rather suggests that this Thr has a structural and functional role in human D1-like receptors though alterations of packing interactions of TM residues forming the agonist binding pocket. Interestingly, among the GPCR family, this Thr is only found in vertebrate D1-like receptors (http://www.gpcr.org). In fact, all catecholamine receptors including the dopamine D2-like subtypes harbor at this position a positively charged residue (Arg, Lys or His) or Gln reinforcing the view that distinct structural mechanisms regulate D1-like receptor activation.

The collapse of "Arg-Glu ionic lock" in activated forms of rhodopsin culminates also in the outward movement of TM6 (Ahuja & Smith 2009, Hofmann et al. 2009, Park et al. 2008a, Scheerer et al. 2008, Choe et al. 2011, Standfuss et al. 2011). This outward TM6 movement is also observed in the crystal structures of agonist-bound human A2A adenosine receptor and nanobody-stabilized active β2-AR (Rasmussen et al. 2011, Xu et al. 2011). We propose that Ser/Thr residues in IL3/TM6 junction of hD1R (Thr273 and Ser275) and hD5R (Thr297 and Ser299) potentially form hydrogen bonds and control packing interactions involved in constraining the outward movement of TM6, which hinders the transition from R to R* of unliganded human D1-like receptors. This view is compatible with the higher basal activity of D1-like receptors harboring mutations of

these residues (**Fig. 4C**). Moreover, mutations of these residues lead to gains in agonist affinity (**Fig. 3A**) suggesting that they are also involved in shaping packing interactions of TM amino acids for agonist binding. These two residues are closer to TM6 relative to Thr268 (hD1R) and Thr292 (hD5R). Intriguingly, in hD5R, mutation of Thr292, which is further away from TM6 relative to Thr297 and Ser299, has a more pronounced impact on agonist affinity while this phenomenon is not observed in hD1R. As sequences of TM6 up to the conserved Glu are virtually identical between hD1R and hD5R, it implies that packing interactions regulating TM6 function in these two subtypes are different. In support of this view, mutations of Thr273 and Ser275 in hD1R lead to a drastic increase of DA-induced responsiveness (**Fig. 5**). In striking contrast, similar mutations in hD5R had no effect on DA-mediated stimulation of AC (**Fig. 6**). Therefore, our results may hint for marked differences in orientation and outward movement of TM6 during activation of hD1R and hD5R.

In conclusion, the present study has delineated three fully conserved and distinctly located Ser/Thr clusters along IL3 of human D1-like receptors. Two clusters respectively located in IL3/TM5 and IL3/TM6 junctions control agonist affinity and agonist-induced coupling to Gαs in an opposite manner. The last conserved Ser/Thr cluster located in the cytoplasmic extension of IL3/TM6 junction positively controls R* formation and stabilization of agonist-R* complex of hD1R and hD5R. Our study unravels that mutations of some of the highly conserved Ser/Thr between D1-like subtypes lead to strikingly different effects on ligand binding and G protein coupling properties. This suggests distinct mechanisms in agonist binding and activation of hD1R and hD5R. Alternatively, these mutations may have disclosed specific sub-states of inactive and

active forms of hD1R and hD5R as suggested by β1 and β2-AR studies (Kobilka & Deupi 2007, Dror et al. 2009, Moukhametzianov et al. 2011). Additionally, our work using PD3-hD1R and PD3-hD5R potentially hints for a combinatorial effect of these Ser/Thr clusters in regulating functional and structural properties of human D1-like receptor through alterations in the network of intramolecular and packing interactions. Furthermore, as IL3 flexibility has been associated with structural instability of solubilized GPCRs, it is possible that Ser/Thr may have a role in controlling the degree of flexibility/rigidity of IL3 in their native environment. In fact, we noted that some of the Ser/Thr mutations promoted changes in $B_{max}$ values of hD1R and hD5R. Future experiments that are beyond the scope of this study may be useful in assessing how phosphorylation of these residues modulates IL3-mediated functional and structural properties of human D1-like receptors. Ideas put forward in this study remains to be further investigated and validated using biophysical and in silico studies. Meanwhile, we believe that our study provides new structural and functional insights into the role of IL3 Ser and Thr (and more specifically the impact of their hydroxyl group) in the regulation of GPCR conformation for agonist binding and activation.

**Research highlights of chapter 3-manuscript 2**

In this second manuscript, I demonstrated that three Ser/Thr clusters conserved among hD1R and hD5R and one unique to hD5R play a critical role in shaping the structural and functional properties of human D1-like receptors.

- Mutations of Ser/Thr clusters located in IL3/TM5 junction and the cytoplasmic extension of IL3/TM6 junction decrease agonist affinity, agonist-dependent and independent coupling with G protein of hD1R and hD5R. These findings suggest that these two Ser/Thr clusters are involved in the stabilization of R*.
- Mutation of the Ser/Thr cluster located in IL3/TM6 junction leads to an increase of agonist affinity and constitutive activation of hD1R and hD5R. The mutation of this Ser/Thr cluster also culminates in a drastic increase of agonist-induced stimulation of hD1R, while having no effect on agonist-activation of hD5R. This Ser/Thr cluster thus facilitates the stabilization of R state.
- A unique Ser cluster in hD5R located in the central portion of IL3 plays an important role in precluding a higher constitutive activation of hD5R. This Ser cluster is potentially implicated in favoring the stabilization of R state of hD5R.

Importantly, the demonstration of the functional expression of these single-point mutants suggest that they can be used as tools to delineate the potential PKC phosphorylation sites involved in the PMA-mediated hD1R sensitization and hD5R desensitization. This issue is addressed in the following manuscript (manuscript 3).

# Chapter 4

# Manuscript 3

# Molecular mechanisms underlying the opposite PKC-induced regulation of dopamine D1 and D5 receptor coupling to cAMP pathway

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**Running title**: Subtype-specific mechanisms control D1-like receptor modulation by PMA

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**Statement of author contributions:**

BP and MT conceived and designed research. BP performed all experiments except dose-response curves for hD1-AANT-SSSS, hD1-ΔQ224-T245, hD5-ΔQ255-S275, hD5-SSS-DDDD and hD5-SSSS-EEEE which were done with the help of XY and BL. BP and MT analyzed data, wrote and edited the manuscript.

## Summary

In the present study, we probed the role of the numerous Ser and Thr of the third intracellular loop (IL3) of human dopamine (DA) D1 and D5 receptors (hD1R and hD5R) in promoting the opposite PKC-induced regulation of D1-like G protein-coupled receptor (GPCR) coupling to stimulation of cAMP. Our mutational studies demonstrate that the PMA-mediated increase of constitutive activation and DA-induced maximal responsiveness of hD1R are achieved through a potential receptor phosphorylation-independent mechanism. In striking contrast, the drastic PMA-induced reduction of constitutive activity of hD5R was controlled by two Ser residues (Ser261 and Ser271). We also report that the PMA-induced desensitization of DA-mediated hD5R activation is dependent on four Ser residues (Ser260, Ser261, Ser271 and Ser274). Different combinations of mutations of these sites suggest that phosphorylated Ser261 and Ser271 are the respective priming sites for the PKC-dependent phosphorylation of Ser260 and Ser274, which is fully expressed following DA-mediated hD5R activation. Phosphomimetic mutations of these four residues in hD5R lead to a receptor exhibiting a desensitized status in the absence of PMA treatment. Moreover, replacement of matching residues in IL3 of hD1R with Ser culminates in a significant inhibition of the PMA-induced hD1R sensitization. Intriguingly, rather than abrogating the PMA-induced regulation of hD5R, simultaneous inactivating Ser-to-Ala mutations of these four residues in hD5R fully switched the PMA-induced desensitization into a PMA-induced hD5R sensitization, which is indistinguishable from the PMA-induced hD1R sensitization. Truncation of the central IL3 region delimited by residues Gln255 to Ser275 in hD5R and Gln225 to Thr245 in hD1R completely abrogates the PMA-induced

regulation of human D1-like receptors. This suggests that the central region of IL3 of D1-like receptors is critical for PMA and PKC regulation. Overall, our study suggests a new paradigm in regulation of Gs-linked GPCRs by PKC, whereby the prevailing PMA/PKC-induced GPCR sensitization is potentially controlled in an IL3-dependent and receptor phosphorylation-independent fashion. Most importantly, phosphorylation of specific Ser residues strategically located on IL3 switches the PKC-dependent and receptor phosphorylation-independent GPCR sensitization into a PKC phosphorylation-dependent GPCR desensitization.

## Introduction

The dopamine (DA) receptors are G protein-coupled receptors (GPCRs) divided into two classes: D1-like (D1R and D5R) and D2-like ($D2R_{short}$, $D2R_{long}$, D3R and D4R). D1-like receptors are coupled to Gαs subunit while D2-like receptors are coupled to Gαi subunit to stimulate or inhibit adenylyl cyclase (AC) activity, respectively (Missale et al. 1998). Dysfunction of central D1-like dopaminergic receptor neurotransmission has been implicated in numerous pathological states such as seizures, Parkinson's and Huntington's diseases, attention deficit hyperactivity disorder, Tourette's syndrome, depression, bipolar disorder, schizophrenia and drug addiction (O'Sullivan *et al.* 2008, Tang et al. 2007, Lasky-Su *et al.* 2007, Ferrari *et al.* 2008, Papakostas 2006, Pantazopoulos et al. 2004, Koob *et al.* 1987, Caine *et al.* 1999). Thus, studies showing that phenotypic expression of brain pathogenesis is potentially linked to aberrant D1-like receptor signaling reinforce the importance in understanding the molecular mechanisms underlying the regulation of D1-like receptor responsiveness.

Two different mechanisms modulate GPCR responsiveness. The first one, termed homologous desensitization, is an agonist-dependent mechanism. Upon agonist binding to the receptor, the activated GPCR is recognized by G protein-coupled receptor kinases (GRKs) leading to its phosphorylation and β-arrestin recruitment to phosphorylated receptors (Pitcher *et al.* 1998, Krupnick & Benovic 1998). This process culminates in the inhibition of GPCR-mediated G protein activation. β-arrestin recruitment also initiates GPCR endocytosis. The second mechanism modulating GPCR responsiveness is called heterologous desensitization, which is mediated through receptor phosphorylation by second messengers-dependent kinases such as protein kinase A (PKA) and protein kinase

C (PKC). Importantly, GPCR phosphorylation by these kinases classically leads to receptor uncoupling from G proteins independently of β-arrestins and agonist occupancy.

PKA-mediated GPCR regulation is somewhat less complex than that controlled by PKC. Indeed, the existence of several PKC isozymes highlights the more complex nature underlying regulation of GPCRs by these Ser/Thr kinases. PKC isozymes are grouped into three classes based upon structural and regulation features: conventional (α, βI, βII, γ), novel (δ, ε, θ, η) and atypical (ξ, τ/λ) (Newton 1995). Activity of conventional PKCs (cPKC) requires $Ca^{2+}$, diacylglycerol (DAG), phosphatidylinositol-4,5-biphosphate ($PIP_2$) and anionic lipids such as phosphatidylserine (PS). In contrast to cPKC, novel PKCs (nPKC) are not $Ca^{2+}$-dependent. Consequently, nPKC are directly activated by an increase of DAG. The atypical PKCs (aPKC) are insensitive to $Ca^{2+}$ and DAG. DAG-dependent PKCs (cPKC and nPKC) can be artificially activated using phorbol esters like phorbol-12-myristate-13-acetate (PMA). Phorbol esters mimic the action of DAG by increasing the enzyme affinity for anionic phospholipids-containing membranes, leading to PKC translocation from the cytosol to the cellular membrane containing receptors (Mosior & Newton 1995). However, in contrast to DAG, phorbol esters are not rapidly metabolized, leading to constitutive activation of PKC upon phorbol ester treatment (Goel *et al.* 2007).

PKC has been established as a key desensitizing molecule for numerous GPCRs through phosphorylation of Ser or Thr residues encompassed in specific amino acid sequences forming PKC substrate motifs (Nishikawa *et al.* 1997). Like several GPCRs, the responsiveness of dopaminergic receptors is also regulated by PKC. Ser residues located in IL3 region of D2R and D3R are critical for PKC-induced desensitization of

D2-like receptors (Morris et al. 2007, Namkung & Sibley 2004, Liu et al. 1992a, Rogue *et al.* 1990, Cho et al. 2007b). We recently demonstrated that D5R expressed in human embryonic kidney 293 (HEK293) cells undergoes a PMA-mediated desensitization (Jackson et al. 2005). In striking contrast, and in spite of the very similar primary structure shared between D1-like receptors, we showed that D1R in HEK293 cells is instead robustly sensitized following PMA treatment. We also demonstrated using pharmacological inhibitors that PMA effects on D1R and D5R were potentially mediated by nPKCs (Jackson et al. 2005). Interestingly, in the striatal neurons expressing predominantly D1R, PMA treatment potentiates DA-induced cAMP formation (Schinelli et al. 1994, Surmeier et al. 1996). Additionally, activation of group I metabotropic glutamate and M1 muscarinic receptors promotes PKC-induced sensitization of D1R in striatal neurons and slices (Paolillo et al. 1998, Sanchez-Lemus & Arias-Montano 2006). Therefore, our studies in HEK293 cells essentially recapitulate results obtained in D1R-expressing neurons. Nevertheless, the physiological relevance of these differential PKC effects on D1R and D5R is unclear.

Meanwhile, several studies have reported that ethanol stimulates the translocation and activation of PKC isozymes in various cellular models (Skwish & Shain 1990, Messing *et al.* 1991, DePetrillo & Liou 1993, Kharbanda *et al.* 1993, Gordon *et al.* 1997, Steiner *et al.* 1997, Jones *et al.* 1998, Chen *et al.* 1999, Satoh *et al.* 2006, Zhang *et al.* 2007b). Interestingly, ethanol reward and self-administration have been positively correlated with increased D1R and PKC activities. On one hand, rats treated with the D1-like agonist SKF81297 self-administrate greater amounts of ethanol compared to saline-treated rats (D'Souza *et al.* 2003). On the other hand, administration of the D1-like

antagonist ecopipam (SCH39166) to C57BL/6 mice reduces ethanol reward (Price & Middaugh 2004). Moreover, nPKCε null mice also demonstrate a marked reduction in ethanol self-administration (Hodge *et al.* 1999, Olive *et al.* 2000). Altogether, these studies suggest that ethanol-mediated increase in PKC activity potentially leads to PKC-induced D1R sensitization (in particular through nPKC actions) and this regulation pathway could thus play a central part in the implementation of ethanol reward and addiction.

These findings highlight the importance of investigating the molecular mechanisms underlying the regulation of D1-like receptors by phorbol esters and PKC. Studies in cortical neurons have shown that methoxamine, an alpha 1-adrenergic agonist, promotes an acceleration of the resensitization of the D1-like mediated response via a PKC-dependent mechanism (Trovero *et al.* 1994). Our group recently identified the IL3 of D1-like receptors as the critical structural determinant dictating the PMA-mediated sensitization and desensitization of D1R and D5R, respectively (Plouffe & Tiberi, 2011; see thesis paper 1). However, studies have yet to address in a mechanistic fashion how PKC activation promotes a differential regulation of D1R and D5R responsiveness. Herein, comprehensive mutational studies were performed to delineate the potential Ser and Thr residues of IL3 of D1R and D5R involved in PKC-induced regulation. Overall, our data demonstrate that four D5R-specific Ser residues on IL3 regulate PMA-mediated D5R desensitization while an IL3-dependent and receptor phosphorylation-independent process controls PKC-induced D1R sensitization.

## Experimental procedures

### Drugs and chemicals

Minimal essential media (MEM), phosphate-buffered saline (PBS), gentamicin, trypsin-EDTA and sterile 1M HEPES buffer were purchased from Invitrogen (Burlington, Ontario, Canada). Fetal bovine serum (FBS) was from PPA Laboratories Inc. (Etobicoke, Ontario, Canada). DA, cAMP, *cis*-flupenthixol, dimethyl sulfoxide (DMSO) and 1-methyl-3-isobutylxanthine (IBMX) were obtained from Sigma/RBI (Oakville, Ontario, Canada). Phorbol-12-myristate-13-acetate (PMA) was acquired from Calbiochem (La Jolla, CA, USA). N-[methyl-$^{3}$H]-SCH23390 (~65-91 Ci/mmol) and [$^{3}$H]-adenine (~20 Ci/mmol) were bought from PerkinElmer (Boston, MA, USA) [$^{14}$C]-cAMP (252 mCi/mmol) was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA, USA). Biodegradable Biosafe II liquid scintillation cocktail was from RPI Corp. (Mount Prospect, IL, USA).

### Preparation of mutated receptors

The construction of single-point mutations in IL3 of human D1R (hD1-T243V, hD1-T244V, hD1-T245V, hD1-S254A, hD1-S258A, hD1-S259A, hD1-S263A, hD1-T268A) and D5R (hD5-S260A, hD5-S261A, hD5-S271A, hD5-S274A, hD5-S275A, hD5-T282V, hD5-S283A, hD5-S287A and hD5-T292V) were previously described (Plouffe *et al.*, 2011, see thesis paper 2). Human receptor constructs with all Ser and Thr of IL3 mutated (phosphodeficient IL3 or PD3) referred to as PD3-hD1R and PD3-hD5R were also described before (Plouffe *et al.*, 2011, see thesis paper 2). In the present study, we also constructed double and quadruple-point mutations in IL3 of human D1R (hD1-

AANT-SSSS) and human D5R (hD5-S261A-S271A, hD5-S260A-S261A, hD5-S271A-S274A, hD5-SSSS-AAAA, hD5-SSSS-DDDD, hD5-SSSS-EEEE) using a polymerase chain reaction (PCR)-based overlap extension approach as described in **Supplementary Methods**. The design of truncated IL3 forms of human D1R (hD1-ΔQ224-T245) and D5R (hD5-ΔQ255-T275) is reported elsewhere (Charrette *et al.*, 2011, in preparation). Wild type human D1R (hD1R) and D5R (hD5R) cloned in the mammalian expression vector pCMV5 were utilized as PCR DNA templates DNA oligonucleotides were custom made by Sigma Genosys (Burlington, Ontario, Canada). PCR primer sequences are listed in supplementary Table S1. Identity of receptor mutants were confirmed using automated fluorescent DNA sequencing (Applied Biosystems 3730 DNA Analyzer) performed at the StemCore Laboratories of the Ottawa Genomics Innovation Centre (Ottawa, Ontario, Canada).

**Supplemental Methods**

*Construction of multiple-point mutant of hD1R and hD5R.*

The human D1R and D5R subcloned in the expression vector pCMV5 were used as PCR templates. Two PCR products, A and B, were amplified separately using P1-P2 and P3-P4 primer pairs, respectively. An overlapping region between A and B allowed the amplification of the final PCR product using primers P5-P6. PCR reactions were carried out with Taq DNA polymerase in an Eppendorf thermal Mastercycler using the following conditions: 1 cycle (94°C for 3 min, 50°C for 1 min, 72°C for 3 min), 25 cycles (94°C for 45 s, 50°C for 1 min, 72°C for 1 min) completed by an anneal extension step at 72°C for 8 min. Overlap PCR reactions were done with the following conditions: 1 cycle

(94°C for 3 min, 50°C for 1 min, 72°C for 10 min), 25 cycles (94°C for 45 s, 50°C for 1 min, 72°C for 1 min) completed by an anneal extension step at 72°C for 8 min. PCR products were separated on 1% agarose gel, excised and purified on QIAEX resin (Qiagen). Purified DNA bands were digested with appropriate restriction enzymes and ligated in-frame with the expression vector pCMV5 containing wild type human D1R or D5R DNA sequences. Mutant hD1R were subcloned in hD1R-pCMV5 (HindIII-XbaI) and mutant hD5R were subcloned in hD5R-pCMV5 (BsmI-EagI).

**Cell culture and transfection**

HEK293 cells (CRL-1573, American Type Culture Collection, Manassas, VA, USA) seeded into 100-mm dishes ($2.5 \times 10^6$ cells/dish) were grown in complete MEM (supplemented with 10% heat-inactivated FBS and 40 μg/ml gentamicin) at 37°C and 5% $CO_2$. For DA dose-response curves experiments, cells were transiently transfected with 0.02-0.04 μg of receptor construct DNA/dish to obtain moderate receptor levels (0.5-2.5 pmol per mg of membrane proteins) using a modified calcium phosphate precipitation procedure (Plouffe et al. 2010). Empty pCMV5 vector was used to normalize the total amount of DNA (5 μg) transfected per 100-mm dish. To study the PMA effects on constitutive (agonist-independent) activity of wild type and mutant hD1R and hD5R, cells were transfected with 5 μg of receptor construct DNA/dish to obtain maximal achievable receptor expression. After an overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells were washed with PBS, trypsinized, reseeded in 100-mm dishes (radioligand binding) and 12-well plates (dose-response curves) or 6-well plates (constitutive activity experiments) and grown for an additional 48 h.

**Whole cell cAMP assay**

Regulation of AC activity by wild type and mutant human D1-like receptors was assessed in the presence of IBMX (1 mM) using a whole cell cAMP assay as previously described (Plouffe et al. 2010). Cells seeded in 6- or 12-well plates were labeled with [$^3$H]-adenine (1-2 μCi/ml) at 37ºC overnight. The next day, cells were incubated with 0.1 mM ascorbic acid alone (DA vehicle) or with increasing concentrations of DA in the absence (DMSO) or presence of PMA (1 μM) for 30 min at 37ºC. Each experiment was done using triplicate determinations. At the end of incubation period, plates were put on ice tray, media aspirated and cells incubated with 1 ml of lysis solution containing 2.5% (v/v) perchloric acid, 0.1 mM cAMP and [$^{14}$C]-cAMP (~10,000 dpm, internal standard for column recovery) for 30 min. Lysates were neutralized with 4.2 M KOH, clarified by low-speed centrifugation and [$^3$H]-cAMP purified by sequential chromatography using Dowex and alumina columns (Johnson et al. 1994, Plouffe et al. 2010). Alumina eluates were collected in plastic vials and mixed with 17 ml of biodegradable Biosafe II scintillation cocktail. Dual counting of $^3$H and $^{14}$C counts was performed in a liquid scintillation counter (Beckman, LS 6500). The amount of intracellular [$^3$H]-cAMP formed (CA) over the total uptake of [$^3$H]-adenine (TU) was calculated to determine the relative AC activity and initially expressed as CA/TU × 1000 (Plouffe et al. 2010).

**Membrane Preparation and Radioligand Binding**

Transfected HEK293 cells seeded in 100-mm dishes were washed with cold PBS, harvested in ice-cold lysis buffer (10mM Tris-HCl, pH 7.4, 5 mM EDTA), and centrifuged at 40,000 *g* for 20 min at 4°C (Plouffe et al. 2010). The membrane pellet was

resuspended in lysis buffer using a Brinkmann Polytron (velocity of 17,000 rpm for 15 sec) and centrifuged at 40,000 *g* for 20 min at 4°C. The final pellet was homogenised in resuspension buffer (62.5 mM Tris-HCl, pH 7.4; 1.25 mM EDTA) and membranes were used immediately. Fresh membranes (100 µl) were incubated in a total volume reaction of 500 µl (final in assays: 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM EDTA) with a saturating concentration of N-[methyl-$^3$H]-SCH23390 (7-9 nM) in the absence or presence of *cis*-flupenthixol (10 µM) to delineate total and nonspecific binding using duplicate determinations. Binding reactions were carried out for 90 min at room temperature. At the end of incubation, membranes were harvested on glass fiber filters (GF/C, Whatman) using rapid filtration and filters were washed three times with 5 ml of cold washing buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl) and mixed with 5 ml of Biosafe II scintillation liquid. Bound radioactivity was counted using a liquid scintillation counter (Beckman, LS 6500). Protein concentrations were measured using Bio-Rad assay kit with bovine serum albumin as standard to determine the maximal binding capacity of N-[methyl-$^3$H]-SCH23390 ($B_{max}$ expressed in pmol/mg membrane proteins).

**Statistical analysis**

Results are expressed as arithmetic means ± S.E. unless stated otherwise. Statistical differences between experimental conditions tested were assessed by one-sample t test, unpaired t test and one-way ANOVA (with Newman-Keuls multiple comparison post-hoc test) using GraphPad Prism 5.03 for Windows (GraphPad Software, San Diego; www.graphpad.com). The level of significance was set at $p < 0.05$. Dose-

response curves of DA for wild type and mutant receptors obtained under control (DMSO) and PMA conditions were simultaneously analyzed by a four-parameter logistic equation from GraphPad Prism software as described below.

**Equation 1:** $Y = Basal + (Maximal - Basal) / (1 + 10^{((LogEC50 - X) \times Slope)})$

Curves were first analyzed using raw data (CA/TU × 1000) using a shared slope factor to obtain the best-fitted values for the effective concentration producing 50% of maximal response ($EC_{50}$) and DA-mediated maximal stimulation ($E_{max}$) in cells expressing similar levels of wild type and mutant receptors under control and PMA conditions. Then, raw intracellular cAMP levels measured at each experimental point for wild type and mutant receptors under DMSO (control) and PMA conditions were expressed as percent of their respective best-fitted control $E_{max}$ value. Individual curves expressed as percent of best-fitted control $E_{max}$ value were averaged and simultaneously analyzed to determine best-fitted parameters for $EC_{50}$ and $E_{max}$ using a shared slope factor. $EC_{50}$ with 95% confidence intervals and $E_{max}$ with approximate S.E. are reported as computed by GraphPad Prism software. Statistical differences between control and PMA conditions for $EC_{50}$ and $E_{max}$ of wild type and mutant receptors were established by comparing computed F values with unconstrained, shared and constrained curve fitting. $EC_{50}$ shift (PMA/control) values were determined from individual best-fitted dose-response curves of DA in the absence and presence of PMA.

**Table S1. Sequences of oligonucleotide primers for the construction of hD1R and hD5R mutants.**

| Construct | Oligonucleotide primers (5'-3') |
|---|---|
| hD1-AANT-SSSS | **P1**: TTCATCCCAGTGCAGCTC |
| | **P2**:ACCTGTGGTCGACTGGCAACTCTTGGCGTGGACTGC TGCCCTCTCCAAGGACGAAATGCGCCGTATTTGTTT |
| | **P3**:CGGCGCATTTCGTCCTTGGAGAGGGCAGCAGTCCAC GCCAAGAGTTGCCAGTCGACCACAGGTAATGGAAAG |
| | **P4**: TTAGGACAAGGCTGGTGG |
| | **P5**: ACTGTGACTCCAGCC |
| | **P6**: GGCCAGGAGAGGCA |
| hD5-S260A-S261A | **P1**: TACGGTGGGAGG |
| | **P2**: GTGCTCTGCAGCCCTCTCCAGGGCGGCAATCCTGCG GATCTGCAC |
| | **P3**: CGCAGGATTGCCGCCCTGGAGAGGGCTGCAGAGCAC GCGCAG |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |
| hD5-S261A-S271A* | **P1**: TACGGTGGGAGG |
| | **P2**: GCAGGCTGCGCTGCTCCGGCAGGCCTGCGCGTGCTC TGCGGC |
| | **P3**: CACGCGCAGGCCTGCCGGAGCAGCGCAGCCTGCGC GCCCGAC |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |
| hD5-S271A-S274A | **P1**: TACGGTGGGAGG |
| | **P2**: GCAGGCTGCGCTAGCCCGGCAGGCCTGCGCGTGCT CTGCGGC |
| | **P3**: CACGCGCAGGCCTGCCGGGCTAGCGCAGCCTGCGC GCCCGAC |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |
| hD5-SSSS-AAAA** | **P1**: TACGGTGGGAGG |
| | **P2**: GTGCTCTGCAGCCCTCTCCAGGGCGGCAATCCTGCG GATCTGCAC |
| | **P3**: CGCAGGATTGCCGCCCTGGAGAGGGCTGCAGAGCA CGCGCAG |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |

**Table S1.** ***Continued.***

| Construct | Oligonucleotide primers (5'-3') |
|---|---|
| hD5-SSSS-DDDD | **P1**: TACGGTGGGAGG |
| | **P2**:GCAGGCTGCGCTGTCCCGGCAGTCCTGCGCATGCTCTGC GGCCCTCTCCAGGTCGTCAATCCTGCGGATCTGCACCTG |
| | **P3**:CGCAGGATTGACGACCTGGAGAGGGCCGCAGAGCATGC GCAGGACTGCCGGGACAGCGCAGCCTGCGCGCCCGACACC |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |
| hD5-SSSS-EEEE | **P1**: TACGGTGGGAGG |
| | **P2**:GCAGGCTGCGCTCTCCCGGCACTCCTGCGCGTGCTCTGCGG CCCTCTCGAGCTCCTCAATCCTGCGGATCTGCACCTG |
| | **P3**:CGCAGGATTGAGGAGCTCGAGAGGGCCGCAGAGCACGCGC AGGAGTGCCGGGAGAGCGCAGCCTGCGCGCCCGACACC |
| | **P4**: TCATGTGGATGTAGGCAG |
| | **P5**: ACCTGGCCAACTGGA |
| | **P6**: TGTTCACCGTCTCCA |

* hD5-S261A was used as template instead of wild type hD5R
** hD5-S271A-S274A was used as template instead of wild type hD5R

## Results

PMA-induced sensitization of hD1R coupling to cAMP production in HEK293 cells is highlighted by a robust increase in $E_{max}$ of DA and higher constitutive (agonist-independent) activation of the receptor (Jackson et al. 2005). Meanwhile, PMA-mediated desensitization of hD5R coupling to stimulatory Gs proteins is depicted by a strong decrease in DA potency (as indexed by the robust $EC_{50}$ rightward shift) and almost complete abrogation of its agonist-independent activity (Jackson et al. 2005). Recently, we have established that the opposite regulation of hD1R and hD5R responsiveness by PMA/PKC pathway depends exclusively on D1-like subtype-specific structural determinants of IL3 (Plouffe & Tiberi, 2011; see thesis paper 1). Interestingly, upon examination of the IL3 primary sequences of human D1-like receptors, these subtype-specific structural determinants, notably Ser and Thr residues, are likely to be found within or in close proximity to the least conserved region between IL3 of hD1R and hD5R (**Fig. 1**). Specifically, the divergent region within IL3 is bordered by Lys223-Arg266 in hD1R and Val254-Lys290 in hD5R (**Fig. 1**). Herein, we employed single-point mutations (Ser-to-Ala and Thr-to-Val) to delineate the potential PKC sites controlling PMA-induced regulation of hD1R and hD5R responsiveness. The single-point mutant forms of hD1R (8) and hD5R (9) are functionally expressed in HEK293 cells as described (Plouffe *et al*. 2011; see thesis paper 2).

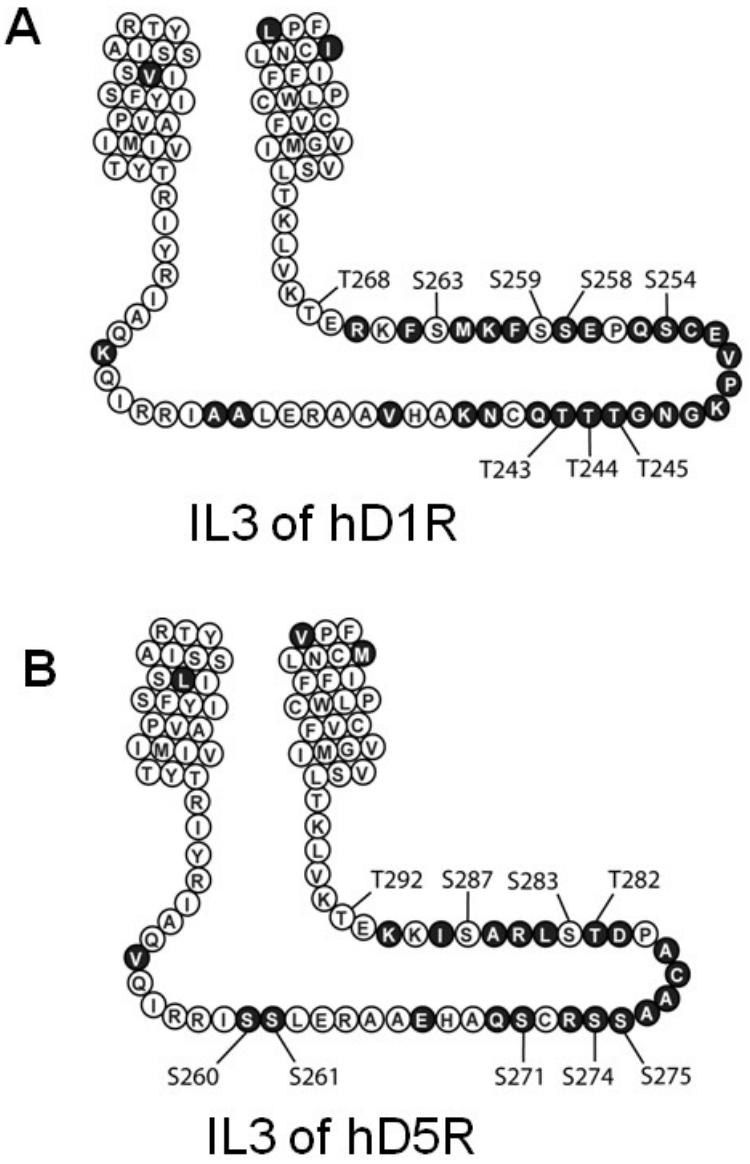


**Figure 1. Schematic representation of IL3 of hD1R and hD5R.**
Ser and Thr residues of IL3 of hD1R (**A**) and hD5R (**B**) tested in the PMA-induced regulation are indicated. Ser and Thr were mutated into Ala and Val, respectively. Conserved residues between human D1-like receptors are represented using empty circles. Primary amino acid sequence of transmembrane region 5 and 6 is also shown.

**PMA-induced sensitization of DA-mediated hD1R activation is potentially modulated in an IL3 phosphorylation-independent manner.**

As previously shown by our group, there is a robust increase of $E_{max}$ in hD1R-transfected cells treated with PMA i.e. 200-250% (**Fig. 2A** and **Table 1**). Interestingly, no change in the extent of PMA-induced increase of $E_{max}$ was detected in cells expressing the single-point hD1R mutants when compared with wild type receptor (**Fig. 2A** and **Table 1**). Similarly, the small rightward shift in $EC_{50}$ (1.4 - 3 fold) produced by PMA treatment remains essentially unchanged by single-point mutations relative to wild type hD1R. We then reasoned that PMA effects on hD1R are potentially mediated through the PKC-induced phosphorylation of multiple Ser and Thr residues. To test this hypothesis, we assess the effect of PMA on the responsiveness of PD3-hD1R, a receptor construct harbouring mutations of all Ser/Thr in IL3 of hD1R. It is worth mentioning that PD3-hD1R does not exhibit major changes in functional expression, DA affinity and G protein coupling properties (Plouffe et al., 2011; see thesis paper 2). Intriguingly, mutation of all Ser and Thr residues of IL3 had no impact on the PMA-mediated increase of $E_{max}$ (**Fig. 2B** and **Table 1**). While IL3 of hD1R is critical for PKC-induced sensitization of hD1R, our current results clearly show that Ser and Thr found in this cytoplasmic loop are not implicated in the robust increase of $E_{max}$ of DA in hD1R-expressing cells treated with PMA. Overall, our data strongly suggests that the PMA-induced regulation of DA-induced activation of hD1R is potentially controlled through IL3 phosphorylation-independent mechanism.

**Table 1. PMA-mediated effects on DA-dependent stimulation of AC in HEK293 cells transfected with wild type and mutant hD1R.**

| Receptors | $B_{max}$ (pmol/mg membrane prot.) | $E_{max}$ (% of ctrl) | | $E_{max}$ (PMA/ctrl) | $EC_{50}$ (nM) | | $EC_{50}$ shift (PMA/ctrl) |
|---|---|---|---|---|---|---|---|
| | | control | PMA | % | control | PMA | Fold |
| hD1R | 1.06 ± 0.37 | 102 ± 6 | 240 ± 8 | 234 ± 1 | 23 [8-64] | 46 [29-75] | 2.1 ± 0.26 |
| hD1-T243V | 0.90 ± 0.12 | 103 ± 6 | 245 ± 8 | 237 ± 13 | 19 [7-55] | 49 [30-79] | 3.1 ± 0.58 |
| hD1R | 1.97 ± 0.15 | 99 ± 5 | 242 ± 7 | 247 ± 7 | 12 [5-30] | 25 [17-37] | 2.5 ± 0.66 |
| hD1-T244V | 2.41 ± 0.85 | 99 ± 5 | 241 ± 6 | 245 ± 13 | 8 [3-19] | 20 [13-29] | 2.7 ± 0.57 |
| hD1R | 1.12 ± 0.32 | 98 ± 4 | 221 ± 5 | 228 ± 9 | 14 [7-27] | 27 [20-37] | 2.2 ± 0.45 |
| hD1-T245V | 1.67 ± 0.88 | 98 ± 4 | 227 ± 5 | 233 ± 9 | 15 [8-29] | 26 [19-35] | 2.1 ± 0.53 |
| hD1R | 1.97 ± 0.15 | 99 ± 7 | 244 ± 9 | 247 ± 7 | 12 [3-45] | 26 [15-45] | 2.5 ± 0.66 |
| hD1-S254A | 2.49 ± 1.08 | 99 ± 7 | 236 ± 9 | 237 ± 16 | 6 [2-25] | 18 [10-33] | 3.0 ± 0.97 |
| hD1R | 0.63 ± 0.21 | 100 ± 4 | 231 ± 5 | 232 ± 2 | 22 [12-41] | 44 [32-59] | 2.0 ± 0.26 |
| hD1-S258A | 0.45 ± 0.12 | 100 ± 4 | 228 ± 5 | 229 ± 5 | 27 [14-53] | 68 [49-95] | 2.9 ± 0.98 |
| hD1R | 1.55 ± 0.56 | 99 ± 6 | 240 ± 7 | 245 ± 9 | 12 [5-34] | 25 [16-38] | 2.4 ± 0.67 |
| hD1-S259A | 2.08 ± 1.11 | 99 ± 5 | 247 ± 6 | 251 ± 20 | 5 [2-16] | 14 [9-21] | 2.5 ± 0.60 |
| hD1R | 1.04 ± 0.14 | 100 ± 5 | 228 ± 5 | 227 ± 11 | 14 [7-29] | 28 [20-40] | 2.4 ± 0.67 |
| hD1-S263A | 1.24 ± 0.27 | 100 ± 5 | 227 ± 6 | 226 ± 13 | 43 [20-92] | 59 [42-84] | 2.5 ± 0.60 |
| hD1R | 2.67 ± 1.09 | 99 ± 6 | 226 ± 7 | 226 ± 19 | 9 [3-27] | 28 [20-40] | 1.5 ± 0.28 |
| hD1-T268V | 1.56 ± 0.49 | 99 ± 8 | 239 ± 10 | 238 ± 26 | 155 [48-497] | 306 [183-510] | 1.9 ± 0.32 |
| hD1R | 1.32 ± 0.27 | 102 ± 4 | 220 ± 4 | 218 ± 5 | 9 [5-17] | 16 [11-21] | 1.7 ± 0.17 |
| PD3-hD1R | 1.18 ± 0.34 | 101 ± 4 | 212 ± 6 | 210 ± 7 | 35 [17-68] | 66 [46-94] | 2.0 ± 0.32 |

Best-fitted and unconstrained parameters for $EC_{50}$ and $E_{max}$ obtained by simultaneous curve fitting of averaged dose-response curves of DA (shown in **Fig. 2**) are listed. $EC_{50}$ values are expressed with 95% confidence intervals in brackets while $E_{max}$ values are reported as percent of control $E_{max}$ value ± approximate S.E. as determined by GraphPad Prism. $E_{max}$ ratios and $EC_{50}$ shifts were computed for each individual dose-response curve using best-fitted $EC_{50}$ and $E_{max}$ values obtained in the absence and presence of PMA. Receptor levels ($B_{max}$), $E_{max}$ ratios and $EC_{50}$ shift values are expressed as arithmetic means ± S.E. No statistical differences between $B_{max}$, $E_{max}$ ratios and $EC_{50}$ shift values of wild type and mutated receptors were detected using unpaired t test.

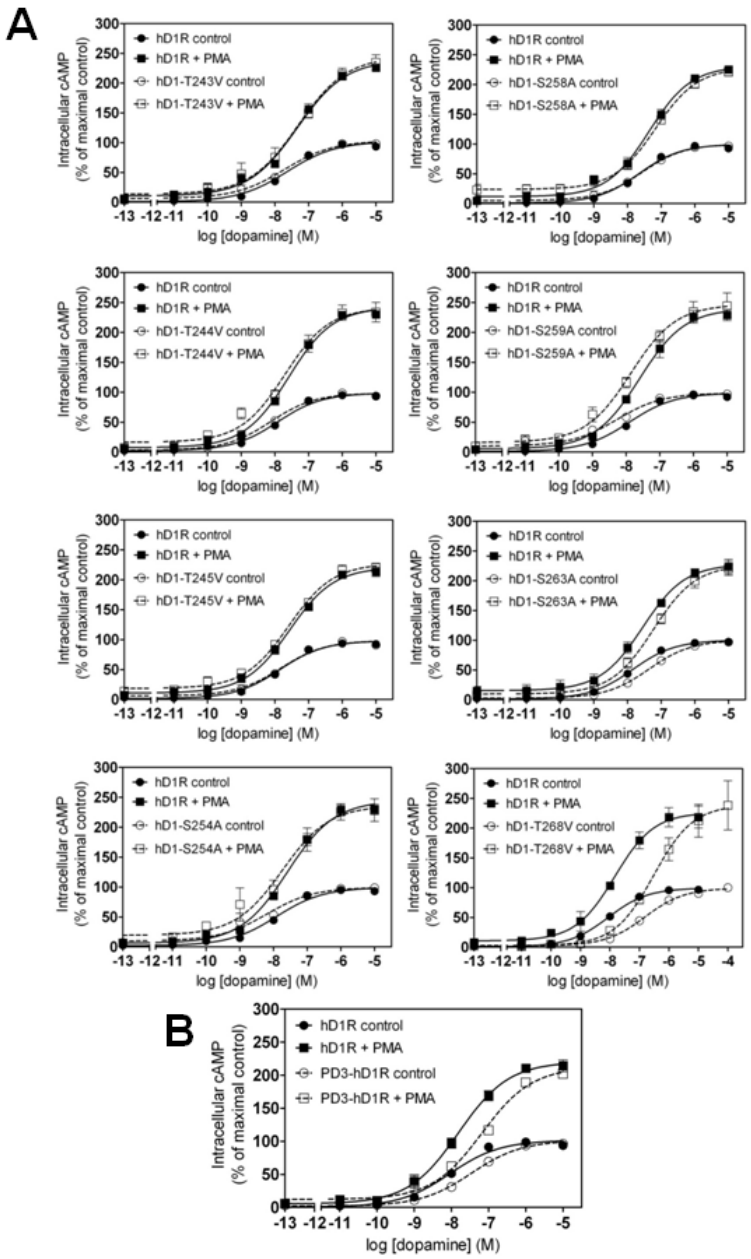


**Figure 2. DA-mediated stimulation of AC by wild type and mutant hD1R in the absence and presence of PMA.**

Transfected HEK293 cells were seeded in 12-well plates and labeled with [$^3$H]-adenine as described in Experimental Procedures. Cells treated with vehicle (DMSO, 0.02% (v/v); control) or 1 μM PMA were incubated with increasing DA concentrations for 30 min at 37°C. Intracellular cAMP levels were plotted as a function of log of DA concentrations and expressed as percentage of best-fitted $E_{max}$ obtained under control condition for each receptor. For each mutant receptor, experiments were repeated at least 3 times in triplicate determinations and done in parallel with wild type hD1R as the control receptor. Best-fitted curves obtained from single-point mutations and PD3-hD1R are represented in **A** and **B**, respectively. Values of PMA-mediated effects on $E_{max}$ and $EC_{50}$ for each receptor are reported in **Table 1**.

**Mutation of Ser and Thr of IL3 does not alter PMA-induced increase of agonist-independent activity of hD1R**

The Phe264 in IL3 of hD1R is important in maintaining a lower agonist-independent activity relative to hD5R (Charpentier et al. 1996). Owing to the presence of Ser and Thr residues surrounding Phe264, we assessed whether the increase of constitutive activation of hD1R in PMA-treated cells is potentially regulated by the PKC-induced phosphorylation of these IL3 residues. However, as shown in **Fig. 3A**, no significant difference was found between the extent of the PMA-induced increase of basal activity (~1.5 fold over control) of wild type and single-point mutant forms of hD1R (**Fig. 3B**). Likewise, mutations of all Ser and Thr of IL3 had no effect on the extent of PMA-induced increase of hD1R constitutive activation (**Fig. 3B**). As a control, we showed that mock-transfected cells do not display any elevation in basal levels of intracellular cAMP following PMA treatment. Altogether, our results reinforce the notion that PMA-induced sensitization of hD1R observed in the absence and presence of DA may not require IL3 phosphorylation. In the following series of experiments, we investigated if a similar mechanism underlies the PMA-induced hD5R desensitization.

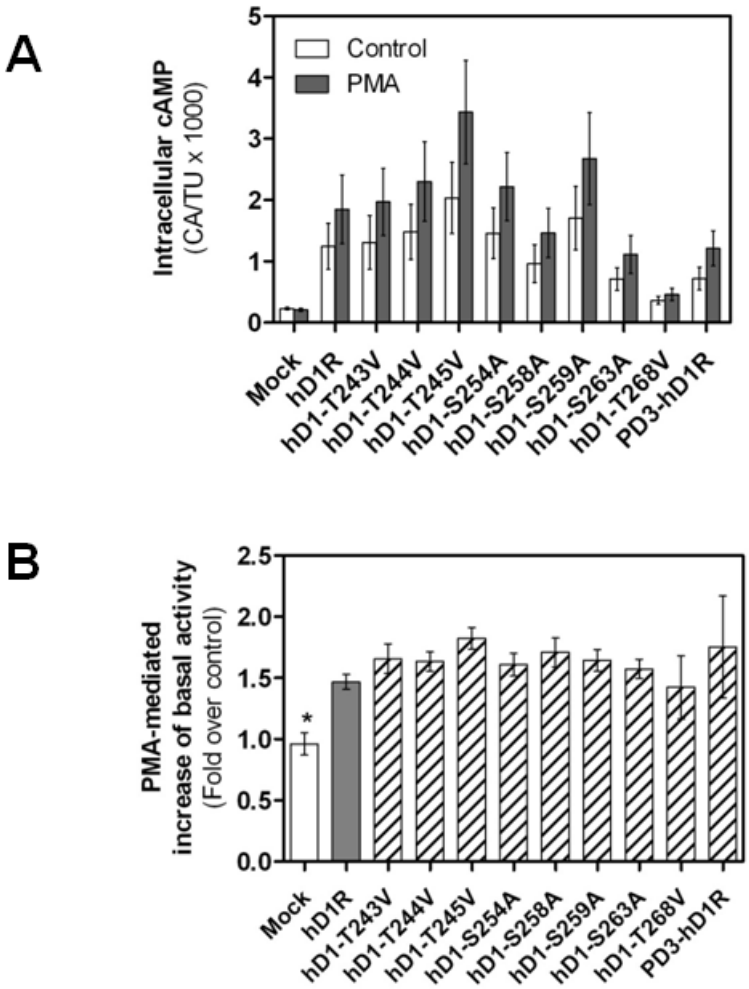


**Figure 3. Effect of PMA treatment on constitutive activity of wild type and mutant hD1R.**

HEK293 cells transfected with 5 μg of receptor DNA construct per dish were seeded in 6-well plates and labeled with [$^3$H]-adenine as described in Experimental Procedures. Cells were treated with vehicle (DMSO, 0.02% (v/v)) (control) or 1 μM PMA for 30 minutes at 37°C. **(A)** Intracellular cAMP levels were determined and expressed as CA/TU x 1000. Raw data are expressed as the arithmetic mean ± SE of at least 5 experiments done in triplicate determinations. **(B)** For each experiment, the ratio of the amount of intracellular cAMP levels produced under PMA relative to control was computed and expressed as the arithmetic mean ± S.E. Statistical difference between ratios obtained in mock-transfected cells or cells expressing hD1R mutants were compared with wild type hD1R ratio using one-way ANOVA with Newman-Keuls multiple comparison post-test. $B_{max}$ values (pmol/mg membrane prot.) are as follows: 9.07 ± 1.09 (hD1R), 15.6 ± 0.73 (hD1-T243V), 17.8 ± 0.92 (hD1-T244V), 26.1 ± 1.44 (hD1-T245V), 15.63 ± 0.63 (hD1-S254A), 9.99 ± 0.89 (hD1-S258A), 18.8 ± 1.21 (hD1-S259A), 20.6 ± 0.99 (hD1-S263A), 14.6 ± 1.51 (hD1-T268V) and 20.2 ± 0.82 (PD3-hD1R). * $p < 0.05$ vs. hD1R.

**Four unique Ser residues in IL3 regulate the PMA-induced desensitization of DA-mediated activation of hD5R.**

As previously reported by our group (Jackson et al. 2005), hD5R undergoes a robust desensitization upon PMA treatment resulting in a ~40 fold rightward shift in $EC_{50}$ value for DA (**Fig. 4A** and **Table 2**). In contrast to our hD1R studies, our mutational approach delineated four single-point hD5R mutants exhibiting a drastic decrease in the PMA-induced rightward shift of $EC_{50}$ for DA when compared with wild type hD5R (**Figs. 4A, B** and **Table 2**). Specifically, single-point mutations of Ser260, Ser261, Ser271 and Ser274 lead to a significant reduction of the PMA-mediated rightward shift of $EC_{50}$ for DA when compared with wild type receptor value that ranges from ~40% to 85% (**Fig. 4B**). Notably, mutation Ser271 produces the strongest inhibition of PMA-induced desensitization of DA-mediated hD5R activation (**Figs. 4A, B** and **Table 2**). Based on the differences in the extent of percent inhibition of the $EC_{50}$ rightward shift, our data suggest that these four unique hD5R Ser residues differentially contribute to the PMA-induced desensitization of DA-activated hD5R.

Surprisingly, each mutation of these Ser residues is also associated with a concomitant PMA-induced increase of $E_{max}$ (**Figs. 4A, C** and **Table 2**). In fact, the percent augmentation of PMA-induced increase of DA-mediated maximal activation of hD5R mutants was in line with the extent of inhibition of PMA effect on the EC50 rightward shift produced by each Ser mutation. For instance, mutation of Ser271 mediates the most powerful effect (~300% augmentation) with respect to increase in DA-associated $E_{max}$ following PMA treatment (**Fig. 4C**). Mutation of Ser260, Ser261 and Ser274 promote an augmentation of $E_{max}$ in PMA-treated cells ranging from 160% to

250% relative that of hD5R-expressing cells following PMA treatment. Interestingly, the small $EC_{50}$ rightward shift and higher $E_{max}$ potentiation obtained in cells expressing hD5-S260A, hD5-S261A, hD5-S271A and hD5-S274A following PMA treatment are reminiscent of the PMA sensitization pattern elicited in cells transfected with hD1R (Tables 1-2). Importantly, these Ser residues are replaced with either Ala, Asn or Thr in IL3 of hD1R (**Fig. 1**). This suggests the PKC-induced phosphorylation of these four Ser residues in IL3 of hD5R potentially turn on a desensitization microswitch. We then tested this hypothesis using PD3-hD5R, in which all Ser/Thr of IL3 are mutated, and hD5-SSSS-AAAA harbouring the quadruple-point mutations of Ser260, Ser261, Ser271 and Ser274 in IL3. It is worth mentioning that PD3-hD5R (Plouffe et al., 2011; see thesis paper 2) and hD5-SSSS-AAAA (data not shown) do not display drastic changes in DA affinity and G protein coupling properties in comparison with wild type hD5R.

**Table 2. PMA-mediated effects on DA-dependent stimulation of AC in HEK293 cells transfected with wild type and mutant hD5R.**

| Receptors | $B_{max}$ (pmol/mg membrane prot.) | $E_{max}$ (% of ctrl) | | $E_{max}$ (PMA/ctrl) | $EC_{50}$ (nM) | | $EC_{50}$ shift (PMA/ctrl) |
|---|---|---|---|---|---|---|---|
| | | control | PMA | % | control | PMA | Fold |
| hD5R | 1.91 ± 0.28 | 101 ± 3 | 138 ± 5 | 135 ± 4 | 0.61 [0.30-1.2] | 26 [15-44] | 43 ± 7 |
| hD5-S260A | 2.08 ± 0.22 | 100 ± 3 | 161 ± 4 | 157 ± 7* | 0.41 [0.19-0.89] | 13 [8-20] | 24 ± 5* |
| hD5R | 1.62 ± 0.19 | 100 ± 2 | 130 ± 4 | 128 ± 4 | 1.1 [0.61-1.8] | 36 [24-54] | 32 ± 3 |
| hD5-S261A | 1.65 ± 0.24 | 101 ± 2 | 167 ± 3 | 165 ± 5* | 0.98 [0.57-1.7] | 9.9 [7.3-13] | 11 ± 2* |
| hD5R | 1.59 ± 0.22 | 100 ± 2 | 131 ± 3 | 131 ± 5 | 0.95 [0.58-1.5] | 35 [24-50] | 37 ± 3 |
| hD5-S271A | 1.69 ± 0.22 | 100 ± 2 | 181 ± 3 | 180 ± 4* | 0.97 [0.57-1.7] | 5.0 [3.9-6.4] | 5 ± 1* |
| hD5R | 1.71 ± 0.22 | 100 ± 2 | 134 ± 3 | 134 ± 4 | 0.84 [0.59-1.2] | 32 [25-43] | 43 ± 6 |
| hD5-S274A | 1.60 ± 0.27 | 100 ± 2 | 148 ± 2 | 149 ± 5* | 0.87 [0.60-1.3] | 10 [7.9-13] | 13 ± 2* |
| hD5R | 1.66 ± 0.30 | 100 ± 2 | 131 ± 3 | 132 ± 4 | 0.80 [0.54-1.2] | 33 [24-44] | 46 ± 7 |
| hD5-S275A | 1.75 ± 0.31 | 100 ± 2 | 141 ± 3 | 142 ± 6 | 0.69 [0.46-1.0] | 21 [16-28] | 38 ± 7 |
| hD5R | 1.81 ± 0.42 | 100 ± 3 | 133 ± 4 | 133 ± 4 | 0.89 [0.54-1.5] | 29 [20-43] | 34 ± 4 |
| hD5-T282V | 1.57 ± 0.35 | 100 ± 2 | 122 ± 2 | 123 ± 7 | 0.85 [0.50-1.5] | 35 [23-54] | 51 ± 10 |
| hD5R | 1.54 ± 0.21 | 100 ± 2 | 133 ± 3 | 133 ± 5 | 0.49 [0.29-0.81] | 20 [14-29] | 43 ± 8 |
| hD5-S283A | 1.73 ± 0.39 | 100 ± 2 | 140 ± 3 | 141 ± 4 | 0.33 [0.19-0.57] | 14 [9.9-20] | 41 ± 5 |
| hD5R | 0.93 ± 0.19 | 100 ± 2 | 139 ± 4 | 140 ± 9 | 1.0 [0.64-1.7] | 34 [24-49] | 35 ± 1 |
| hD5-S287A | 0.99 ± 0.22 | 100 ± 2 | 140 ± 4 | 141 ± 7 | 1.6 [0.97-2.6] | 62 [43-88] | 45 ± 10 |
| hD5R | 1.77 ± 0.41 | 100 ± 2 | 133 ± 3 | 133 ± 4 | 0.75 [0.48-1.2] | 30 [21-44] | 39 ± 3 |
| hD5-T292V | 1.54 ± 0.45 | 100 ± 3 | 145 ± 4 | 145 ± 10 | 4.1 [2.5-6.6] | 103 [73-146] | 31 ± 6 |

Best-fitted and unconstrained parameters for $EC_{50}$ and $E_{max}$ obtained by simultaneous curve fitting of averaged dose-response curves of DA (shown in **Fig. 4**) are listed. $EC_{50}$ values are expressed with 95% confidence intervals in brackets while $E_{max}$ values are reported as percent of control $E_{max}$ value ± approximate S.E. as determined by GraphPad Prism. $E_{max}$ ratios and $EC_{50}$ shifts were computed for each individual dose-response curve using best-fitted $EC_{50}$ and $E_{max}$ values obtained in the absence and presence of PMA. Receptor levels ($B_{max}$), $E_{max}$ ratios and $EC_{50}$ shift values are expressed as arithmetic means ± S.E. Statistical differences between $B_{max}$, $E_{max}$ ratios and $EC_{50}$ shift values of wild type and mutated receptors were assessed using unpaired t test. * $p < 0.05$ vs. hD5R.

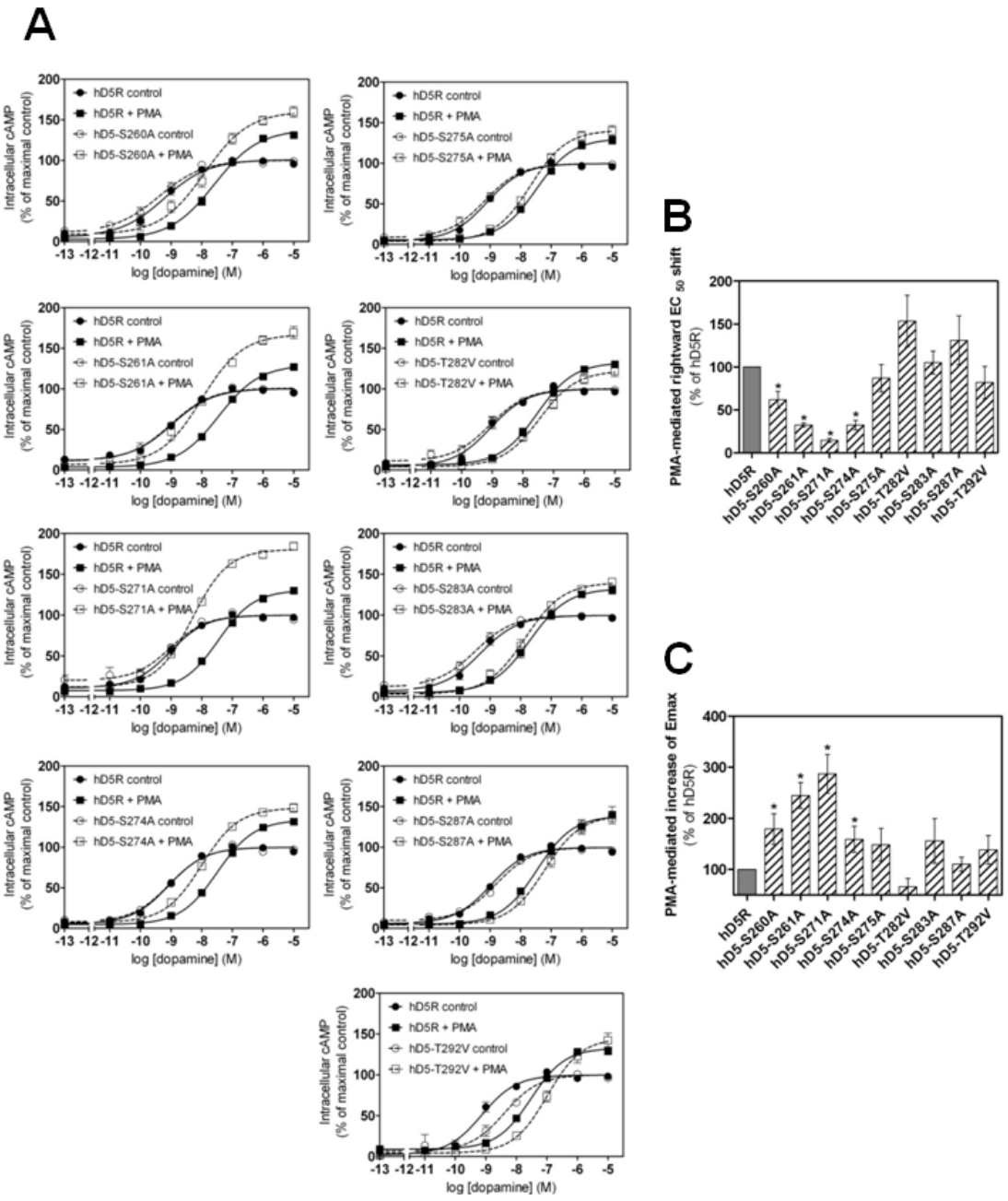


**Figure 4. DA-mediated stimulation of AC by wild type and mutant hD5R in the absence and presence of PMA.**

Transfected HEK293 cells were seeded in 12-well plates and labeled with [$^3$H]-adenine as described in Experimental Procedures. Cells treated with vehicle (DMSO, 0.02% (v/v); control) or 1 μM PMA were incubated with increasing DA concentrations for 30 min at 37°C. Intracellular cAMP levels were plotted as a function of log of DA concentrations and expressed as percentage of best-fitted $E_{max}$ obtained under control condition for each receptor. For each mutant receptor, experiments were repeated at least 4 times in triplicate determinations and done in parallel with wild type hD5R as the control receptor. Best-fitted curves obtained from single-point mutations are represented in **A**. Values of PMA-mediated effects on $E_{max}$ and $EC_{50}$ obtained with each receptor are reported in Table 2. The arithmetic means ± S.E. of PMA-mediated $EC_{50}$ rightward shift (**B**) and PMA-mediated change of $E_{max}$ (**C**) expressed relative to wild type hD5R are shown. Statistical differences between percent values of mutant hD5R and control value of wild type hD5R (set at 100%) were determined using one sample t test. * $p < 0.05$ vs. hD5R.

**Full switch of PMA-induced desensitization into PMA-induced sensitization following the quadruple-point mutations of Ser260, Ser261, Ser271 and Ser274 in IL3 of hD5R**

We first assessed the DA-mediated PD3-hD5R responsiveness following PMA treatment. Interestingly, mutations of all Ser/Thr residues located in IL3 of hD5R completely abrogate the PMA-induced desensitization process as indexed by the slight $EC_{50}$ rightward shift (1.9 fold) as compared with ~40-fold rightward shift in $EC_{50}$ of DA in PMA-treated cells expressing wild type hD5R (**Fig. 5A** and **Table 3**). Additionally, in striking contrast to cells expressing wild type hD5R, cells transfected with PD3-hD5R display a robust increase in $E_{max}$ of DA following PMA treatment (**Fig. 5A**, **Table 3**). Interestingly, the PMA-induced potentiation of $E_{max}$ of DA in cells transfected with PD3-hD5R is indistinguishable from that measured in PMA-treated cells harbouring wild type hD1R (**Fig. 5A**, **Table 3**).

To address whether results obtained with PD3-hD5R are explained by the mutations of the four unique Ser if IL3 of hD5R, we compared the PMA regulation of PD3-hD5R with that of quadruple-point mutant form of hD5R, hD5-SSSS-AAAA. As shown in **Fig. 5B** and **Table 3**, there is no significant difference in the extent of PMA-mediated increase of $E_{max}$ in cells expressing hD5-SSSS-AAAA and PD3-hD5R. In addition, PD3-hD5R and hD5-SSSS-AAAA display a similar small rightward shift in $EC_{50}$ of DA (<2 fold) upon PMA treatment (**Table 3**). These studies may suggest a critical role of Ser260, Ser261, Ser271 and Ser274 phosphorylation in dictating the PMA-mediated desensitization of hD5R-induced cAMP synthesis.

To test further this hypothesis, we engineered two phosphomimetic forms of these four Ser residues using Ser-to-Asp and Ser-to-Glu mutations. In the absence of PMA treatment, the phosphomimetic hD5R mutants, named hD5-SSSS-DDDD and hD5-SSSS-EEEE, exhibit a strong rightward shift in $EC_{50}$ of DA when compared with that of control cells expressing wild type hD5R (**Fig. 5C**, **Table 4**). However, the rightward shift in $EC_{50}$ is not as striking as the one measured in cells transfected with wild type hD5R upon PMA treatment. These results are potentially explained by differences in the overall negative charge (one from Asp or Glu vs two for organic phosphate). Therefore, as opposed to sensitization of hD1R by PMA, our results suggest that the PMA-induced desensitization of DA-mediated activation of hD5R is controlled in phosphorylation-dependent fashion through four unique Ser residues in IL3.

**Table 3. PMA-mediated effects on DA-dependent stimulation of AC in HEK293 cells transfected with hD1R, hD5R, PD3-hD5R and hD5-SSSS-AAAA**

| Receptors | $B_{max}$ (pmol/mg membrane prot.) | $E_{max}$ (% of ctrl) | | $E_{max}$ (PMA/ctrl) | $EC_{50}$ (nM) | | $EC_{50}$ shift (PMA/ctrl) |
|---|---|---|---|---|---|---|---|
| | | control | PMA | % | control | PMA | Fold |
| hD1R | 1.78 ± 0.22 | 101 ± 3 | 217 ± 4 | 216 ± 3* | 10 [5.9-17] | 20 [15-26] | 1.9 ± 0.12* |
| hD5R | 1.67 ± 0.22 | 100 ± 3 | 131 ± 4 | 132 ± 3 | 0.91 [0.5-1.5] | 32 [21-48] | 37 ± 5 |
| PD3-hD5R | 1.69 ± 0.27 | 100 ± 3 | 211 ± 3 | 212 ± 3* | 4.8 [2.9-8.0] | 7.6 [5.9-9.8] | 1.8 ± 0.46* |
| PD3-hD5R | 2.42 ± 0.26 | 101 ± 5 | 198 ± 6 | 197 ± 6 | 11 [5.0-24] | 13 [8.5-19] | 1.2 ± 0.15 |
| hD5-SSSS-AAAA | 2.20 ± 0.18 | 100 ± 4 | 213 ± 4 | 213 ± 4 | 1.8 [0.5-7.0] | 2.0 [1.2-3.4] | 1.5 ± 0.45 |

Best-fitted and unconstrained parameters for $EC_{50}$ and $E_{max}$ obtained by simultaneous curve fitting of averaged dose-response curves of DA (shown in **Figs. 5A, B**) are listed. $EC_{50}$ values are expressed with 95% confidence intervals in brackets while $E_{max}$ values are reported as percent of control $E_{max}$ value ± approximate S.E. as determined by GraphPad Prism. $E_{max}$ ratios and $EC_{50}$ shifts were computed for each individual dose-response curve using best-fitted $EC_{50}$ and $E_{max}$ values obtained in the absence and presence of PMA. Receptor levels ($B_{max}$), $E_{max}$ ratios and $EC_{50}$ shift values are expressed as arithmetic means ± S.E. Statistical differences between $B_{max}$, $E_{max}$ ratios and $EC_{50}$ shift values of hD1R, hD5R and PD3-hD5R were assessed using one-way ANOVA followed by Newman-Keuls post-test. No statistical differences between $B_{max}$, $E_{max}$ ratios and $EC_{50}$ shift values of PD3-hD5R and hD5-SSSS-AAAA were detected using unpaired t test. * $p < 0.05$ vs. hD5R.

**Table 4. Best-fitted $EC_{50}$ of DA-dependent stimulation of AC in HEK293 cells transfected with hD5R (± PMA) and phosphomimetic mutant hD5-SSSS-DDDD and hD5-SSSS-EEEE.**

| **Receptors** | **$B_{max}$** (pmol/mg membrane prot.) | **$EC_{50}$** (nM) |
|---|---|---|
| hD5R control | 1.26 ± 0.22 | 1.1 [0.63-1.8]$^{\#}$ |
| hD5R + PMA | | 60 [33-108]$^{*}$ |
| hD5-SSSS-DDDD control | 0.88 ± 0.04 | 19 [11-30]$^{*\#}$ |
| hD5-SSSS-EEEE control | 1.08 ± 0.18 | 9.1 [5.5-15]$^{*\#}$ |

Best-fitted and unconstrained parameters for $EC_{50}$ obtained by simultaneous curve fitting of averaged dose-response curves of DA (shown in **Fig. 5C**) are reported. $EC_{50}$ values are expressed with 95% confidence intervals in brackets as determined by GraphPad Prism. $E_{max}$ ratios and $EC_{50}$ shifts were computed for each individual dose-response curve using best-fitted $EC_{50}$ and $E_{max}$ values obtained in the absence and presence of PMA. Receptor levels ($B_{max}$) are expressed as arithmetic means ± S.E. Statistical differences between $EC_{50}$ values of hD5R (± PMA), hD5-SSSS-DDDD (control) and hD5-SSSS-EEEE (control) were established by comparing goodness of fit obtained using unconstrained and shared $EC_{50}$ value. No statistical differences between $B_{max}$, were detected using one-way ANOVA followed by Newman-Keuls post-test. * $p < 0.05$ vs. hD5R (control); # $p < 0.05$ vs. hD5R (PMA).

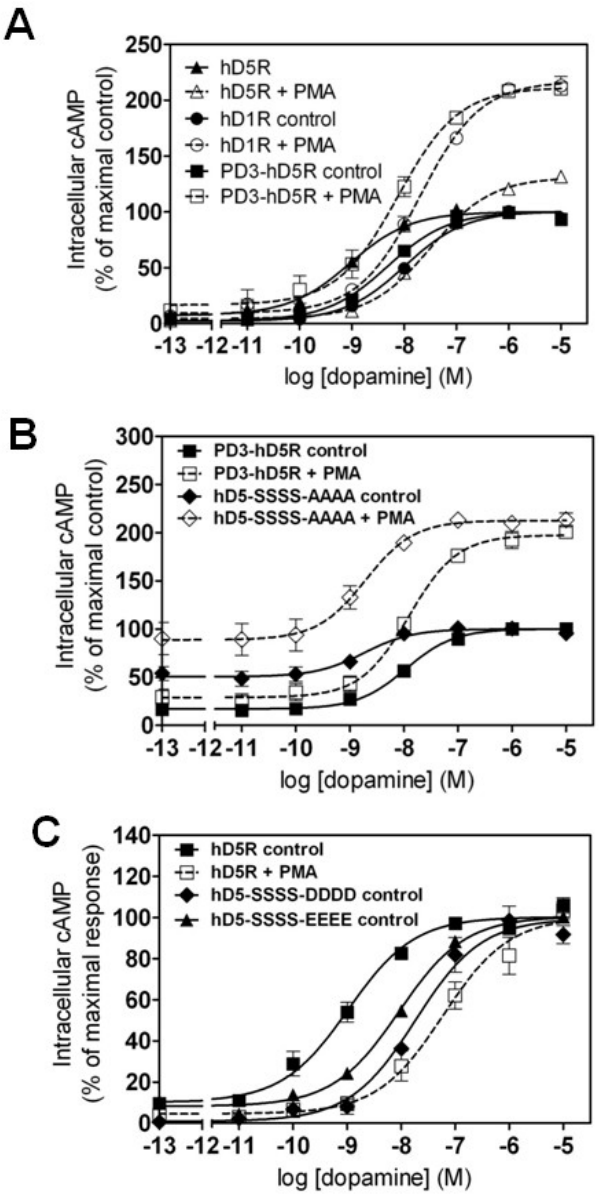


**Figure 5. DA-mediated stimulation of AC by wild type human D1-like receptors, PD3-hD5R, hD5-SSSS-AAAA, hD5-SSSS-DDDD and hD5-SSSS-EEEE in the absence and presence of PMA.**

Transfected HEK293 cells were seeded in 12-well plates and labeled with [$^3$H]-adenine as described in Experimental Procedures. Cells treated with vehicle (DMSO, 0.02% (v/v); control) or 1 μM PMA were incubated with increasing DA concentrations for 30 min at 37°C. Intracellular cAMP levels were plotted as a function of log of DA concentrations and expressed as percentage of best-fitted $E_{max}$ obtained under control condition for each receptor (**A** and **B**) or as percent of the best-fitted $E_{max}$ obtained in each experimental condition to facilitate the graphical representation of $EC_{50}$ shift between phosphomimetic mutants and hD5R (**C**). For each mutant receptor, experiments were repeated at least 4 times in triplicate determinations and done in parallel with wild type hD5R (**A** and **C**) and PD3-hD5R (**B**) as control receptors. Best-fitted curves obtained from parallel experiments using hD5R, hD1R and PD3-hD5R (**A**), PD3-hD5R and hD5-SSSS-AAAA (**B),** and hD5R, hD5-SSSS-DDDD and hD5-SSSS-EEEE (**C**) are shown. Values of PMA-mediated effects on $E_{max}$ and $EC_{50}$ for each receptor are reported in **Table 3** (hD1R, hD5R, PD3-hD5R and hD5-SSSS-AAAA) and **Table 4** (hD5R, hD5-SSSS-DDDD and hD5-SSSS-EEEE).

**Ser261 and Ser271 act as potential priming phosphorylation sites in PMA-induced desensitization of DA-dependent activation of hD5R**

So far, we have demonstrated that single-point mutant receptors (hD5-S260A, hD5-S261A, hD5-S271A and hD5-S274A) display a strong reduction in the PMA-induced desensitization of DA-dependent receptor stimulation as indexed by the smaller rightward shift of $EC_{50}$ and higher $E_{max}$ potentiation. Furthermore, rather than being fully inhibited, the PMA-induced desensitization of DA-mediated activation of PD3-hD5R and hD5-SSSS-AAAA is converted into a PMA-induced sensitization of receptor responsiveness similar to that of hD1R. Our data reveal that Ser261 and Ser271 play the most important role in promoting the desensitization of DA-dependent hD5R stimulation upon PMA treatment. Meanwhile, the mutation of Ser260 or Ser274 mediates also a significant diminution of PMA-induced desensitization of DA-dependent hD5R stimulation (**Fig. 4**). We speculate that Ser260 and Ser274 phosphorylation may require the PKC-induced phosphorylation of Ser261 and Ser271. If true, the double-point mutation of Ser261 and Ser 271 may recapitulate the PMA-induced regulation of DA-mediated stimulation of hD5-SSSS-AAAA. To test this hypothesis, we generated a new hD5R construct named hD5-Ser261A-Ser271A, which contains the double-point mutation of Ser261 and Ser271 in IL3. Our data obtained with this mutant clearly show that there is no significant difference between the extent of PMA-induced sensitization of hD5-S261A-S271A and hD5-SSSS-AAAA (**Fig. 6A** and **Table 5**). This indicates that mutation of Ser261 and Ser271 is sufficient to abrogate totally the PMA-induced desensitization of DA-dependent stimulation of hD5R. Thus, results of these experiments

may point to a role of PMA-mediated phosphorylation of Ser260 and Ser274 in controlling phosphorylation status of Ser261 and Ser271 by PKC.

Mutation of Ser261 alone or mutations of Ser260 and Ser261 would lead to similar PMA effects on hD5R responsiveness in the presence of DA. Likewise, we expect that the PMA-induced regulation of hD5R upon mutating Ser271 alone or both Ser271 and Ser274 will be equivalent. To address this issue, we performed PMA experiments using two new mutant forms of hD5R, hD5-S260A-S261A and hD5-S271A-S274A (**Figs. 6B, C**). While mutations of both Ser261 and Ser271 (as measured using hD5-S261A-S271A) fully switch the PMA-induced desensitization into PMA-induced sensitization, our results clearly show that double-point mutations of Ser260-Ser261 or Ser271-Ser274 partially recapitulate the PMA-induced sensitization of hD5-S261A-S271A in the presence of DA (**Figs 6B, C**). Additionally, there are no statistically detectable differences in $E_{max}$ potentiation and $EC_{50}$ rightward shift mediated by PMA between hD5-S260A-S261A and hD5-S261A and between hD5-S271A-S274A and hD5-S271A (**Figs. 6B, C** and **Table 5**). These findings may suggest a complete loss of PMA-induced desensitization of the DA-dependent hD5R stimulation following the mutation of both Ser261 and Ser271. Additionally, our data may suggest a recruitment of Ser260 and Ser274 in PKC-induced desensitization of the DA-mediated hD5R that is dependent on the phosphorylation status of Ser261 and Ser271.

**Table 5. PMA-mediated effects on DA-dependent stimulation of AC in HEK293 cells transfected with hD5-SSSS-AAAA, hD5-S261A-S271A, hD5-S260A-S261A, hD5-S271A-S274A, hD5-S261A and hD5-S271A.**

| **Receptors** | **$B_{max}$** (pmol/mg membrane prot.) | ***$E_{max}$*** (% of ctrl) | | ***$E_{max}$*** (PMA/ctrl) | **$EC_{50}$** (nM) | | **$EC_{50}$ shift** (PMA/ctrl) |
|---|---|---|---|---|---|---|---|
| | | **control** | **PMA** | **%** | **control** | **PMA** | **Fold** |
| hD5-SSSS-AAAA | 2.20 ± 0.18 | 100 ± 4 | 212 ± 5 | 213 ± 4 | 1.8 [0.50-7.4] | 2.0 [1.1-3.4] | 1.5 ± 0.45 |
| hD5-S261A-S271A | 2.46 ± 0.36 | 100 ± 5 | 206 ± 5 | 207 ± 2 | 1.8 [0.48-6.7] | 2.5 [1.5-4.3] | 1.4 ± 0.20 |
| hD5-S261A-S271A | 1.48 ± 0.26 | 100 ± 3 | 217 ± 4 | 218 ± 10*# | 1.7 [0.77-3.5] | 2.2 [1.6-3.0] | 1.3 ± 0.08*# |
| hD5-S260A-S261A | 1.63 ± 0.29 | 100 ± 4 | 153 ± 4 | 154 ± 9 | 1.9 [0.89-4.1] | 6.5 [4.3-9.9] | 3.5 ± 0.65 |
| hD5-S261A | 1.99 ± 0.52 | 100 ± 4 | 147 ± 4 | 148 ± 6 | 1.8 [0.71-4.4] | 9.1 [5.8-15] | 5.7 ± 1.3 |
| hD5-S271A-S274A | 1.73 ± 0.46 | 100 ± 4 | 158 ± 4 | 159 ± 4 | 2.2 [0.99-5.0] | 7.4 [5.0-11] | 3.6 ± 0.73 |
| hD5-S271A | 1.97 ± 0.33 | 100 ± 3 | 172 ± 4 | 172 ± 6 | 1.2 [0.53-2.6] | 5.0 [3.3-7.5] | 4.4 ± 0.75 |

Best-fitted and unconstrained parameters for $EC_{50}$ and $E_{max}$ obtained by simultaneous curve fitting of averaged dose-response curves of DA (shown in **Fig. 6**) are listed. $EC_{50}$ values are expressed with 95% confidence intervals in brackets while $E_{max}$ values are reported as percent of control $E_{max}$ value ± approximate S.E. as determined by GraphPad Prism. $E_{max}$ ratios and $EC_{50}$ shifts were computed for each individual dose-response curve using best-fitted $EC_{50}$ and $E_{max}$ values obtained in the absence and presence of PMA. Receptor levels ($B_{max}$), $E_{max}$ ratios and $EC_{50}$ shift values are expressed as arithmetic means ± S.E. Statistical differences between $B_{max}$, $E_{max}$ ratios and $EC_{50}$ shift values of hD5-SSSS-AAAA, hD5-S261-S271A, hD5-S260-S261A, hD5-S271-S274A, hD5-S261 and hD5-S271A were assessed using one-way ANOVA followed by Newman-Keuls post-test. No statistical differences between $B_{max}$, $E_{max}$ ratios and $EC_{50}$ shift values of hD5-SSSS-AAAA and hD5-S261-S271A were detected using unpaired t test. * $p < 0.05$ vs. hD5-S261A; # $p < 0.05$ vs. hD5-S271A.

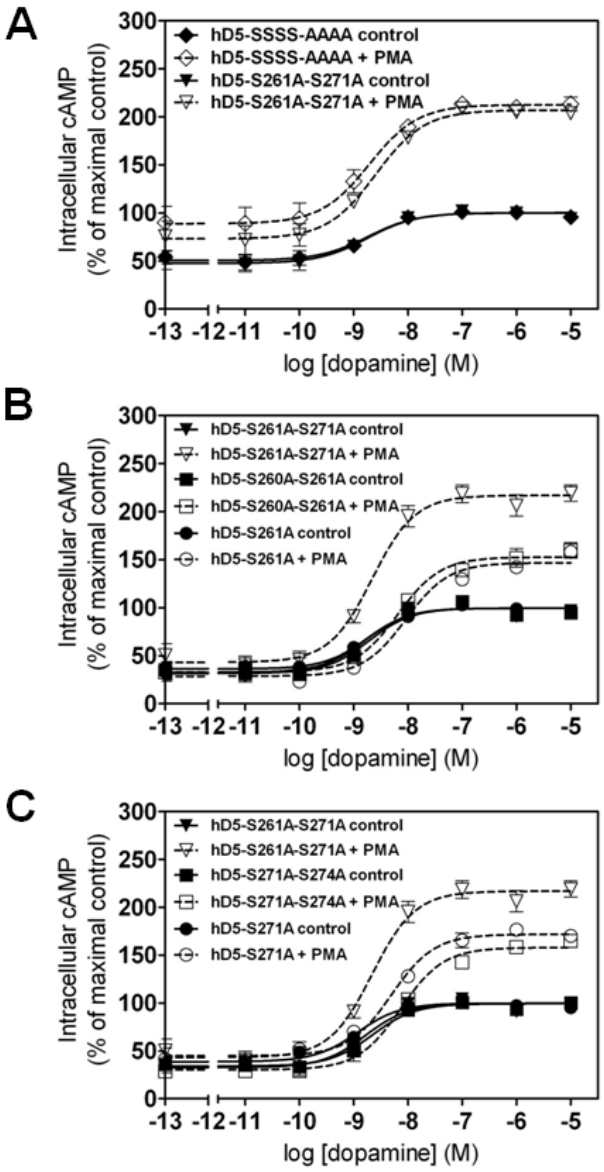


**Figure 6. DA-mediated stimulation of AC by hD5R, hD5-SSSS-AAAA, hD5-S261A-S271A, hD5-S260A-S261A, hD5-S271A-S274A, hD5-S261A and hD5-S271A in the absence and presence of PMA.**

Transfected HEK293 cells were seeded in 12-well plates and labeled with [$^3$H]-adenine as described in Experimental Procedures. Cells treated with vehicle (DMSO, 0.02% (v/v); control) or 1 μM PMA were incubated with increasing DA concentrations for 30 min at 37°C. Intracellular cAMP levels were plotted as a function of log of DA concentrations and expressed as percentage of best-fitted $E_{max}$ obtained under control condition for each receptor. For each mutant receptor, experiments were repeated at least 4 times in triplicate determinations and done in parallel with hD5-SSSS (**A**) and hD5-S261-S271A (**B**, **C**) as control receptors. Best-fitted curves obtained from parallel experiments using hD5-SSSS-AAAA, hD5-S261A-S271A (**A**), hD5-S261A-S271A, hD5-S260A-S261A, hD5-S261A, hD5-S2671A-S274A and hD5-S271A (**B**, **C**) are shown. To facilitate graphical comparison the best-fitted curves for hD5-S261A-S271A in the absence and presence of PMA are reported in B and C. Values of PMA-mediated effects on $E_{max}$ and $EC_{50}$ for each receptor are reported in **Table 5**.

**Potential role of Ser261 and Ser271 phosphorylation in promoting PMA-induced inhibition of constitutive (DA-independent) activity of hD5R**

Our group previously reported that, in contrast to hD1R, the constitutive activity of hD5R is drastically decreased by PMA treatment (Jackson et al. 2005). We have also shown that the PMA effect on constitutive activity of hD5R is mediated through IL3 (Plouffe & Tiberi, 2011; see thesis paper 1). Thus, this raises an important question about which Ser and/or Thr of IL3 controls PMA-induced inhibition of the constitutive activity of hD5R. Specifically, we wanted to establish whether Ser260, Ser261, Ser271 and Ser274 also play a role in PMA-induced inhibition of constitutive activation of hD5R. Importantly, we have recently demonstrated that Ser to Ala mutations of residues 260, 261 and 271 lead to an increase of basal activity of hD5R (Plouffe et al., 2011; see thesis paper 2). This indicates that these Ser critically shape the extent of constitutive activation of hD5R. Additionally, Iso288 in IL3 has an important role in facilitating the allosteric transition of hD5R from inactive (R) to active (R*) state (Charpentier et al. 1996). Interestingly, several Ser and/or Thr are located close to Iso288 (**Fig. 1B**). Notably, recent data from our lab show that mutation of Thr292 and Ser287 decrease the extent of hD5R constitutive activity (Plouffe et al., 2011; see thesis paper 2). Thus, PMA-induced phosphorylation of Ser and Thr in IL3 may culminate in a structural rearrangement of Iso288 and stabilization of hD5R in a more inactive state. Herein, we show that no single-point mutation completely abrogated the PMA-induced inhibition of hD5R constitutive activity (**Figs. 7A, B**). In fact, the single-point mutations of Ser261 and Ser271 were the only mutations that significantly reduce the extent of PMA-induced inhibition of receptor constitutive activity (**Fig. 7B**). Indeed, constitutive activity of hD5-

S261A and hD5-S271A is respectively reduced by 55% and 40% in comparison to the 80% inhibition of agonist-independent activity of wild type hD5R following PMA treatment (**Fig. 7B**). Intriguingly, the double-point mutation of Ser261 and Ser271 not only completely blocked PMA-induced inhibition of receptor constitutive activity but switched it into a PMA-induced augmentation of the agonist-independent activity (**Fig. 7C**). Most importantly, the extent of PMA-induced increase of the basal activity of hD5-S261A-S271A is undistinguishable from that of hD5-SSSS-AAAA, PD3-hD5R and hD1R (**Fig. 7C**). Altogether, these findings imply a key role of Ser261 and Ser271 in the regulation of PMA-induced inhibition of hD5R constitutive activity.

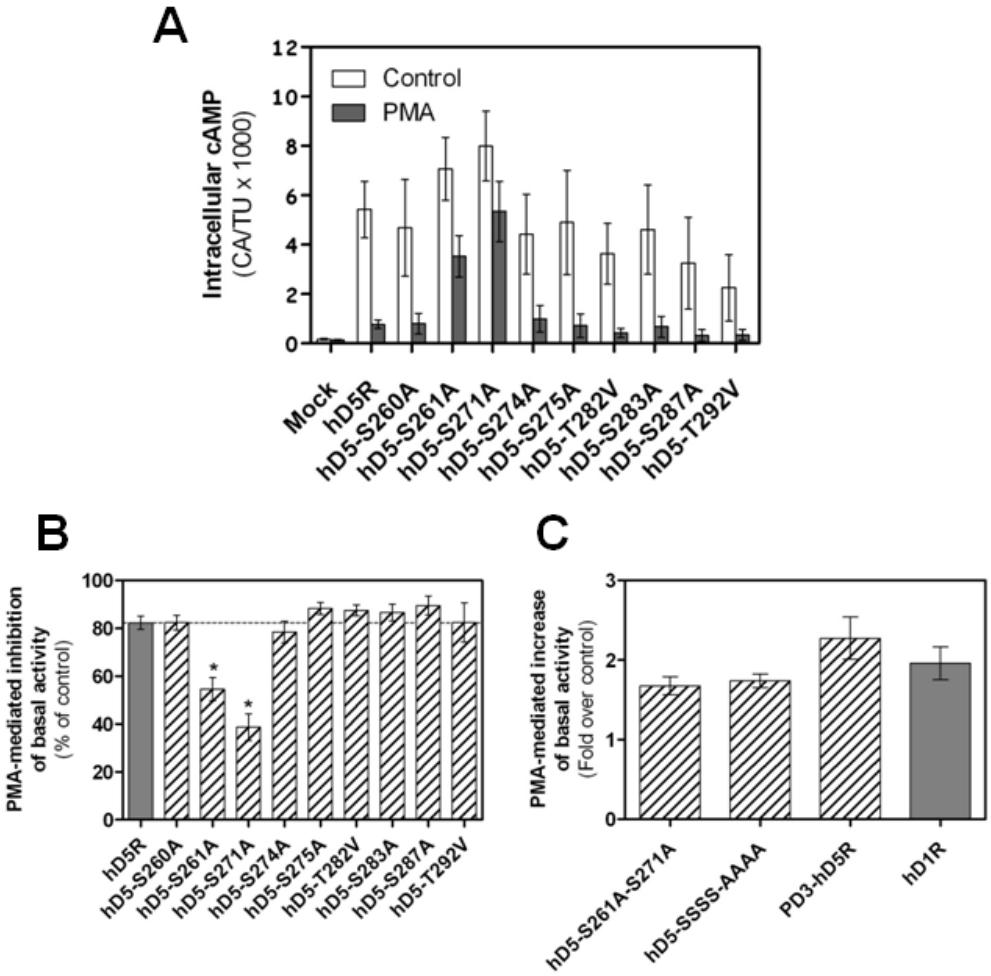


**Figure 7. Effect of PMA treatment on constitutive activity of wild type and mutant hD5R.**

HEK293 cells transfected with 5 μg of receptor DNA construct per dish were seeded in 6-well plates and labeled with [$^3$H]-adenine as described in Experimental Procedures. Cells were treated with vehicle (DMSO, 0.02% (v/v)) (control) or 1 μM PMA for 30 minutes at 37°C. **(A)** Intracellular cAMP levels were determined and expressed as CA/TU x 1000. Raw data are expressed as the arithmetic mean ± S.E. of at least 7 experiments done in triplicate determinations. **(B)** For each experiment, the ratio of the amount of intracellular cAMP levels produced under PMA relative to control in cells expressing wild type and single-point mutant forms of hD5R was computed and expressed as the arithmetic mean ± S.E. (**C**) PMA-induced effect on constitutive activity of hD5-S261-S271A, hD5-SSSS-AAAA, PD3-hD5R and hD1R computed as fold over control are expressed as the arithmetic mean ± S.E. of at least 4 experiments done in triplicate determinations. Statistical difference between ratios was compared with wild type hD5R ratio (**B**) or hD5-S261-S271A (**C**) using one-way ANOVA with Newman-Keuls multiple comparison post-test. $B_{max}$ values (pmol/mg membrane proteins) were as follows: 10.0 ± 0.91 (hD5R), 8.15 ± 1.35 (hD5-S260A), 11.3 ± 0.92 (hD5-S261A), 12.0 ± 0.98 (hD5-S271A), 13.2 ± 0.93 (hD5-S261A-S271A), 13.2 ± 0.76 (hD5-SSSS-AAAA), 9.67 ± 1.08 (hD5-S274A), 9.77 ± 1.24 (hD5-S275A), 9.64 ± 1.18 (hD5-T282V), 8.98 ± 1.19 (hD5-S283A), 10.2 ± 1.44 (hD5-S287A), 13.5 ± 1.83 (hD5-T292V), 13.2 ± 0.95 (PD3-hD5R) and 10.8 ± 0.92 (hD1R). * $p < 0.05$ vs. hD5R.

**"Transplantation" of unique hD5R Ser residues into IL3 of hD1R blunts PMA-induced sensitization of hD1R**

Results obtained until now suggest that the outcome of D1-like receptor regulation by PMA treatment is dependent on the absence or presence of Ser residues strategically located in IL3. Specifically, Ser260, Ser261, Ser271 and Ser274 of hD5R are required to trigger PMA-mediated desensitization of this D1-like subtype while mutations of these residues allows a sensitization process of DA-independent and dependent hD5R responsiveness upon PMA treatment. These four hD5R Ser residues match with Ala229, Ala230, Asn240 and Thr243 in IL3 of hD1R (**Fig. 1**). To validate this model, these four residues in IL3 of hD1R were replaced with Ser residues leading to a new hD1R mutant referred to as hD1-AANT-SSSS. The hD1-AANT-SSSS mutant was tested in parallel with hD1R and a chimeric hD1R in which the IL3 was exchanged for the one from hD5R (called hD1-$IL3_{D5}$). We show that hD1-$IL3_{D5}$ chimera essentially behave as hD5R with respect to PMA-induced desensitization of receptor responsiveness (Plouffe & Tiberi, 2011; see thesis paper 1). This "transplantation" strategy leads to a significant reduction of PMA-induced sensitization of hD1R responsiveness (i.e. increases receptor desensitization) as suggested by the bigger $EC_{50}$ rightward shift (from 1.3 to 7 fold) and reduced $E_{max}$ potentiation upon PMA treatment (**Fig. 8** and **Table 6**). However, it is worth mentioning the PMA-induced $EC_{50}$ rightward shift for DA measured with hD1-AANT-SSSS is smaller than that of hD1-$IL3_{D5}$ (13 fold). Residues neighbouring the "transplanted" Ser in the context of IL3 of hD1R potentially explain these differences between hD1-AANT-SSSS and hD1-$IL3_{D5}$. Alternatively, agonist binding to these mutant receptors may promote distinct active receptor conformations, which are

differentially regulated by PMA treatment as suggested by our recent work (Plouffe *et al.* 2012). Notwithstanding this issue, our data strongly suggest that the transplantation of the key desensitizing Ser residues from IL3 of hD5R into IL3 of hD1R at matching positions can promote a PMA-induced desensitization of hD1R responsiveness.

**Table 6. PMA-mediated effects on DA-dependent stimulation of AC in HEK293 cells transfected with hD1R, hD1-IL3$_{D5}$ and hD1-AANT-SSSS.**

| Receptors | $B_{max}$ (pmol/mg membrane prot.) | $E_{max}$ (% of ctrl) | | $E_{max}$ (PMA/ctrl) | $EC_{50}$ (nM) | | $EC_{50}$ shift (PMA/ctrl) |
|---|---|---|---|---|---|---|---|
| | | control | PMA | % | control | PMA | Fold |
| hD1R | 1.57 ± 0.31 | 100 ± 4 | 223 ± 4 | 217 ± 12$^{\#}$ | 13 [7.4-25] | 16 [12-21] | 1.3 ± 0.23$^{\#}$ |
| hD1-IL3$_{D5}$ | 1.29 ± 0.27 | 100 ± 4 | 159 ± 6 | 158 ± 16$_{*}$ | 10 [5.5-19] | 122 [81-184] | 13 ± 2$^{*}$ |
| hD1-AANT-SSSS | 1.30 ± 0.31 | 100 ± 4 | 187 ± 5 | 186 ± 5$^{*}$ | 10 [5.6-19] | 73 [52-102] | 7.1 ± 1.3$^{*\#}$ |

Best-fitted and unconstrained parameters for $EC_{50}$ and $E_{max}$ obtained by simultaneous curve fitting of averaged dose-response curves of DA (shown in **Fig. 8**) are listed. $EC_{50}$ values are expressed with 95% confidence intervals in brackets while $E_{max}$ values are reported as percent of control $E_{max}$ value ± approximate S.E. as determined by GraphPad Prism. $E_{max}$ ratios and $EC_{50}$ shifts were computed for each individual dose-response curve using best-fitted $EC_{50}$ and $E_{max}$ values obtained in the absence and presence of PMA. Receptor levels ($B_{max}$), $E_{max}$ ratios and $EC_{50}$ shift values are expressed as arithmetic means ± S.E. Statistical differences between $B_{max}$, $E_{max}$ ratios and $EC_{50}$ shift values of hD1R, hD1-IL3$_{D5}$ and hD1-AANT-SSSS were assessed using one-way ANOVA followed by Newman-Keuls post-test. No statistical differences were detected between $B_{max}$ values of hD1R, hD1-IL3$_{D5}$ and hD1-AANT-SSSS. * $p < 0.05$ vs. hD1R; # $p < 0.05$ vs. hD1-IL3$_{D5}$.

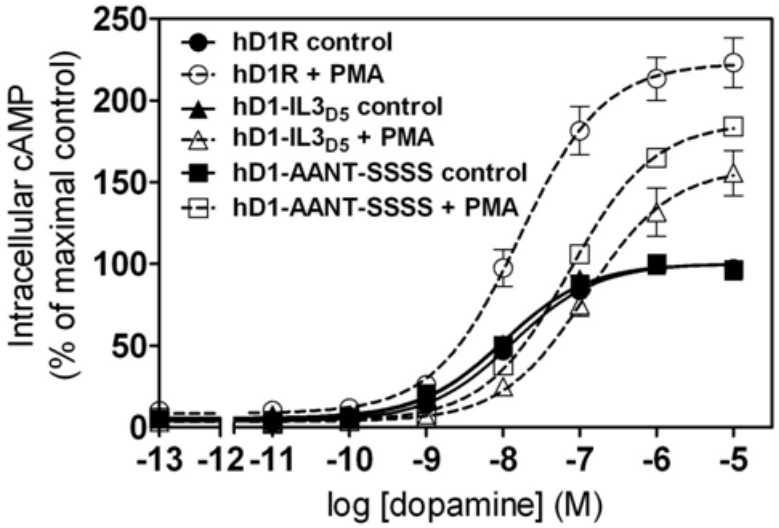


**Figure 8. DA-mediated stimulation of AC by hD1R, hD1-IL3$_{D5}$ and hD1-AANT-SSSS in the absence and presence of PMA.**

Transfected HEK293 cells were seeded in 12-well plates and labeled with [$^3$H]-adenine as described in Experimental Procedures. Cells treated with vehicle (DMSO, 0.02% (v/v); control) or 1 μM PMA were incubated with increasing DA concentrations for 30 min at 37°C. Intracellular cAMP levels were plotted as a function of log of DA concentrations and expressed as percentage of best-fitted $E_{max}$ obtained under control condition for each receptor. For each mutant receptor, experiments were repeated 4 times in triplicate determinations and done in parallel with hD1R as the control receptor. Best-fitted curves obtained from parallel experiments using hD1R, hD1-IL3$_{D5}$ and hD1-AANT-SSSS are shown. Values of PMA-mediated effects on $E_{max}$ and $EC_{50}$ for each receptor are reported in **Table 6**.

**PMA-induced hD1R sensitization and hD5R desensitization are lost following truncation of the central part of IL3**

Our recent study using a chimeric approach show that IL3 is the key region for hD1R potentiation upon PMA treatment (Plouffe & Tiberi, 2011; see thesis paper 1). Additionally, this study provided evidence that PMA-induced effects are mediated directly through receptors and not on AC activity (Plouffe & Tiberi, 2011; see thesis paper 1). Herein, results obtained with PD3-hD1R imply that PMA-induced sensitization of hD1R is also controlled by an IL3 phosphorylation-independent mechanism. Arguably, one could raise an issue about the potential role of Ser or Thr located in the CT of hD1R, which could have been overlooked in the context of studies using chimeras. Indeed, hD5R harbours numerous Ser and Thr on its CT region. However, preliminary studies in our laboratory using an hD1R lacking Ser and Thr residues in the CT still display an unaltered PMA-induced sensitization (B. Plouffe, data not shown). In light of all these findings, we would like to propose that a potential structural motif located in IL3 of hD1R is required for the action of PMA and PKC on this receptor. Moreover, the PMA-induced sensitization observed for PD3-hD5R and hD5-SSSS-AAAA suggests that such motif(s) may also be found in hD5R. Notably, our previous studies also hint that residues located in N- and C-terminal parts of IL3 (adjacent to TM5 and TM6) are unlikely to be involved as they are fully conserved between hD1R and hD5R (**Fig. 1**). We reasoned that motifs for the PMA and PKC-induced regulation are potentially located within the central part of IL3 delimited by Lys223 and Arg266 in hD1R, and Val254 and Lys290 in hD5R (**Fig. 1**). In the present study, we investigated the role of structural determinants of the central part of IL3 using a truncation approach. Truncations of IL3 region encompassing

Gln224 to Thr245 in hD1R (mutant termed hD1-ΔQ224-T245) and the corresponding Gln255 to Ser275 in hD5R (mutant termed hD5-ΔQ255-S275) were generated. Importantly, the IL3 truncated receptors express in HEK293 cells and display D1-like receptor affinity for DA (Charrette et al., 2011, in preparation). Large truncations of IL3 yield to non-functional receptors (data not shown). Interestingly, hD1-ΔQ224-T245 is insensitive to PMA treatment when compared with wild type hD1R (**Fig. 9A** and **Table 7**). Additionally, hD5-ΔQ255-S275 did not undergo PMA-induced desensitization as indexed by the absence of rightward shift in $EC_{50}$ for DA. These results are in line with those obtained with PD3-hD5R and hD5-SSSS-AAAA harbouring mutated Ser260, Ser261, Ser271 and Ser274. However, as opposed to PD3-hD5R and hD5-SSSS-AAAA, hD5-ΔQ255-S275 exhibits no $E_{max}$ potentiation upon PMA treatment (**Fig. 9B** and **Table 7**). Overall, these findings highlight that the central part of IL3 of hD1R and hD5R contains potential motifs that lack or harbour Ser residues responsible for the sensitization and desensitization of human D1-like subtypes by PMA and PKC. Further studies are underway to delineate more precisely the amino acid composition of these IL3 motifs.

**Table 7. PMA-mediated effects on DA-dependent stimulation of AC in HEK293 cells transfected with hD1R, hD5R, hD1-ΔQ224-T245 and hD5-ΔQ255-S275.**

| Receptors | $B_{max}$ (pmol/mg membrane prot.) | $E_{max}$ (% of ctrl) | | $E_{max}$ (PMA/ctrl) | $EC_{50}$ (nM) | | $EC_{50}$ shift (PMA/ctrl) |
|---|---|---|---|---|---|---|---|
| | | control | PMA | % | control | PMA | Fold |
| hD1R | 1.02 ± 0.17 | 100 ± 5 | 197 ± 6 | 199 ± 8# | 17 [6.6-45] | 21 [13-33] | 1.5 ± 0.38# |
| hD1-ΔQ224-T245 | 0.93 ± 0.16 | 99 ± 7 | 114 ± 7 | 115 ± 5* | 227 [78-661] | 272 [106-695] | 1.2 ± 0.19# |
| hD5R | 1.21 ± 0.14 | 100 ± 6 | 112 ± 7 | 113 ± 6* | 1.4 [0.40-4.7] | 18 [6.7-48] | 15 ± 3* |
| hD5-ΔQ255-S275 | 1.10 ± 0.21 | 99 ± 8 | 115 ± 7 | 126 ± 18* | 192 [48-776] | 31 [11-87] | 0.24 ± 0.05# |

Best-fitted and unconstrained parameters for $EC_{50}$ and $E_{max}$ obtained by simultaneous curve fitting of averaged dose-response curves of DA (shown in **Fig. 9**) are listed. $EC_{50}$ values are expressed with 95% confidence intervals in brackets while $E_{max}$ values are reported as percent of control $E_{max}$ value ± approximate S.E. as determined by GraphPad Prism. $E_{max}$ ratios and $EC_{50}$ shifts were computed for each individual dose-response curve using best-fitted $EC_{50}$ and $E_{max}$ values obtained in the absence and presence of PMA. Receptor levels ($B_{max}$), $E_{max}$ ratios and $EC_{50}$ shift values are expressed as arithmetic means ± S.E. Statistical differences between $B_{max}$, $E_{max}$ ratios and $EC_{50}$ shift values of hD1R, hD5R, hD1-ΔQ224-T245 and hD5-ΔQ255-S275 were assessed using one-way ANOVA followed by Newman-Keuls post-test. No statistical differences were detected between $B_{max}$ values of hD1R, hD5R, hD1-ΔQ224-T245 and hD5-ΔQ255-S275.
* $p < 0.05$ vs. hD1R; # $p < 0.05$ vs. hD5R

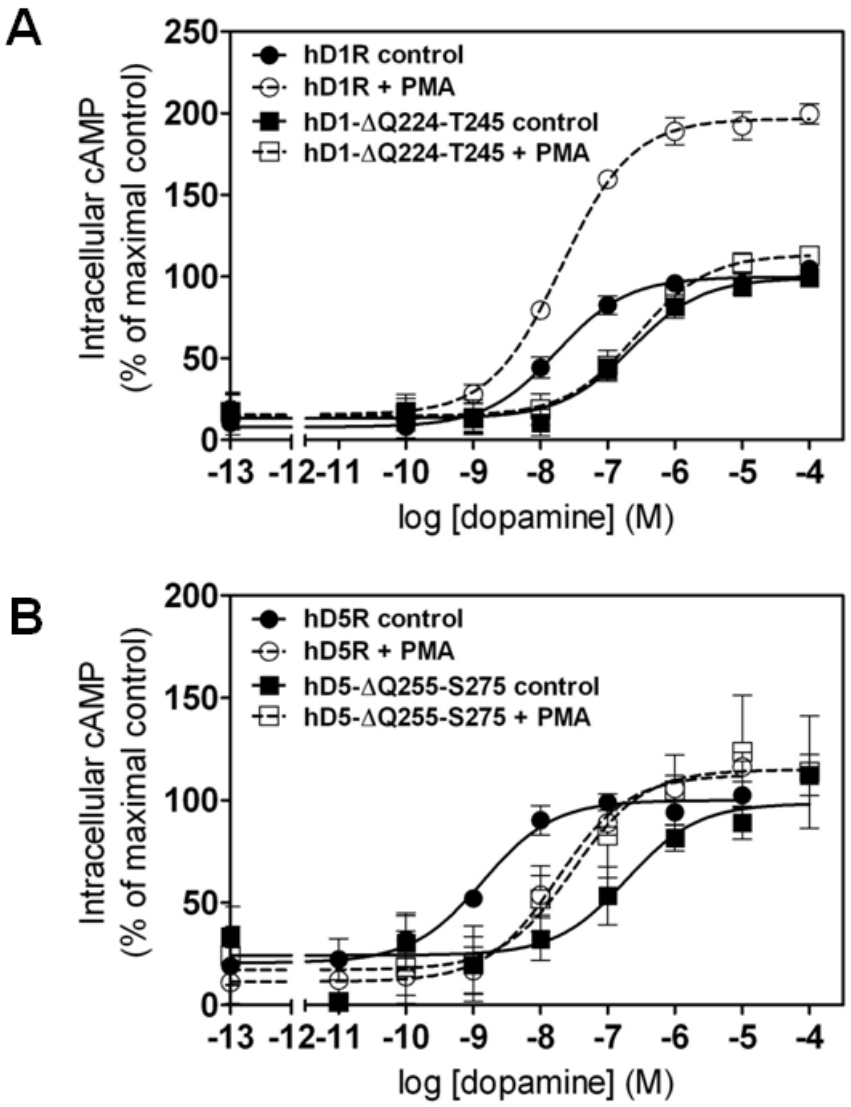


**Figure 9. DA-mediated stimulation of AC by wild type and IL3 truncated forms of hD1R and hD5R in the absence and presence of PMA.**
Transfected HEK293 cells were seeded in 12-well plates and labeled with [$^3$H]-adenine as described in Experimental Procedures. Cells treated with vehicle (DMSO, 0.02% (v/v); control) or 1 μM PMA were incubated with increasing DA concentrations for 30 min at 37°C. Intracellular cAMP levels were plotted as a function of log of DA concentrations and expressed as percentage of best-fitted $E_{max}$ obtained under control condition for each receptor. For each mutant receptor, experiments were repeated 4 times in triplicate determinations and done in parallel with hD1R (**A**) or hD5R (**B**) as control receptors. Best-fitted curves obtained from parallel experiments using hD1R and hD1-ΔQ224-T245 (**A**) and hD5R and hD5-ΔQ255-S275 (**B**) are shown. Values of PMA-mediated effects on $E_{max}$ and $EC_{50}$ for each receptor are reported in **Table 7**.

## Discussion

### Insight into sequences composing PKC phosphorylation sites of IL3 of hD5R

Studies have determined the classic hallmark of a consensus recognition and phosphorylation site motif for PKC (Pearson & Kemp 1991). Notably, the presence of Arg or Lys (two basic amino acids) at position -3 and hydrophobic amino acid at position +1 seems to be required for the phosphorylation of Ser (position 0) by PKC isoforms (Nishikawa et al. 1997, Newton 2001). Findings also suggest PKC-induced regulation of proteins can be mediated through variant sequences of this consensus phosphorylation motif (Pearson & Kemp 1991, Nishikawa et al. 1997, Newton 2001). With respect to hD5R, the stretch of sequence containing Ser261 (Arg257-Arg258-Iso259-**Ser260-Ser261**-Leu262) fulfils the consensus amino acid requirement for an optimal PKC substrate. In agreement with this, we show that Ser261 plays a predominant role in PMA-induced desensitization of hD5R. The other most important residue for PMA-induced desensitization of hD5R is Ser271. The surrounding amino acids of Ser271 (Glu267-His268-Ala269-Gln270-**Ser271**-Cys272) are not fully compliant with residues that form an optimal PKC substrate sequence. Nevertheless, at position -3 the standard Arg or Lys is replaced with His, another basic amino acid. Additionally, studies have shown that the PKC site on the elongation factor 1α and neuromodulin has a Gln residue neighbouring the phosphor-acceptor Ser/Thr. Thus, the stretch of amino acids around Ser271 may represent a new PKC phosphorylation site motif. Ser260 and Ser274, which are implicated only in the PMA-induced desensitization of DA-dependent activation of hD5R, meets partially the criteria for the standard optimal PKC site as suggested by the presence of Arg at position -3 and -1, respectively. However, the full compliance of an

optimal PKC substrate site may not be necessary for the PKC-induced phosphorylation of Ser260 and Ser274. Indeed, as the recruitment of Ser260 and Ser274 in the PMA-induced hD5R desensitization depends on PKC-phosphorylation of their respective adjacent Ser (Ser261 and Ser271) and DA binding to receptors, we believe that structural rearrangements within the IL3 region containing Ser260 and Ser274 lead to the formation of optimal PKC substrate sites for these two residues. Altogether, previous findings using pharmacological inhibitors and present data strongly point to a role of these four unique hD5R Ser residues as potential phosphorylation sites for nPKCs (Jackson et al. 2005).

**Critical role of IL3 central region in PMA-induced hD1R sensitization**

In contrast to hD5R, mutations of single or multiple Ser and Thr in IL3 of hD1R had no significant impact on the PMA-induced sensitization of constitutive and DA-dependent activation. We have previously established that IL3 dictates the fate of hD1R and hD5R responsiveness upon PMA treatment (Plouffe et al. 2012). Findings reported in this paper potentially suggest that the IL3-dependent PMA-induced hD1R sensitization does not require IL3 phosphorylation. Additionally, deletion of the central region of IL3 of hD1R completely abolishes the PMA-induced sensitization. Collectively, the results may point to a novel GPCR mechanism whereby PKC regulates PMA-induced receptor sensitization through a potential IL3/PKC interaction-dependent and phosphorylation-independent process. Alternatively, regulation of hD1R and hD5R by PKC may be controlled through the PKC-induced regulation of intermediary partners of hD1R and hD5R signaling complex.

**Presence of putative PKC pseudosubstrate site on IL3 of hD1R?**

Our assertion about the requirement of an interaction between PKC and IL3 in PMA-induced sensitization of hD1R is based on evidence showing that removal of IL3 central region of hD1R abolishes the PMA effect. Thus, this raises an important question about how PKC interacts with IL3 of hD1R. Interestingly, PKC has been shown to interact also with pseudosubstrate site motifs (House & Kemp 1987, Nishikawa et al. 1997, Newton 2001). The PKC pseudosubstrate site motif is identical to the substrate site with the exception that an Ala residue replaces the phospho-acceptor Ser/Thr residues. A comparison of the amino acid sequence within the central region of IL3 shows that Ser260 and Ser261 in hD5R are replaced in hD1R with Ala residues (Ala229 and Ala230). Potentially, this sequence in IL3 of hD1R may act as pseudosubstrate PKC sites. Meanwhile, Ser271 and Ser274 of hD5R correspond to Asn240 and Thr243 in hD1R. This apparent mismatch with Ala requirement for pseudosubstrate site may not be irreconcilable with the idea that Asn and Thr encompass amino acid sequences for PKC pseudosubstrate sites. In fact, a study has shown that replacement of Ala with Val or Asp does not interfere with the functionality of the PKC pseudosubstrate site in IL3 of the long isoform of human D2R (Morris et al. 2007). Moreover, while Ser and Thr can serve as phospho-acceptors, studies have demonstrated that in vertebrate cells, Thr residues undergo a ~10-fold lower phosphorylation rate than Ser residues (Mann *et al.* 2002). It is then possible that Thr243 is not phosphorylated by PKC. In fact, we demonstrated herein that substitution of these four hD1R residues with Ser (to mimic hD5R PKC sites) interfere with the expression of a full-scale PMA-induced sensitization of hD1R. Further studies are required to address in a more mechanistic fashion whether Asn240 and

Thr243 are involved in the formation of potentially new type of PKC pseudosubstrate sites in IL3 of hD1R.

**Putative mechanism of PMA-induced sensitization of hD1R: a working model**

Classically, PKC pseudosubstrate sites act as decoys, reducing phorbol ester sensitivity and inhibition of protein activity by PKC (House & Kemp 1987, Newton 2001). For instance, the pseudosubstrate site present in IL3 of human $D2R_{long}$ isoform promotes a reduced sensitivity to phorbol esters and weaker PKC-induced desensitization relative to $D2R_{short}$ isoform by competing with the binding of PKC to conserved Ser phospho-acceptor sites (Morris et al. 2007). In contrast to the PKC pseudosubstrate of $D2R_{long}$ isoform, we propose that the PKC pseudosubstrate sites (Arg226-Arg227-Iso228-**Ala229-Ala230**-Leu231) in IL3 of hD1R potentially interact with PKC to form a signaling complex mediating the PMA-induced sensitization of receptor responsiveness. To support this idea, PKC not only binds to lipids but also promote protein-protein interactions through its regulatory and catalytic domains (Ron & Kazanietz 1999). In this context, we then hypothesize that, as opposed to hD5R in which PKC may rapidly dissociate from IL3 following Ser phosphorylation, the hD1R PKC pseudosubstrate sites provide a more stable interaction with PKC owing to the lack of production of negative charges by PKC. The stable interaction between IL3 of hD1R and PKC alone with its PKC binding partner(s) may underlie the formation of a signaling complex that enhances hD1R coupling to Gs proteins. On the contrary, we think that PKC dissociates more readily from hD5R following PKC-mediated IL3 phosphorylation, a process linked to receptor conformational changes hindering the constitutive and agonist-dependent

coupling to Gs. Interestingly, studies have demonstrated that phosphorylation-independent activation of phospholipase D by PKC is controlled through direct protein-protein interactions (Conricode *et al.* 1992, Chen & Exton 2004). We hypothesize that the modality of regulation of human D1-like receptor responsiveness by PMA (sensitization vs. desensitization) is potentially dependent on the strength of interaction between PKC and unphosphorylated/phosphorylated state of IL3.

Additionally, because PMA is a potent activator of PKC, we infer that PMA-induced hD5R desensitization and hD1R sensitization is controlled by PKC. However, pharmacological inhibitors of PKC reduce PMA-induced potentiation of $E_{max}$ in hD1R-expressing cells by only 50% while fully blocking PMA-induced hD5R desensitization (Jackson et al. 2005). These findings thus raise a question about whether PKC activation and binding to IL3 solely promote the IL3 phosphorylation-independent PMA-induced hD1R sensitization. Several other non-PKC proteins have been described as phorbol ester receptors (Ron & Kazanietz 1999, Yang & Kazanietz 2003, Springett *et al.* 2004). However, a role of non-PKC phorbol ester receptors in the regulation of GPCR responsiveness has yet to be demonstrated.

## Conclusions

In the present study, we have demonstrated that the opposite regulation of the highly homologous hD1R and hD5R by PMA is mediated through strikingly different IL3-dependent mechanisms. Inactivating and phosphomimetic mutations have highlighted the critical role of four Ser residues (Ser260, Ser261, Ser271 and Ser274) located in the central region of IL3 of hD5R in triggering the PMA-induced desensitization of constitutive and DA-dependent activation of hD5R. Our study also suggests that the extent of PMA/PKC-induced desensitization of hD5R is dependent on the phosphorylation status of four unique Ser residues. Finally, as hD1R and hD5R are co-expressed in neurons with GPCR coupled to PKC activation, we believe that this regulation of human D1-like receptors by PMA may be of physiological importance in the fine-tuning of DA-associated motor and cognitive functions under normal and pathological conditions. Meanwhile, future studies using cell phosphorylation assays will be required to confirm the IL3 phosphorylation-independent hD1R potentiation upon PMA treatment as well as the phosphorylation of hD5R by PKC. Mass spectrometry also needs to be used to validate the hierarchichal phosphorylation mechanism possibly involved in PMA-induced hD5R desensitization.

**Research highlights of chapter 4-manuscript 3**

In this third manuscript, I demonstrated that:

- PMA-mediated hD1R sensitization may be IL3 phosphorylation-independent while requiring the IL3 central region.
- Desensitization of DA-mediated hD5R responsiveness by PMA treatment is potentially dependent on IL3 phosphorylation of Ser260, Ser261, Ser271 and Ser274.
- Phosphorylation of Ser261 and Ser271 act as potential priming sites for phosphorylation of Ser260 and Ser274, respectively.
- Mutation of Ser261 and Ser271 prevents PMA-mediated decrease of hD5R constitutive activity.
- DA binding to hD5R potentially mediates agonist-induced conformational change in IL3 of hD5R controlling Ser260 and Ser274 phosphorylation which is necessary for complete hD5R desensitization in presence of dopamine.
- Presence or absence of four PKC sites control whether D1-like receptors are desensitized or sensitized.

The potential mechanisms involved in the opposite regulation of hD1-like receptors by PMA were described in this third manuscript. However, novel PKC isoforms regulating the PMA-induced D1-type receptor regulation are unknown. The next manuscript (manuscript 4) addresses this question using shRNAs.

# Chapter 5

# Manuscript 4

# Role of novel PKC isoforms in dopamine D1 and D5 receptor signaling

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**Running title**: Regulation of D1R and D5R by nPKC isoforms.

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**Statement of author contributions:**

BP and MT conceived and designed research. BP performed research. BP and MT analyzed data, wrote and edited the manuscript.

## Summary

Previously, we demonstrated that protein kinase C (PKC) stimulation by phorbol-12-myristate-13-acetate (PMA) robustly desensitizes the human dopamine D5 receptor (hD5R) in HEK293 cells. In contrast, PMA treatment strongly sensitizes the human dopamine D1 receptor (hD1R), which is highly homologous to hD5R. Additionally, related studies using pharmacological inhibitors indicate that PMA-induced hD1R sensitization and hD5R desensitization is controlled by novel PKCs (nPKCs) isoforms (δ, ε, θ, η). In the present study, we used a short hairpin RNA (shRNA) approach to test the role of three nPKC isoforms (δ, ε and θ) in the opposite regulation of hD1R and hD5R responsiveness by PMA. ShRNA transfections in HEK293 cells indicate that each targeted nPKC tested (δ, ε and θ) was reduced by at least 70% in an isozyme-specific shRNA manner without any significant nPKC isoform off target knockdowns. Surprisingly, knockdown studies strongly suggest that PKCδ and PKCθ promote a positive tonic regulation of dopamine-mediated activation of hD1R and hD5R in HEK293 cells. We further investigated the effect of PKCδ, PKCε and PKCθ knockdowns on the PMA-mediated regulation of hD1R and hD5R responsiveness. Our data indicate that a decrease of PKCε expression significantly reduces PMA-induced hD1R sensitization. In contrast, the PMA-induced hD5R desensitization is inhibited by PKCδ knockdown. Overall, our studies demonstrate that human D1-like receptors are positively regulated by a tonic action of PKCδ and PKCθ. Moreover, the opposite PMA-induced regulation of hD1R and hD5R responsiveness is achieved through specific nPKC isoforms. Most importantly, our studies highlight for the first time that the regulation of

Gs-coupled G protein-coupled receptor responsiveness can be modulated by an intricate interplay between receptors and different PKC isoforms.

## Introduction

Dopaminergic receptors are G protein-coupled receptors (GPCRs) divided into two classes: D1-like (D1R and D5R) and D2-like ($D2R_{short}$, $D2R_{long}$, D3R and D4R) subtypes. The $G\alpha_s$-linked D1-like and $G\alpha_{i/o}$-linked D2-like receptors stimulate and inhibit adenylyl cyclase (AC) activity, respectively (Missale et al. 1998). Studies have implicated aberrant D1-like receptor responsiveness in several neuropsychiatric disorders such as schizophrenia, Parkinson's and Huntington's diseases (O'Sullivan et al. 2008, Tang et al. 2007, Lasky-Su et al. 2007, Papakostas 2006, Pantazopoulos et al. 2004, Caine *et al.* 2007). Therefore, a better understanding of the molecular mechanisms underlying the regulation of human D1-like receptors may help in designing novel therapeutic approaches.

Classically, GPCR activity is tightly modulated by two main regulatory pathways. The first one, referred to as homologous regulation, is triggered upon agonist binding to the receptor. Following agonist binding to GPCRs, two main players, G protein-coupled receptor kinases (GRKs) and β-arrestins, are recruited to the agonist-bound receptor to promote its desensitization and internalization (Gainetdinov *et al.* 2004). In contrast, the second regulatory pathway, called heterologous regulation, is mediated through the phosphorylation of a given receptor by the activation of second messenger-dependent serine/threonine kinases (protein kinase A or PKA; protein kinase C or PKC) following the stimulation of other GPCRs. Typically, GPCR phosphorylation by these Ser/Thr kinases impairs the ability of the receptor to stimulate their G proteins (Pitcher et al. 1992a).

GPCR regulation through PKA is relatively straightforward whereas PKC-induced modulation of GPCR is more complex by virtue of the presence of multiple PKC isoforms. There are ten PKC isoforms divided into three groups (conventional, novel and atypical), according to their activation cofactors. Stimulation of conventional PKC (cPKC) isoforms (α, γ, βI, and βII) depends on anionic phospholipids such as phosphatidylserine (PS), but also $PIP_2$, DAG and calcium. As opposed to cPKC, activation of novel PKCs (nPKC) (δ, ε, θ, η) is calcium-independent. Meanwhile, activation of atypical PKC (aPKC) isoforms (composed of ζ and τ) is calcium- and DAG-insensitive and requires only presence of phospholipids. Conventional and novel PKC (but not aPKC) isoforms have a functional cysteine-rich C1 regulatory domain (Mott *et al.* 1996). This membrane-targeting domain binds to DAG. Phorbol esters such as PMA mimic the action of DAG by increasing the PKC affinity for membranes. This leads to PKC translocation from the cytosol to the cellular membrane. Lipid binding to regulatory domains provides enough energy to release the PKC pseudosubstrate domain from the kinase substrate-binding pocket (Johnson et al. 2000). Hence, in absence of DAG/PMA, PKC kinase domain is kept silent by binding its own PKC pseudosubstrate site (House & Kemp 1987). In addition, as opposed to nPKCs, cPKCs require calcium binding to another regulatory domain (called C2) to efficiently bind membrane lipids. For nPKC isoforms, this domain is referred as novel C2 domain and binds membranes in a calcium-independent manner (Sossin & Schwartz 1993). When PKC is activated by DAG/PMA, the C2 domain is also exposed and can interact with scaffolding or anchoring proteins (Kheifets & Mochly-Rosen 2007). In addition to the C2 domain, several proteins are also

known to interact with the C1 domain, pseudosubstrate site and regions where the sequence is variable among PKC isoforms (Ron & Kazanietz 1999).

Studies have shown that dopaminergic receptors are regulated by PKC. Both, D2R and D3R are desensitized by PKC (Morris et al. 2007, Namkung & Sibley 2004, Liu et al. 1992a, Rogue et al. 1990, Cho et al. 2007b). In contrast, PMA treatment leads to a potentiation of dopamine-induced cAMP formation promoted by activation of D1-like receptors in cultured striatal neurons (Schinelli et al. 1994). Furthermore, mGluR5 activation also promotes the potentiation of dopamine-mediated cAMP production via PKC in cultured striatal neurons (Paolillo et al. 1998). We recently undertook a study to resolve the issue of which D1-like subtype undergoes PKC-induced potentiation, as striatal preparations express predominantly D1R but also D5R (Jackson et al. 2005, Sunahara et al. 1990, Dearry et al. 1990, Zhou *et al.* 1990, Tiberi et al. 1991, Surmeier et al. 1996). In agreement with studies performed in striatal preparations, we have shown a potentiation of dopamine-mediated cAMP production in hD1R-transfected HEK293 cells upon PMA treatment (Jackson et al. 2005). Interestingly, in contrast to hD1R, we also established that the highly homologous hD5R is desensitized in HEK293 cells treated with PMA (Jackson et al. 2005). Meanwhile, it remains to be determined which PKC isozymes mediate the divergent regulation of human D1-like receptors by PMA. Studies using cultured striatal neurons suggest that potentiation of dopamine-mediated increase of cAMP production is not dependent on elevation of intracellular calcium level (Schinelli et al. 1994, Paolillo et al. 1998). In agreement with these studies, preliminary results from our lab demonstrate that treatment of HEK293 cells with the calcium chelator BAPTA-AM does not alter the extent of PMA-induced hD1R sensitization (Jackson and Tiberi,

unpublished data). Additionally, we previously reported that Gö6976, the typical pharmacological inhibitor of cPKCs, did not reduce the extent of PMA-mediated hD1R sensitization and hD5R desensitization (Jackson et al. 2005). Meanwhile, treatment with Gö6983 and bis-indolylmaleimide, which can block cPKC and nPKC, completely abolish the PMA-mediated hD5R desensitization while inhibiting the PMA-induced hD1R sensitization by 50% (Jackson et al. 2005). Altogether, results from HEK293 cells and striatal neurons hint suggest a potential role of nPKC isoforms in mediating the opposite regulation of hD1R and hD5R responsiveness by PMA.

HEK293 cells express all nPKC isoforms (Jackson et al. 2005). In the present study, we aimed to identify which nPKC isoforms are involved in hD1R sensitization and hD5R desensitization after PMA treatment. To do so, we use a short hairpin RNAs (shRNAs) transfection approach to knockdown individually PKCδ, PKCε and PKCθ in HEK293 cells (which are also highly expressed in neurons) and then assess the effect of nPKC knockdown on the regulation of human D1-like receptor responsiveness by PMA. It is worth mentioning that PKCη was not studied herein because this isoform is only expressed in heart, lungs and skin (Battaini 2001). In these tissues, there are no detectable or very low expression of D1R or D5R (Sunahara et al. 1990, Dearry et al. 1990, Zhou et al. 1990, Tiberi et al. 1991, Sunahara et al. 1991, Gingrich *et al.* 1992). Our results indicate that PMA-induced hD1R sensitization and hD5R desensitization involve the activation of PKCε and PKCδ isoforms, respectively. Moreover, they demonstrate that PKCδ and PKCθ contribute to a tonic regulation facilitating hD1R and hD5R coupling to Gs and cAMP formation in HEK293 cells.

## Experimental procedures

### Drugs and chemicals

Minimal essential media (MEM), gentamicin, trypsin-EDTA and HEPES buffer were from Invitrogen (Burlington, ON, Canada). Phosphate-buffered saline (PBS) was purchased from Wisent (St-Bruno, QC, Canada). Fetal bovine serum (FBS) was obtained from VWR (Mississauga, ON, Canada). Dopamine (DA), cAMP, aprotinin, benzamidine, leupeptin, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor, pepstatin A, *cis*-flupenthixol, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA) and 1-methyl-3-isobutylxanthine (IBMX) were from Sigma/RBI (Oakville, ON, Canada). Phorbol-12-myristate-13-acetate (PMA) was obtained from Calbiochem (La Jolla, CA, USA). [$^{14}$C]-cAMP (56.2 mCi/mmol) was obtained from Moravek Biochemicals and Radiochemicals (Brea, CA, USA). [$^{3}$H]-adenine (~25 Ci/mmol) and [$^{3}$H]-SCH23390 (~65-91 Ci/mmol) were from PerkinElmer (Boston, MA, USA). Bio-Safe II liquid scintillation cocktail was purchased from RPI (Mount Prospect, IL, USA). Short hairpin RNAs (shRNA, 29 mer) were bought from Origene Technologies, Inc. (Rockville, MD, USA).

### Cell culture and transfection

HEK293 cells (CRL-1573, ATCC Manassas, VA, USA) were grown in MEM supplemented with 10% heat-inactivated FBS and gentamicin (40 μg/ml) at 37°C and 5% $CO_2$ and seeded into 100-mm dishes (2.5 x $10^6$ cells/dish). For dose-response curves experiments, cells were transiently transfected with 10 μg of nPKC shRNA in pRS vector (Origene Technologies, Inc., Rockville, MD, USA) or empty pRS alone or with 0.005-

0.025 μg of human D1-like receptor (hD1R and hD5R) in mammalian expression pCMV5 vector per dish using a modified calcium phosphate precipitation procedure (Plouffe et al. 2010). Empty pCMV5 was used to have the same total amount of hD1R and hD5R DNA transfected per 100-mm dish. After an overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells were washed with phosphate-buffered saline, trypsinized, reseeded in 100-mm dishes (radioligand binding and western samples) and 12-well plates (dose-response curves) and grown for an additional 48 h. All experiments were performed with cells from 40 to 52 passages.

**Whole cell cAMP assay**

G protein-mediated activation of adenylyl cyclase by D1-like receptors was assessed using a whole cell cAMP assay as previously described (Plouffe et al. 2010). Briefly, the day before experiment, medium of transfected cells was replaced with fresh MEM containing FBS (5% v/v), gentamicin (40 μg/ml) and [$^3$H]-adenine (1 μCi/ml) and cells were incubated overnight at 37°C and 5% $CO_2$. The next day, the media was removed and HEK293 cells were incubated in 20 mM HEPES-buffered MEM containing IBMX (1 mM) in the absence (0.02% v/v DMSO; vehicle) or presence of PMA (1 μM) and increasing concentrations of DA (prepared in ascorbic acid solution at a final concentration of 0.1 mM in assays) for 30 min at 37°C in an humidified 5% $CO_2$ incubator. At the end of incubation period, medium was aspirated and each well filled with 1 ml of lysis solution (2.5% v/v perchloric acid, 0.1 mM cAMP and ~3 nCi of [$^{14}$C]-cAMP) and cells lysed for 30 min at 4°C. The lysates were then transferred to tubes containing 0.1 ml of 4.2 M KOH neutralizing solution, mixed by vortex and centrifuged

(~450 g, 15 min) at 4°C to pellet down salt precipitates. The amount of intracellular [$^3$H]cAMP was determined from clarified supernatants using a sequential chromatography over Dowex (Bio-Rad Laboratories Inc, Mississauga, ON, Canada) and alumina columns (MP Biochemicals Canada, Montréal, QC, Canada) as described (Plouffe et al. 2010). The amount of intracellular [$^3$H]-cAMP formed (CA) over the total amount of intracellular [$^3$H]-adenine (TU) was calculated to determine the relative AC activity and expressed as CA/TU × 1000.

**Radioligand Binding Assays**

The total number of receptor binding sites ($B_{max}$) was determined as described previously (Plouffe et al. 2010). Transfected HEK293 cells were washed with cold PBS, scraped from the 100-mm dishes in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA) and centrifuged at 40,000 *g* for 20 min at 4°C. Membrane pellets were resuspended in lysis buffer using a Brinkmann Polytron (velocity of 17,000 rpm for 15 sec) and centrifuged at 40,000 *g* for 20 min at 4°C. The final pellet was homegeneized in resuspension buffer (62.5 mM Tris-HCl, pH 7.4, 1.25 mM EDTA), and membranes used immediately. Binding assays were performed with 100 µl of fresh membranes in a final reaction volume of 500 µl containing 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM EDTA. Total and nonspecific binding were determined using a saturating concentration of [$^3$H]-SCH23390 (~6-7 nM) in the absence or presence of *cis*-flupenthixol at a final concentration of 10 µM. Membranes were incubated with the radioligand for 90 min at room temperature and harvested using rapid filtration through glass fiber filters (GF/C, Whatman). The filters were washed three

times with 5 ml of cold washing buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl), and bound radioactivity was determined by liquid scintillation counting (Beckman, LS 6500). Protein concentrations were measured using Bradford protein assay dye (Bio-Rad) with bovine serum albumin (BSA) as standard.

**Immunoblotting analysis of protein kinase C isoforms**

HEK293 cells seeded in 100-mm dishes were washed twice with ice-cold PBS and harvested in 400 µl of cold lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA) containing protease inhibitors (aprotinin 2 µg/ml, benzamidine 10 µg/ml, leupeptin 10 µg/ml, PMSF 20 µg/ml, soybean trypsin inhibitor 10 µg/ml, pepstatin A 1 µg/ml). Cell lysates were sonicated on ice for 25 sec and protein concentrations determined using Bradford Bio-Rad assay dye reagent and BSA as standard. 50 µg of proteins were prepared in 2X sample buffer (25 mM Tris-HCl pH 6.5, 8% w/v SDS, 5% v/v 2-mercaptoethanol, 10% v/v glycerol, 0.001% w/v bromophenol blue) and resolved using 10% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto 0.2 µm polyvinylidene difluoride (PVDF) membranes (Bio-Rad) using semi-dry blotting. Nonspecific binding sites were blocked by incubating PVDF membranes in Blotto solution (50 mM Tris-HCl pH 8, 2 mM $CaCl_2$, 80 mM NaCl, 5% w/v nonfat dry milk, 0.2% v/v NP-40, 0.02% w/v $NaN_3$) for at least 1 hour at 4ºC. Membranes were then cut horizontally in two pieces. The bottom piece was incubated overnight at 4°C with a mouse monoclonal anti-GAPDH antibody (1:20000, Abcam, Cambridge, MA, USA) and the upper part with mouse monoclonal anti-PKCδ (1:250, BD Transduction Laboratories, Mississauga, ON, Canada), anti-PKCε (1:1000, mouse monoclonal, BD Transduction

Laboratories) or anti-PKCθ antibodies (1:100, BD Transduction Laboratories). PVDF membranes were then washed four times in TBS-T (Tris-HCl 20 mM pH 7.4, NaCl 137 mM, Tween 0.2% v/v) for 40 min and incubated 1 hour at room temperature with secondary sheep horseradish peroxydase-conjugated anti-mouse antibody (1:10000 for GAPDH or 1:5000 for nPKCs). PVDF membranes were washed again in TBS-T and immunodetection of GAPDH and nPKC isoforms visualized using enhanced chemiluminescence kit (GE Healthcare, Baie d'Urfé, QC, Canada). nPKC and GAPDH immunoreactive bands were quantified by scanning densitometry using the Scion Imaging software. Scanned bands were first corrected for lane background. Then, nPKC bands were normalized to GAPDH input and nPKC knockdown expressed as percent inhibition relative to pRS vector.

**Statistical analysis**

Arithmetic means ± SE were used to report data. One-way ANOVA (with Dunnett's multiple comparison post-hoc test) or one sample t test were performed using GraphPad Prism, version 5.03 for Windows (GraphPad Software, San Diego; www.graphpad.com). Dose-response curves were averaged and simultaneously analyzed using the GraphPad four-logistic parameter equation to compute best-fitted parameters for DA-mediated maximal activation of AC ($E_{max}$) and half-maximal effective concentration ($EC_{50}$). The statistical significance of differences between $E_{max}$ and $EC_{50}$ values from dose-response curves performed various experimental conditions was determined by simultaneous curve fitting using unconstrained, constrained and shared parameters to establish the best curve fit (i.e. whether constraining a specific parameter to

a constant or shared value worsens the goodness of fit. All statistical analyses were performed with a level of significance established at $p < 0.05$.

## Results and discussion

Previously, we reported that PMA treatment robustly increases DA-mediated stimulation of hD1R while diminishing constitutive and DA-dependent activation of hD5R (Jackson et al. 2005). Additionally, while Gö6976 (a PKC inhibitor preferentially targeting cPKC isoforms) did not reduce the PMA-induced hD1R sensitization, bisindolymaleimide (Bis) and Gö6983 (two potent inhibitors of conventional and novel PKC classes) both diminished by 50% the PMA effect on hD1R responsiveness. Likewise, Gö6976 had no effect on hD5R desensitization by PMA while Bis or Gö6983 pretreatment completely prevented PMA-induced desensitization of DA-dependent stimulation of hD5R (Jackson et al. 2005). These results suggest that nPKC isoforms are potentially involved in the PMA-induced hD1R sensitization and hD5R desensitization. To address this issue we employ a knockdown approach to assess the role of three nPKC isozymes: PKCδ, PKCε and PKCθ.

### Determination of efficaciousness and specificity of nPKC shRNA knockdowns in HEK293 cells

For each nPKC tested (PKCδ, PKCε and PKCθ) in the present study, we first determined which of the four shRNAs (A-D) commercially provided by Origene produced the largest knockdown in HEK293 cells. Details about the assessment of each set of four nPKC shRNAs are described in the **Supplementary Information**. Based on these studies we selected the PKCδ shRNA-A, PKCε shRNA-C and PKCθ shRNA-D (**Fig. S1A** and **Table S1A**). Additionally, our experiments indicate that best knockdowns

are obtained using 10 μg shRNAs per 100-mm dish using our transfection approach (**Fig. S1B** and **Table S2**).

**Supplemental Information**

*shRNA construct screening for nPKC isoform knockdown in HEK293 cells*

For each targeted nPKC isoform, HEK293 cells were transfected with 10 μg DNA/dish of 29-mer shRNA construct A, B, C and D or the empty vector pRS, which were all obtained from Origene Technologies, Inc. Total cell proteins were extracted and immunoblots performed using 50 μg of total proteins as described in Experimental procedures. GAPDH was used as loading control (input) for each lane. Immunoblots of nPKCs from cells transfected with different shRNA constructs (A-D) for each nPKC isoform or empty vector pRS (-) are shown in **Fig. S1A**. nPKC bands were quantified by scanning densitometry and corrected for corresponding lane backgrounds and GAPDH inputs. Percent values for nPKC knockdown were calculated relative to nPKC band intensity measured in cells transfected with the empty vector pRS (negative control) are reported in **Table S1.**

*Determination of the optimal amount of shRNA constructs for transfections of HEK293 cells*

To assess the quantity of shRNA to use in transfections for achieving the highest knockdown of each nPKC isoform, HEK293 cells were transfected with the empty vector pRS or with increasing amounts (0.5, 2, 5 or 10 μg/dish) of shRNA constructs identified in our shRNA screening (**Fig. S1** and **Table S1**). The relative amount of nPKC expressed

in the cells following shRNA transfection was assessed by immunoblotting as described above. Results are shown on **Fig. S1B**. Percentage of knockdown obtained for each condition is reported in **Table S2**. For each nPKC isoform, the highest percentage of knockdown was obtained following a transfection of HEK293 cells using 10 µg of shRNA per dish. This transfection condition was subsequently used in all our experiments.

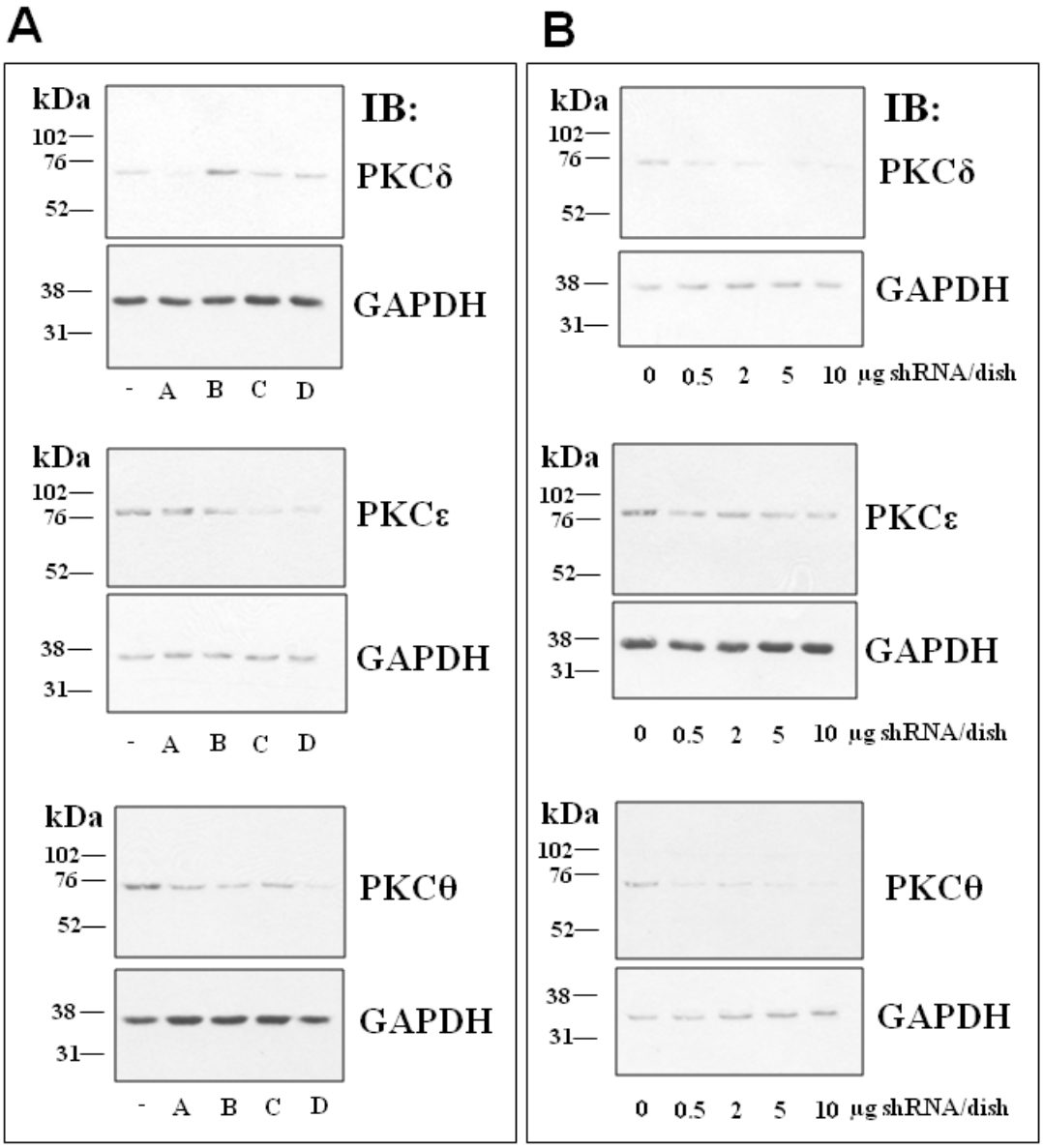


**Figure S1. Determination of optimal conditions for nPKC knockdown using shRNA in HEK293 cells.**

**Table S1. Comparison of knockdown obtained with the four shRNA constructs from Origene Technologies, Inc.**

| nPKC isoform targeted | Constructs | Percentage of PKC knock down obtained |
|---|---|---|
| δ | A | **79** |
| | B | -167 |
| | C | 37 |
| | D | -12 |
| ε | A | 21 |
| | B | 49 |
| | C | **86** |
| | D | 81 |
| θ | A | 69 |
| | B | 80 |
| | C | 74 |
| | D | **92** |

The highest percentage of knockdown obtained is bold and underlined. These shRNA constructs were selected and used in all the subsequent experiments. Negative percent values represent the shRNA constructs for which an increased expression of nPKC was observed.

**Table S2. Determination of the amount of shRNA to transfect in HEK293 cells for an optimal knockdown of each nPKC isoform**.

| nPKC isoform targeted | Amount of transfected shRNA construct (µg/dish) | nPKC Knockdown (%) |
|---|---|---|
| δ | 0.5 | 60 |
| | 2 | 65 |
| | 5 | 83 |
| | 10 | **88** |
| ε | 0.5 | 29 |
| | 2 | 22 |
| | 5 | 68 |
| | 10 | **72** |
| θ | 0.5 | 75 |
| | 2 | 78 |
| | 5 | 89 |
| | 10 | **91** |

The highest percentage of knockdown obtained using different amount of DNA in transfection is bold and underlined. The 10 µg/dish was used as the amount of shRNA construct to transfect to obtain an optimal knockdown of nPKC.

We next assessed the specificity of each selected shRNA with respect to off-target effects towards the other nPKCs investigated in the present study. Equal amount of proteins (50 μg/lane) for each experimental condition was subjected to immunoblotting studies using GAPDH as our loading control for the quantification of knockdown. A representative example of our off-target studies obtained with each nPKC isoform is illustrated in **Fig. 1A**. As suggested by our immunoblotting studies, each selected shRNA specifically and robustly knockdown the targeted nPKC isoform without major alterations in expression levels of other nPKCs in HEK293 cells (**Fig. 1B**). While PKCθ and PKCδ exhibited a slight but significant knockdown when PKCε and PKCθ are targeted, respectively, our results suggest that shRNAs tested are highly specific and provide a useful tool to investigate the role of PKCδ, PKCε and PKCθ in PMA-induced regulation of hD1R and hD5R responsiveness.

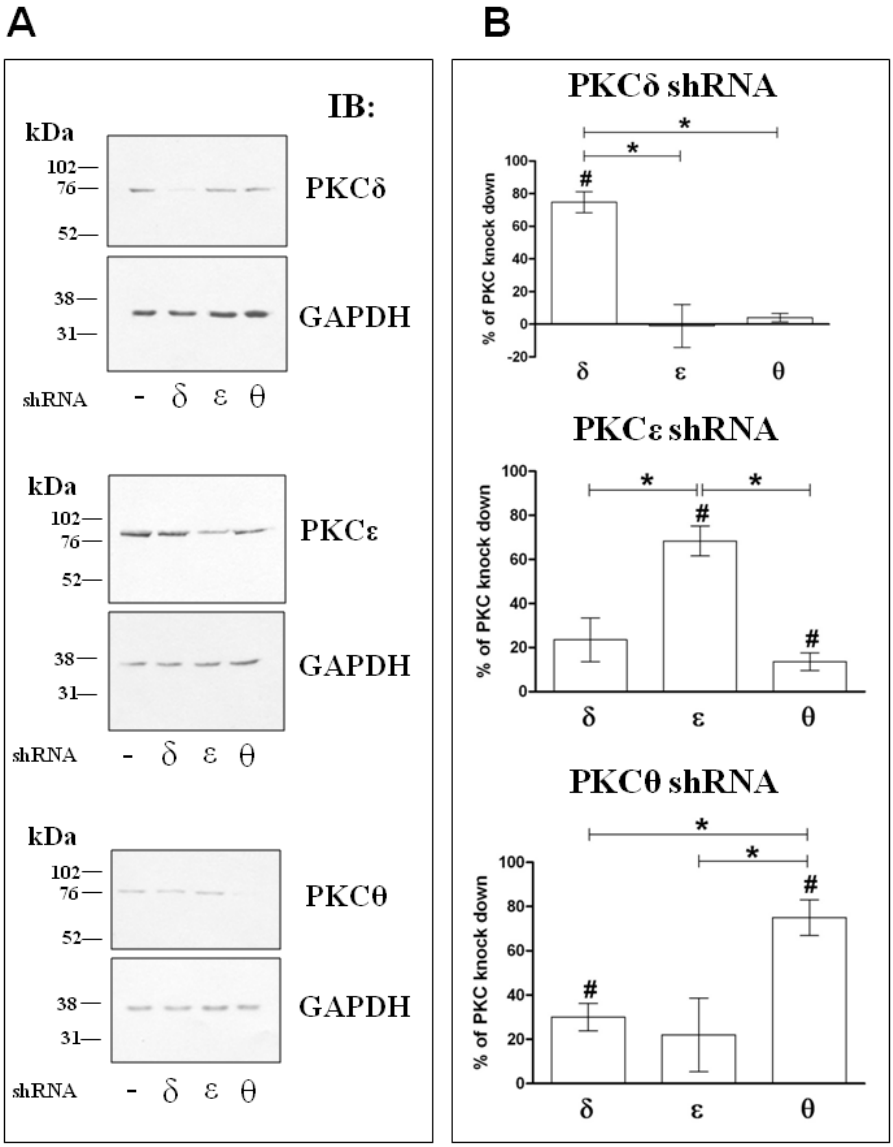


**Figure 1. Efficaciousness and specificity of novel PKC isoform shRNAs in HEK293 cells.** Following an overnight transfection with 10 μg DNA/dish of shRNAs (PKCδ, PKCε and PKCθ) or empty PRS vector) HEK293 cells were reseeded in 100-mm dishes and grown for an additional 48 hours. Aliquots of total cell proteins (50 μg) were resolved using SDS-PAGE on 10% gels, transferred on PVDF membranes and nPKCs and GAPDH detected by immunoblotting and ECL kits. (**A**) A representative example of an experiment repeated 4-5 times is shown. Knockdown of nPKCs obtained with selected targeting shRNA for PKCδ, PKCε and PKCθ is reported in top, middle and bottom panels, respectively. (**B**) Quantification of expression levels of targeted and untargeted nPKC isoforms in percent knockdown relative to cells transfected with empty pRS vector. Densitometry of nPKC bands were corrected for lane backgrounds and GAPDH inputs. Data are expressed as the arithmetic mean ± SEM. The effect of shRNA on knockdown (%) of nPKCs was compared with a value of "0" using one sample t test. Statistical differences between expression levels of nPKC were assessed using one-way ANOVA followed by Dunnett's post-test.

#, $p < 0.05$ when compared with "0"; *, $p < 0.05$ when compared to targeted nPKC.

**Tonic regulation of hD1R and hD5R responsiveness by PKCδ and PKCθ**

The effect of PKC knockdown on the DA-induced stimulation of hD1R and hD5R in HEK293 cells without PMA treatment was first assessed by generating dose-response curves. Surprisingly, at similar hD1R or hD5R expression levels, DA-mediated production of intracellular cAMP by these two receptors is bluntly reduced in HEK293 cells displaying knockdowns of PKCδ (conditions $hD1R^{shPKC\delta}$ and $hD5R^{shPKC\delta}$) and PKCθ (conditions $hD1R^{shPKC\theta}$ and $hD5R^{shPKC\theta}$) relative to cells co-transfected with D1-like receptors and empty pRS (conditions $hD1R^{pRS}$ and $hD5R^{pRS}$) (**Figs. 2A** and **3A**). Indeed, $E_{max}$ of DA for $hD1R^{shPKC\delta}$ and $hD1R^{shPKC\theta}$ reaches only 56% and 69% of $E_{max}$ associated with $hD1R^{pRS}$ (**Table 1**). Similar reductions in $E_{max}$ are observed with $hD5R^{shPKC\delta}$ and $hD5R^{shPKC\theta}$. Specifically, $E_{max}$ of DA for $hD5R^{shPKC\delta}$ and $hD5R^{shPKC\theta}$ is 62% and 68% of $E_{max}$ measured for $hD5R^{pRS}$ (**Table 2**) However, PKCδ and PKCθ knockdowns had no effect on the $EC_{50}$ of DA in cells expressing hD1R and hD5R (**Tables 1 and 2**). Interestingly, no detectable effect on dose-response curve of DA of hD1R or hD5R was observed with PKCε knockdown (**Fig. 2B** and **3B**). Notably, the extent of knockdown obtained with PKCε shRNA was similar to that measured with PKCδ and PKCθ shRNAs. Overall, these results strongly suggest that PKCδ and PKCθ play an important positive role in regulating DA-induced activation of hD1R and hD5R in HEK293 cells. Most importantly, as these effects were detected without addition of external phorbol ester analogs, PKCδ and PKCθ are implicated in the tonic regulation of hD1R and hD5R signaling complexes associated with the formation of intracellular cAMP. In the next series of experiments, we assessed the impact of PKCδ, PKCε and PKCθ knockdowns on PMA effects evoked on human D1-like receptor responsiveness.

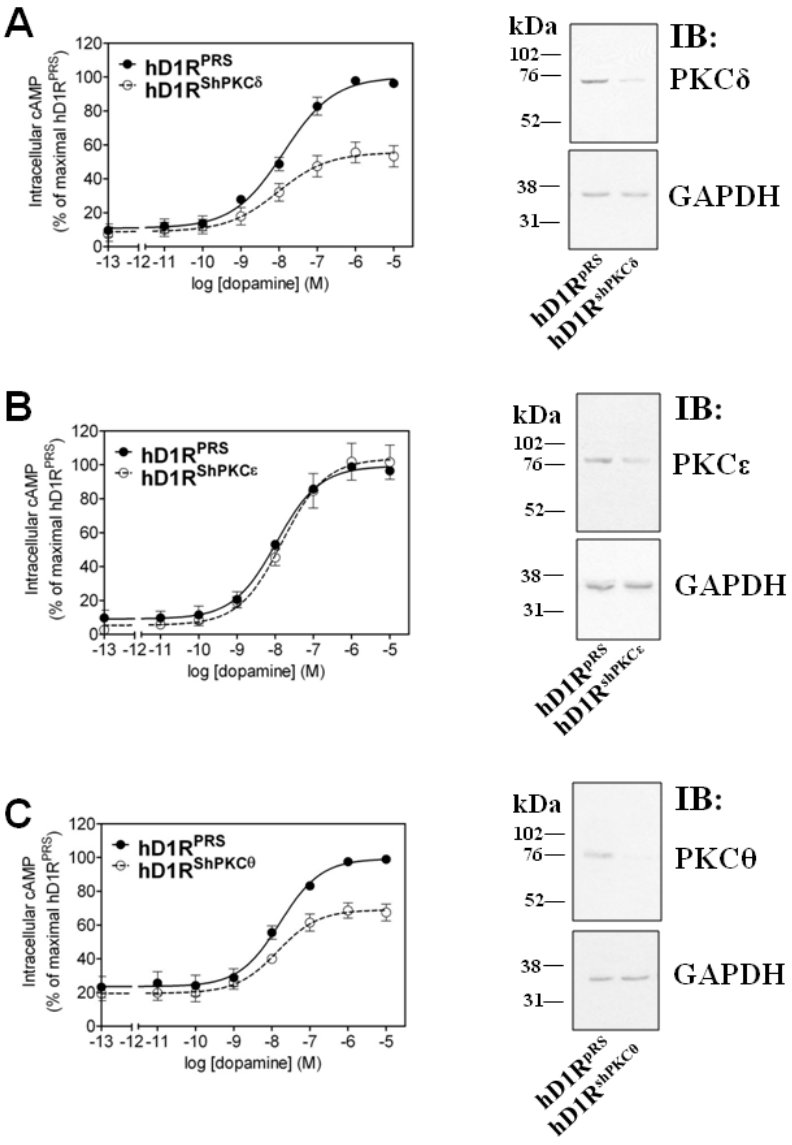


**Figure 2. DA-mediated stimulation of AC in HEK293 cells co-transfected with hD1R alone or with different nPKC shRNAs.**

Transfected HEK293 cells were seeded in 12-well plates and labeled with [$^3$H]-adenine as described in Experimental Procedures. Cells treated with vehicle (DMSO, 0.02% (v/v)) or 1 μM PMA were incubated in the presence of increasing DA concentrations for 30 min at 37°C. To facilitate graphical representation of shRNA effect on DA-induced stimulation by hD1R in the absence of PMA, control curves (DMSO) were analyzed without curves obtained in PMA-treated cells. Intracellular cAMP levels were plotted as a function of log of DA concentrations (**left panels**) and expressed as percentage of best-fitted $E_{max}$ obtained under control condition (empty pRS vector-DMSO). Curves were done in triplicate determinations and repeated 3-5 times. Best-fitted curves using PKCδ shRNA (**A**), PKCε shRNA (**B**) or PKCθ shRNA (**C**) are shown. A representative immunoblot for each isoform is shown (**right panels)**. Best-fitted parameters for dose-response curves, $B_{max}$ and percent knockdown for each nPKC isoform are reported in **Table 1.**

**Table 1. Best-fitted values for dose-response curves of DA in HEK293 cells transfected with hD1R alone or with different nPKC shRNAs.**

| **Conditions** | **$EC_{50}$** (nM) | **$E_{max}$** (% of $hD1R^{pRS}$) | **$B_{max}$** (pmol/mg membrane prot.) | **nPKC knockdown** (%) |
|---|---|---|---|---|
| $hD1R^{pRS}$ | 13 [7-25] | 100 ± 4 | 2.15 ± 0.36 | |
| $hD1R^{shPKC\delta}$ | 9 [3-31] | 56 ± 3* | 1.89 ± 0.28 | 78 ± 8 |
| $hD1R^{pRS}$ | 11 [6-20] | 99 ± 4 | 2.29 ± 0.38 | |
| $hD1R^{shPKC\varepsilon}$ | 15 [9-27] | 104 ± 4 | 1.91 ± 0.12 | 70 ± 11 |
| $hD1R^{pRS}$ | 17 [9-31] | 99 ± 4 | 3.03 ± 0.79 | |
| $hD1R^{shPKC\theta}$ | 14 [6-36] | 69 ± 3* | 2.83 ± 0.91 | 73 ± 9 |

Best-fitted and unconstrained parameters for $EC_{50}$ and $E_{max}$ obtained by simultaneous curve fitting of averaged dose-response curves (shown in **Fig. 2**) are reported. $EC_{50}$ values are expressed with 95% confidence intervals in brackets while $E_{max}$ values are shown as percent of control $E_{max}$ value for pRS ± approximate as determined by GraphPad Prism. $B_{max}$ values for [$^3$H]-SCH23390 in pmol/mg membrane proteins and percent nPKC knockdown are expressed as arithmetic means ± S.E. Statistical differences between best-fitted values for $EC_{50}$ and $E_{max}$ were determined by assessing the goodness of curve fit obtained using unconstrained, shared and constrained parameters. No significant differences were found between $B_{max}$ computed for different experimental sets of $hD1R^{pRS}$ and $hD1R^{shPKC}$. *, $p < 0.05$ vs. $hD1R^{pRS}$

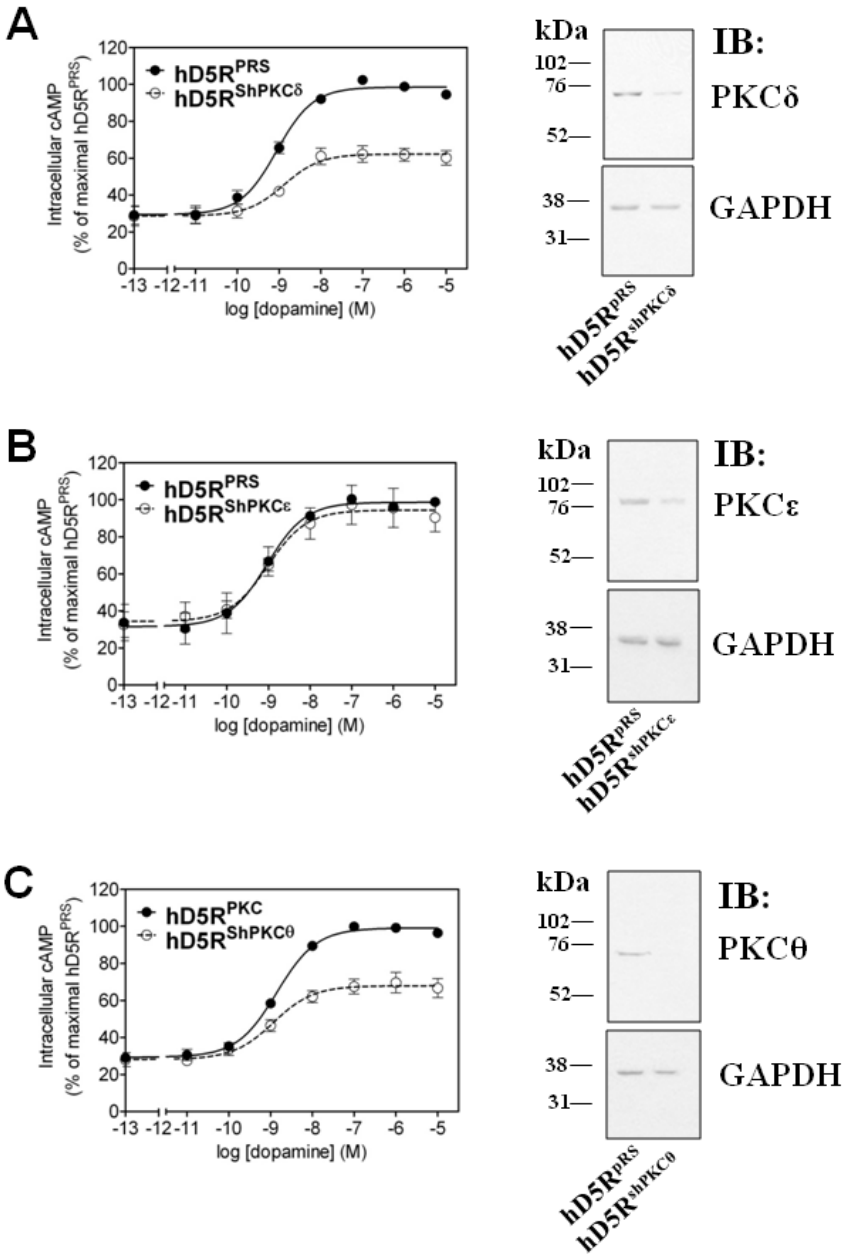


**Figure 3. DA-mediated stimulation of AC in HEK293 cells co-transfected with hD5R alone or with different nPKC shRNAs.**

Transfected HEK293 cells were seeded in 12-well plates and labeled with [$^3$H]-adenine as described in Experimental Procedures. Cells treated with vehicle (DMSO, 0.02% (v/v)) or 1 μM PMA were incubated in the presence of increasing DA concentrations for 30 min at 37°C. To facilitate graphical representation of shRNA effect on DA-induced stimulation by hD5R in the absence of PMA, control curves (DMSO) were analyzed without curves obtained in PMA-treated cells. Intracellular cAMP levels were plotted as a function of log of DA concentrations (**left panels**) and expressed as percentage of best-fitted $E_{max}$ obtained under control condition (empty pRS vector-DMSO). Curves were done in triplicate determinations and repeated 3-5 times. Best-fitted curves using PKCδ shRNA (**A**), PKCε shRNA (**B**) or PKCθ shRNA (**C**) are shown. A representative immunoblot for each isoform is shown (**right panels)**. Best-fitted parameters for dose-response curves, $B_{max}$ and percent knockdown for each nPKC isoform are reported in **Table 2**.

**Table 2. Best-fitted values for dose-response curves of DA in HEK293 cells transfected with hD5R alone or with different nPKC shRNAs.**

| **Conditions** | **$EC_{50}$** (nM) | **$E_{max}$** (% of $hD5R^{PRS}$) | **$B_{max}$** (pmol/mg membrane prot.) | **PKC knockdown** (%) |
|---|---|---|---|---|
| $hD5R^{pRS}$ | 0.88 [0.55-1.4] | 99 ± 2 | 2.47 ± 0.29 | |
| $hD5R^{shPKC\delta}$ | 1.3 [0.50-3.4] | 62 ± 2* | 2.58 ± 0.22 | 75 ± 5 |
| $hD5R^{pRS}$ | 0.92 [0.37-2.3] | 99 ± 4 | 2.99 ± 0.37 | |
| $hD5R^{shPKC\varepsilon}$ | 0.98 [0.35-2.7] | 94 ± 5 | 2.51 ± 0.15 | 63 ± 4 |
| $hD5R^{pRS}$ | 1.4 [0.94-2.1] | 99 ± 2 | 3.27 ± 0.69 | |
| $hD5R^{shPKC\theta}$ | 1.2 [0.57-2.4] | 68 ± 2* | 3.77 ± 0.81 | 74 ± 7 |

Best-fitted and unconstrained parameters for $EC_{50}$ and $E_{max}$ obtained by simultaneous curve fitting of averaged dose-response curves (shown in **Fig. 3**) are reported. $EC_{50}$ is expressed with 95% confidence intervals in brackets while $E_{max}$ is shown as percent of control $E_{max}$ value for pRS ± approximate as determined by GraphPad Prism. $B_{max}$ values for [$^3$H]-SCH23390 in pmol/mg membrane proteins and percent nPKC knockdown are expressed as arithmetic means ± S.E. Statistical differences between best-fitted values for $EC_{50}$ and $E_{max}$ were determined by assessing the goodness of curve fit obtained using unconstrained, shared and constrained parameters. No significant differences were found between $B_{max}$ computed for different experimental sets of $hD5R^{pRS}$ and $hD5R^{shPKC}$. *, $p < 0.05$ vs. $hD5R^{pRS}$

**PMA elicits PKCε-dependent hD1R sensitization and PKCδ-dependent hD5R desensitization**

In a similar fashion to our previous studies, PMA treatment induces a robust increase of $E_{max}$ in all conditions using HEK293 cells co-transfected with hD1R and empty pRS (**Fig. 4A**). Interestingly, our studies show that the PMA-induced receptor sensitization is significantly reduced for $hD1R^{shPKC\varepsilon}$ when compared with $hD1R^{pRS}$ (**Fig. 4A, middle panel**). In fact, the PMA-induced increase of $E_{max}$ for $hD1R^{shPKC\varepsilon}$ is 197% versus the 231% augmentation of $E_{max}$ for $hD1R^{pRS}$ (**Table 3**). The effect of PKCε knockdown culminates in a significant 25% diminution of the extent of PMA-induced hD1R sensitization (**Fig. 4B** and **Table 3**). PKCδ and PKCθ knockdowns have no effect on the PMA-induced sensitization of hD1R (**Figs. 4A, B** and **Table 3**). Next, the role of nPKCs was probed in PMA-induced hD5R desensitization.

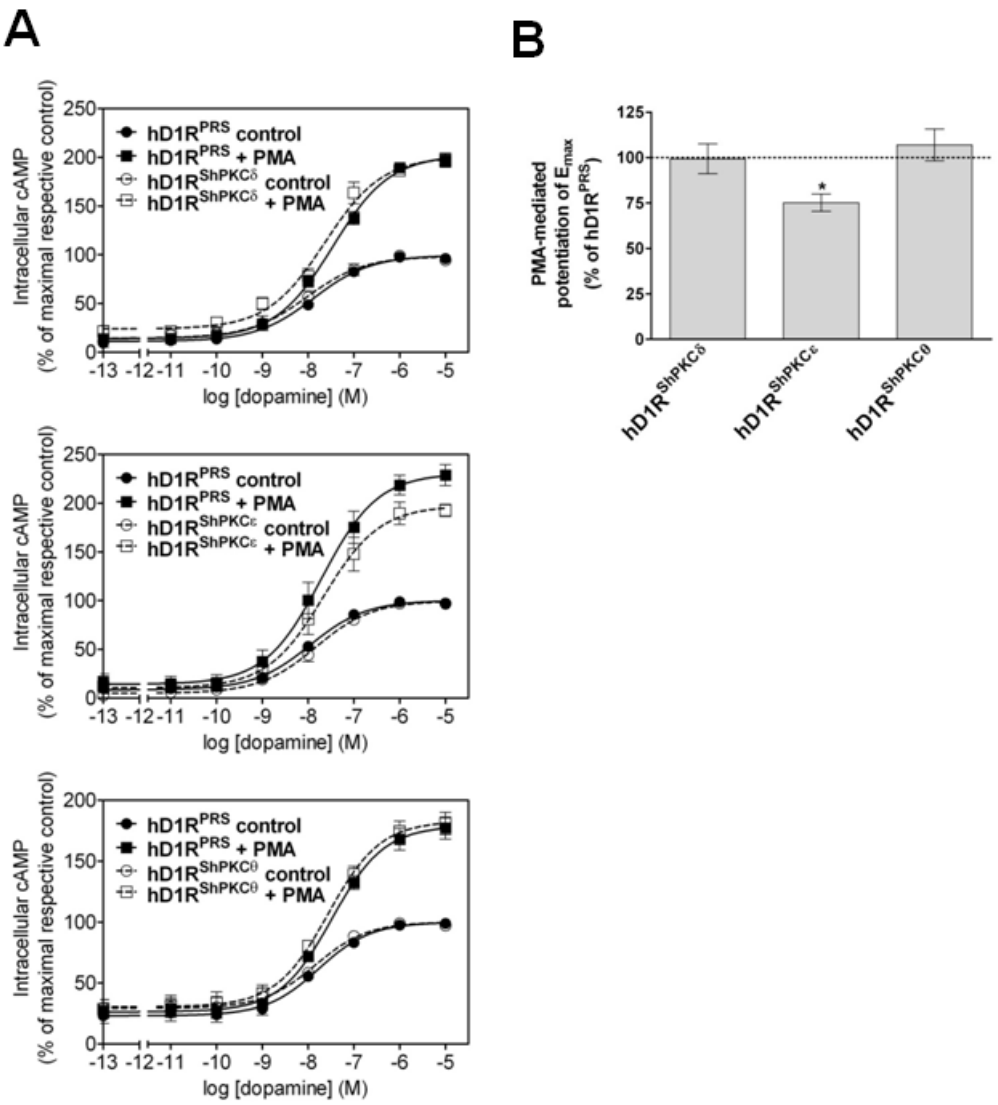


**Figure 4. Effect of nPKC knockdowns on PMA-induced regulation of DA-mediated stimulation by hD1R.**

Transfected HEK293 cells were seeded in 12-well plates and labeled with [$^3$H]-adenine as described in Experimental Procedures. Cells treated with vehicle (DMSO, 0.02% (v/v)) or 1 μM PMA were incubated with increasing DA concentrations for 30 min at 37°C. Intracellular cAMP levels were plotted as a function of log of DA concentrations and expressed as percentage of best-fitted $E_{max}$ obtained under control condition (DMSO). Curves were done in triplicate determinations and repeated 3-5 times. (**A**) Best-fitted curves using PKCδ shRNA (**top**), PKCε shRNA (**middle**) or PKCθ shRNA (**bottom**) are shown. (**B**) Percentages of PMA-mediated $E_{max}$ potentiation obtained under different hD1R$^{shPKC}$ conditions relative to hD1R$^{pRS}$ are expressed as arithmetic means ± S.E. Percent values were statistically compared with a value of 100% using one sample t test. $B_{max}$ values are shown in Table 1. Best-fitted parameters for $E_{max}$ and averaged values for PMA-mediated $E_{max}$ potentiation are reported in Table 3. *, $p < 0.05$ when compared with "100"

**Table 3. Best-fitted parameters for dose-response curves of DA in HEK293 cells transfected with hD1R alone or with different nPKC shRNAs following PMA treatment.**

| Conditions | $E_{max}$ (% of control) | | PMA-induced $E_{max}$ potentiation (% of $hD1R^{PRS}$) |
|---|---|---|---|
| | **control** | **PMA** | |
| $hD1R^{pRS}$ | 100 ± 4 | 203 ± 5 | |
| $hD1R^{shPKC\delta}$ | 99 ± 4 | 201 ± 5 | 99 ± 8 |
| $hD1R^{pRS}$ | 100 ± 6 | 231 ± 7 | |
| $hD1R^{shPKC\varepsilon}$ | 100 ± 6 | 197 ± 7* | 75 ± 5# |
| $hD1R^{pRS}$ | 100 ± 4 | 181 ± 5 | |
| $hD1R^{shPKC\theta}$ | 100 ± 4 | 183 ± 5 | 107 ± 9 |

Best-fitted and unconstrained parameters for $E_{max}$ obtained by simultaneous curve fitting of averaged dose-response curves (shown in **Fig. 4A**) are reported. $E_{max}$ values are expressed as percent of control $E_{max}$ value for pRS ± approximate as determined by GraphPad Prism. Percent values for PMA-induced $E_{max}$ potentiation are expressed as arithmetic means ± S.E. Statistical differences between best-fitted values for $E_{max}$ following PMA treatment were determined by assessing the goodness of curve fit obtained using unconstrained, shared and constrained parameters. Percent values of PMA-induced $E_{max}$ potentiation were compared with a hypothetical value of "100" using one sample t test.

*, $p < 0.05$ vs. $hD1R^{pRS}$ (PMA); #, $p < 0.05$ vs. hypothetical value of "100"

Typically, the PMA-induced hD5R desensitization is depicted by a strong rightward shift in $EC_{50}$ of DA. As shown in **Fig. 5A**, the $EC_{50}$ rightward shift by PMA is observed in HEK293 cells transfected with hD5R and empty pRS. However, the extent of $EC_{50}$ rightward shift is reduced by almost 50% for $hD5R^{shRNA\delta}$ in comparison with $hD5R^{pRS}$ (**Figs. 5A top panel**, **5B** and **Table 4**). This blunting effect on PMA-induced desensitization of hD5R by decreased PKCδ expression is not observed following PKCε and PKCθ knockdowns. Additionally, the slight increase of $E_{max}$ observed for hD5R upon PMA treatment is significantly augmented for $hD5R^{shPKC\delta}$ (**Fig. 5A**, upper panel and **Table 4**). These findings are in agreement with our previous work showing that a loss of hD5R desensitization (reduced $EC_{50}$ rightward shift) is associated with a gain of $E_{max}$ potentiation by PMA treatment in HEK293 cells (Plouffe et al., 2011; see thesis paper 3). These data strongly imply that PKCδ is involved in the PMA-mediated hD5R desensitization.

Overall, our studies suggest that the opposing actions of PMA on hD1R and hD5R responsiveness are regulated in a PKC isoform-manner by PKCε and PKCδ, respectively. Meanwhile, our shRNA studies raise an important issue about the partial inhibition mediated on PMA effects following nPKC knockdowns.

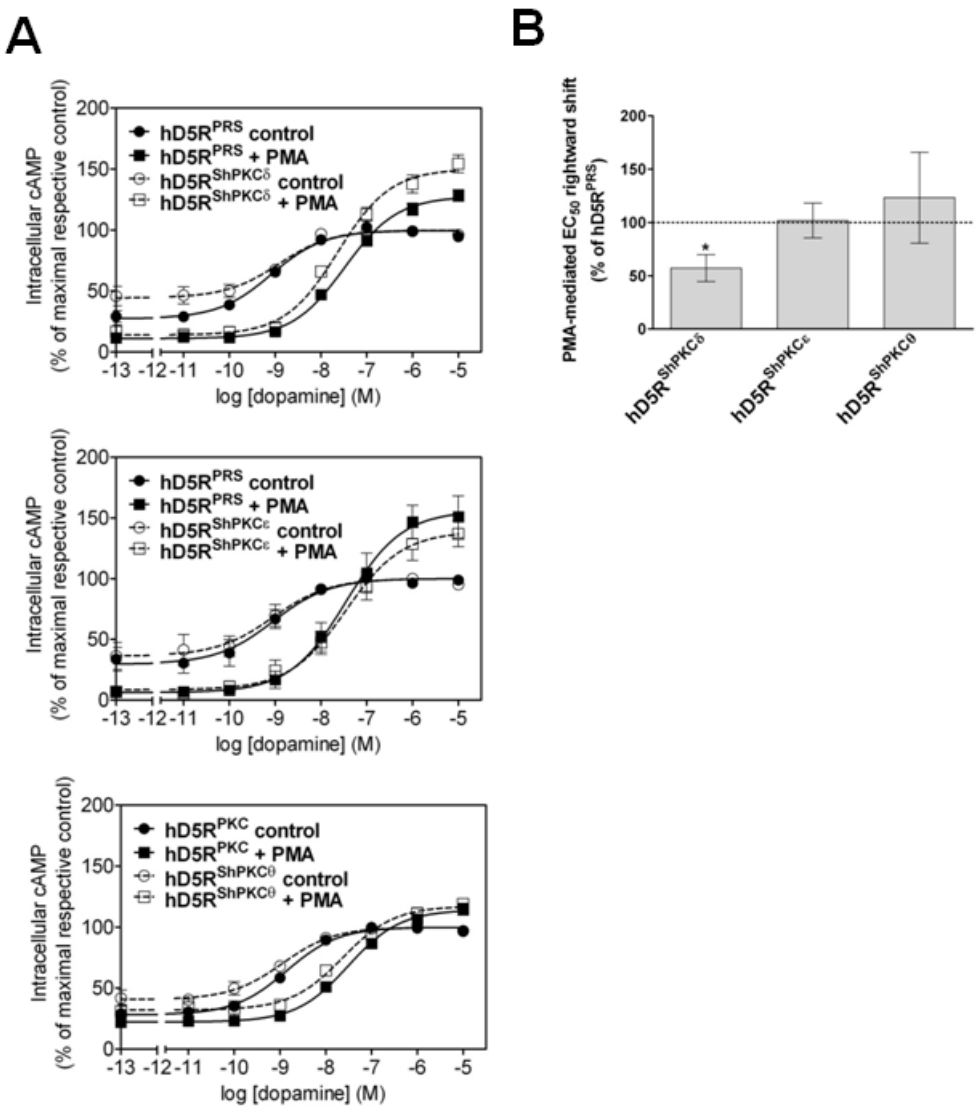


**Figure 5. Effect of nPKC knockdowns on PMA-induced regulation of DA-mediated stimulation by hD5R.**

Transfected HEK293 cells were seeded in 12-well plates and labeled with [$^3$H]-adenine as described in Experimental Procedures. Cells treated with vehicle (DMSO, 0.02% (v/v)) or 1 μM PMA were incubated with increasing DA concentrations for 30 min at 37°C. Intracellular cAMP levels were plotted as a function of log of DA concentrations and expressed as percentage of best-fitted $E_{max}$ obtained under control condition (DMSO). Curves were done in triplicate determinations and repeated 3-5 times. (**A**) Best-fitted curves using PKCδ shRNA (**top**), PKCε shRNA (**middle**) or PKCθ shRNA (**bottom**) are shown. (**B**) Percentages of PMA-mediated $EC_{50}$ rightward shift obtained under different hD5R$^{shPKC}$ conditions relative to hD5R$^{pRS}$ are expressed as arithmetic means ± S.E. Percent values of $EC_{50}$ righward shift were statistically compared with a value of 100% using one sample t test. $B_{max}$ values are shown in **Table 2**. Best-fitted $EC_{50}$ and $E_{max}$ values and averages for PMA-mediated $EC_{50}$ rightward shift are reported in Table 4. *, $p < 0.05$ when compared with "100"

**Table 4. Best-fitted parameters for dose-response curves of DA in HEK293 cells transfected with hD5R alone or with different nPKC shRNAs following PMA treatment.**

| Experimental Conditions | $EC_{50}$ (nM) | | PMA-mediated $EC_{50}$ rightward shift (% of $hD5R^{PRS}$) | $E_{max}$ (% of control) | |
|---|---|---|---|---|---|
| | control | PMA | | control | PMA |
| $hD5R^{pRS}$ | 0.82 [0.41-1.6] | 33 [21-51] | | 100 ± 3 | 128 ± 4 |
| $hD5R^{shPKC\delta}$ | 1.2 [0.50-2.9] | 23 [16-34] | 57 ± 13* | 100 ± 3 | 150 ± 4# |
| $hD5R^{pRS}$ | 0.91 [0.24-3.4] | 35 [18-69] | | 100 ± 5 | 156 ± 8 |
| $hD5R^{shPKC\varepsilon}$ | 0.90 [0.21-3.9] | 35 [16-75] | 102 ± 16 | 100 ± 5 | 139 ± 7 |
| $hD5R^{pRS}$ | 1.4 [0.82-2.4] | 31 [20-48] | | 100 ± 2 | 114 ± 3 |
| $hD5R^{shPKC\theta}$ | 1.1 [0.60-2.2] | 23 [15-37] | 123 ± 43 | 100 ± 2 | 117 ± 3 |

Best-fitted and unconstrained parameters for $EC_{50}$ and $E_{max}$ obtained by simultaneous curve fitting of averaged dose-response curves (shown in **Fig. 5A**) are reported. $EC_{50}$ is expressed with 95% confidence intervals in brackets while $E_{max}$ is shown as percent of control $E_{max}$ value for pRS ± approximate as determined by GraphPad Prism. Percent values for PMA-induced $EC_{50}$ rightward shift are expressed as arithmetic means ± SEM. Statistical differences between best-fitted values for $EC_{50}$ and $E_{max}$ following PMA treatment were determined by assessing the goodness of curve fit obtained using unconstrained, shared and constrained parameters. PMA-induced $EC_{50}$ rightward shift were compared with a hypothetical value of "100" using one sample t test.
*, $p < 0.05$ vs. hypothetical value of "100"; #, $p < 0.05$ vs. $hD5R^{pRS}$ (PMA)

**Potential compensatory mechanisms in nPKC-depleted cells: role of untargeted nPKC isoforms in PMA-induced regulation of hD1R and hD5R responsiveness**

The partial reduction observed in the extent of PMA-induced hD1R sensitization and hD5R desensitization may be explained by the incomplete knockdown of PKCε (70%) and PKCδ (75%) in this series of experiments (**Tables 1 and 2**). If we assume a linear relationship between the extent of knockdown and percent inhibition of PMA effect, it still remains unlikely that a full knockdown these nPKC isoforms would lead to a total blockade of PMA-induced regulation of human D1-like receptors. While the PMA-induced regulation of human D1-like receptor is insensitive to the knockdowns of PKCδ and PKCθ (hD1R) or PKCε and PKCθ (hD5R), we cannot rule out that these untargeted nPKC isoforms may partially mediate the PMA-induced regulation of hD1R and hD5R in the absence of targeted nPKC. Importantly, this potential compensatory role by the untargeted nPKC isoforms is not linked to their expression up-regulation following PKCε and PKCδ knockdowns (**Fig. 1**). Additionally, one has to bear in mind that the incomplete knockdown with shRNA may be explained by the long half-life of nPKC proteins. This implies that the remaining levels of nPKCs may be sufficient to mediate most of PMA effects on hD1R and hD5R. Notably, PKC are involved in several signaling pathways suggesting that the intracellular PKC pool has to be large, of which a small fraction could be more stable and readily available for phorbol ester-dependent signaling pathways. Other potential compensatory mechanisms could explain results obtained with nPKC shRNA as discussed in the following sections.

**Potential compensatory mechanisms in nPKC-depleted cells: role of non-PKC phorbol ester receptors in PMA-induced regulation of human D1-like receptor responsiveness**

It is well established that phorbol esters are potent activator of cPKCs and nPKCs. Meanwhile, non-PKC molecules such as α- and β-chimaerins, RasGRP and Munc-13 proteins have been shown to serve as phorbol ester receptors (Ron & Kazanietz 1999, Kazanietz 2002). Importantly, these proteins are characterized by the presence of a cysteine-rich domain similar to the C1 domain found in PKC. Therefore, PMA-mediated recruitment of non-PKC phorbol ester receptors may be facilitated by reduced expression of phorbol ester-sensitive nPKCs in depleted cells and impact the regulation of hD1R and hD5R responsiveness. For instance, α2- and β2-chimaerins harbor a SH2 domain, which interacts with phosphotyrosine proteins (Leung et al. 1994, Hall et al. 1993). Potentially, phosphorylated tyrosine residues located intracellular regions of human D1-like receptors could bind to chimaerins following PMA treatment. Interestingly, a recent study has shown that tyrosine residues of hD1R can interact with SH2 domain of tyrosine phosphatase Shp-2 (Fiorentini *et al.* 2008). Further studies are required to establish a role for non-PKC phorbol esters in PMA regulation of human D1-like receptors.

**Potential mechanisms underlying the tonic regulation of hD1R and hD5R-induced cAMP formation by nPKCs**

A recent study using RNAi depletion of nPKCs has demonstrated that nPKCs differentially regulate distinctive patterns of gene expression in cancer cells in a stimulus-dependent manner (serum vs. phorbol esters) (Caino *et al.* 2011). Consequently, we

cannot rule out that effects of nPKC knockdowns in the absence of PMA may be also associated with changes in gene expression of protein(s) involved in the control of hD1R and hD5R coupling to Gs and cAMP formation in HEK293 cells. This is particularly true for the tonic regulation of human D1-like receptors by PKCδ and PKCθ. In fact, the demonstration of a tonic regulation of human D1-like receptor responsiveness by PKCδ or PKCθ in the absence of PMA is novel role in GPCR signaling. Studies have clearly demonstrated that GPCR signaling complexes with G proteins and ACs are assembled prior to GPCR trafficking to cell surface (Rebois & Hebert 2003, Hebert *et al.* 2006). It is then possible that organization of hD1R and hD5R signaling complexes requires the assembly with D1-like signaling partners regulated by PKCδ and PKCθ expression. Alternatively, our studies raise also an intriguing possibility that these two nPKCs are intrinsic components directly recruited in the early formation and assembly of D1-like receptor signaling complexes. In this particular case, PKC would not require PMA-mediated activation to be translocated to the plasma membrane because it is already assembled with hD1R and hD5R through direct protein-protein interactions or via other interacting/scaffolding proteins. Another hypothesis is that a pool of PKCδ and PKCθ is constitutively activated by an unknown signaling pathway in HEK293 cells. For instance, this tonic PKC activation could be provided by the stimulation of tyrosine kinase receptors through growth factors in culture medium. Overall, these potential mechanisms may underlie the paradoxical regulation of hD5R responsiveness by PKCδ in the absence (positive) and presence of PMA (negative).

## Conclusions

In the present study, we have highlighted the complexity of GPCR regulation by PKC and PMA. Specifically, we have demonstrated that nPKCs exhibit remarkably different roles (sensitization and desensitization) in the absence or presence of their "activating ligands" (phorbol esters) in regulating GPCR responsiveness in nPKC isoform and receptor subtype-dependent manner. This complex PKC-induced modulation of human D1-like receptors may thus be a prerequisite for ensuring the fine regulation required for normal cell function.

**Research highlights of chapter 5-manuscript 4**

In this fourth manuscript, I demonstrated that:

- PKCε is involved in the PMA-induced hD1R sensitization.
- PKCδ is involved in hD5R desensitization upon PMA treatment.
- PKCδ and PKCθ also contribute tonically to hD1-like receptor responsiveness.

With this fourth manuscript, a global picture describing the mechanisms involved in PMA-mediated hD1R and hD5R regulation can be drawn. However, several functional aspects underlying this regulation are still unknown and the working model has to be further developed. These elements will be discussed in more details in the last section (chapter 6, General Discussion).

# Chapter 6

# General Discussion

## 1. General working model of PKC-mediated regulation of D1-like receptors

Results obtained in this thesis shed light on the molecular mechanism involved in PKC-mediated regulation of hD1R and hD5R. Using a chimeric approach, it was demonstrated that regulation of both human D1-like receptors occurs at the receptor level and that IL3 is the structural determinant involved in receptor modulation upon PMA treatment.

In the second paper (**chapter 3**), the single-point mutations of the potential PKC sites in IL3 of both hD1R and hD5R were first characterized. Results indicate that these mutants do not undergo drastic changes in agonist-binding affinity or dopamine potency and efficacy. Consequently, these mutants were used in the third paper (**chapter 4**) to identify the potential PKC phosphorylation sites on human D1-like receptors. Results obtained in this paper address the third objective, which was to characterize the molecular mechanisms involved in hD1R and hD5R regulation upon PMA treatment. Our results suggest two different mechanisms govern the PKC-mediated hD1R and hD5R sensitization and desensitization, respectively (**Fig. 1**). According to these results, the PMA-induced sensitization of hD1R is mediated by a receptor phosphorylation-independent mechanism. Furthermore, this potentiation requires the presence of a specific region within IL3 of hD1R (**Fig. 1A**). Therefore, the molecular mechanism underlying the regulation of hD1R by PMA is in line with the fourth hypothesis. In contrast, the PKC-mediated hD5R desensitization is in agreement with the phosphorylation-dependent hypothesis (third hypothesis). The sequential phosphorylation of two Ser clusters in IL3 governs the hD5R desensitization by PMA in presence of dopamine (**Fig. 1B, right**

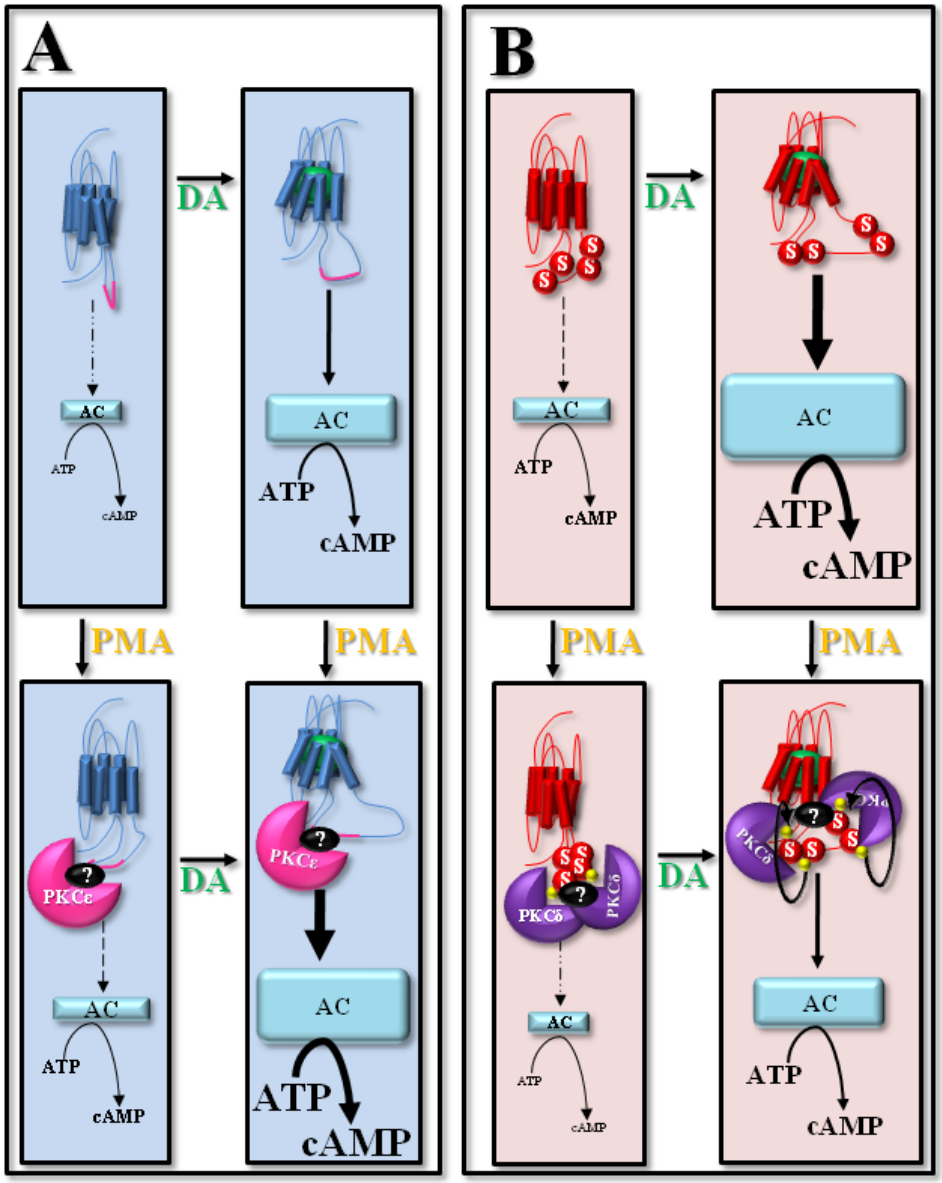


**Figure 1. General working model of PKC-mediated regulation of D1-like receptors.** PMA-mediated hD1R potentiation (**A**) and hD5R desensitization (**B**) are explained by two different mechanisms. The PMA-induced sensitization of hD1R is receptor phosphorylation-independent and requires the presence of a specific amino acids sequence (in pink) within IL3 of hD1R. The potentiation of hD1R activity takes place both in presence (**A, right panels**) or absence (**A, left panels**) of dopamine (DA). In contrast, the PKC-mediated hD5R desensitization is a receptor phosphorylation-dependent mechanism. Reduction of hD5R constitutive activity by PKC is mediated by phosphorylation (yellow circles) of Ser261 and Ser271 (**B, left panels**). Additional phosphorylation of Ser260 and Ser274 is required for the full desensitization of hD5R in presence of DA (**B, right panels**). Given that phosphorylation of Ser260 and Ser274 is conditional to phosphorylation of Ser261 and Ser271, it is hypothesized that upon PKC activation by PMA (leading to phosphorylation of Ser261 and Ser271), DA binding to hD5R promotes a conformational rearrangement of IL3 culminating in the phosphorylation of Ser260 and Ser274 by PKC (**B, bottom panels**). It is also possible that PKC-mediated regulation of both hD1R and hD5R occurs via an intermediary protein linking PKC with IL3 (represented by black oval with interrogation point). The size of arrows and AC boxes are representative of cAMP amount produced by the corresponding receptor.

**panels**). My studies suggest phosphorylation of Ser261 and Ser271 is required for the phosphorylation of Ser260 and Ser274, respectively. In this working model, Ser261 and Ser271 mediate the strong decrease of hD5R constitutive activity following PMA treatment (**Fig. 1B, left panels**). This suggests that upon PKC activation by PMA (leading to phosphorylation of Ser261 and Ser271), binding of dopamine to hD5R promotes a conformational rearrangement of IL3 culminating in the phosphorylation of Ser260 and Ser274 by PKC (**Fig. 1B bottom panels**). This subsequent phosphorylation step is required for the full desensitization of hD5R by PKC in the presence of dopamine.

Results obtained in the third paper (**chapter 4**) using mutant hD5R indicated that progressive Ser to Ala mutation of PKC sites gradually shape the PMA-induced hD5R desensitization into a complete hD5R sensitization. These results suggest a sensitization mechanism involving PKC is an intrinsic property of both D1-like receptors. However, the presence of four specific serine residues on IL3 of hD5R allows PKC to phosphorylate and desensitize this receptor rather than promoting its potentiation. As phosphorylation adds negative charges, it is possible that an increase of negative charges on IL3 of hD5R is responsible for receptor desensitization. By mutations of all four serine residues on IL3 of hD5R, the increase of negative charges on hD5R by PKC phosphorylation is abrogated. Hence, the PMA-mediated receptor phosphorylation-dependent desensitization component is completely removed and the receptor phosphorylation-independent sensitization by PMA is unraveled. In other words, the presence (hD5R) or absence (hD1R) of specific phosphorylation sites dictate if the molecular "switch" turns on either a desensitization or sensitization process triggered by PKC and phorbol esters.

Finally, with the fourth paper using a shRNA approach (**chapter 5**), the fourth objective was reached. PKCδ knock down showed that PKCδ plays a role in the PMA-mediated phosphorylation-dependent hD5R desensitization. In contrast, PKCε regulates the phosphorylation-independent sensitization of hD1R by PMA. Surprisingly, PKCδ and PKCθ isoforms were also identified as major players in the tonic regulation of hD1R and hD5R responsiveness (in absence of PMA treatment) by a mechanism that remains to be further investigated.

## 2. PMA-mediated potentiation of hD1R through PKC activation

Based on results reported in this thesis, IL3 is the main structural determinant for PMA-mediated hD1R potentiation and this receptor regulation occurs through a receptor phosphorylation-independent mechanism. The results in **chapter 4** (**Fig. 9A**) suggest a potential motif located between residues 224 and 245 of IL3 is crucial for the PMA-induced hD1R potentiation. Interestingly, within this region, two of the four Ser residues involved in PMA-mediated hD5R desensitization (Ser260 and Ser261) correspond to alanine residues in hD1R, which are surrounded by residues conserved between the two human D1-like receptors (**see Fig. 1 in chapter 4**). Consequently, this region in hD1R may represent a pseudosubstrate PKC site acting like a "receptor docking site" for PKCε. I hypothesized that PMA-mediated potentiation of hD1R is controlled by a physical interaction between hD1R and PKCε. This interaction (without phosphorylation of IL3 of hD1R by PKCε) could induce a change in the IL3 conformation leading to a better coupling between hD1R and $G\alpha_s$ protein.

## 2.1. Possible players

Alternatively, this PKC docking site could also allow PKCε to be in close proximity to receptor modulators such as GRK, RGS or AGS, allowing a direct modulation by PKCε. Ultimately, the PKC modulation of the regulatory proteins may lead to hD1R potentiation.

### 2.1.1. GRK

As mentioned previously in the **introduction (section 2.1)**, GRK is classically associated with homologous GPCR desensitization. However, there are several mechanisms where PKC can positively or negatively regulate GRK activity. For instance, GRK2 activity is inhibited by PKC activation, which leads to the blockade of GRK2-mediated $\beta_2$-adrenergic receptor desensitization and promotes an enhanced signaling. After stimulation of $\beta_2$-adrenergic receptor, Raf kinase inhibitor protein (RKIP) dissociates from its known target, Raf-1, to associate with GRK2 and block its activity in cardiomyocytes. This RKIP/Raf-1 dissociation is triggered by PKC activation which leads to PKC-induced RKIP phosphorylation (Lorenz *et al.* 2003). It has also been reported that GRK5 phosphorylation by PKC dramatically reduces its ability to phosphorylate light-activated rhodopsin, providing a mechanism to reduce GRK5-mediated desensitization (Pronin & Benovic 1997). Interestingly, D1R is substrate for GRK2, GRK3 and GRK5 (Tiberi *et al.* 1996, Sedaghat & Tiberi 2011). Given that PKC is known to inhibit GRK2 and GRK5 activity, inhibition of GRK-mediated hD1R desensitization by PKC could be a possible mechanism for the PMA-mediated hD1R potentiation. Moreover, a recent publication from our lab show that in the absence of

dopamine, D1R and GRK2 already form a signaling complex in co-transfected HEK293 and striatal cells (Sedaghat & Tiberi 2011). Consequently, the hypothesis of a PMA-mediated GRK2 inhibition is attractive because it could also be applied to the potentiation of hD1R constitutive activity upon PMA treatment.

### 2.1.2. RGS

GRK is one of the kinases mediating GPCR desensitization. In addition to GRK-mediated GPCR desensitization, RGS can also dampen receptor activity by enhancing Gα protein GTPase activity. RGS are known to mediate their inhibitor effect through direct interaction with Gα proteins and effectors. However, recent studies suggest that RGS proteins can also selectively interact with IL3 or CT of numerous GPCRs (Neitzel & Hepler 2006). Consequently, the PMA-mediated recruitment of PKC to IL3 of hD1R could be a mechanism disrupting the possible interaction between hD1R and RGS and hence potentiate hD1R activity. Moreover, binding efficacy and GAP activity of several RGS are known to be modulated by post-translational modifications such as phosphorylation (Benzing *et al.* 2000, Chen *et al.* 2001, Derrien & Druey 2001, Derrien *et al.* 2003, Ogier-Denis *et al.* 2000). Interestingly, strong reductions of RGS5 GAP activity and binding capacity to Gα subunit have been associated with phosphorylation of RGS5 by PKC (Moroi *et al.* 2007). Thus, it is possible that PMA-mediated recruitment of PKC to hD1R could also reduce RGS activity. One of the main pitfalls associated with the RGS hypothesis is that RGS are generally associated to receptors coupled to $G\alpha_i$, $G\alpha_q$ or $G\alpha_{12/13}$ proteins but not $G\alpha_s$-coupled receptors such as hD1R. However, RGS-PX1 has been shown to specifically bind to Gαs subunit in brain lysates and to reduce β2-

adrenergic receptor-induced production of cAMP in transfected HEK293 cells (Zheng *et al.* 2001). Meanwhile, the possible regulation of hD1R signaling by RGS-PX1 remains to be investigated.

### 2.1.3. AGS

Another class of heterotrimeric G protein regulator is AGS. These accessory proteins promote G protein activation through three main mechanisms. Group I (AGS1) behaves as guanine nucleotide exchange factor (GEF) as it promotes the exchange of GDP for GTP (Cismowski 2006). Group II (AGS 3-6) is characterized by the presence of G protein regulatory (GPR) motifs. Binding of GPR motif-containing AGS to heterotrimeric Gαβγ complex enhances the rate of dissociation of this complex (Blumer *et al.* 2007). Additionally, this motif binds to and sequesters GDP-bound Gα subunit before it can rebind to Gβγ. As opposed to group I and group II AGS, group III AGS (AGS2, 7-10) binds to Gβγ subunits. Therefore, AGS proteins from the third group promote dissociation of Gβγ from Gα subunit and decrease Gβγ re-association with Gα subunit. This increases Gβγ-mediated signaling because AGS binding to Gβγ does not affect effector association with Gβγ. Moreover, in contrast to group I and group II AGS that only target $G\alpha_{i2}$- or $G\alpha_{i3}$-coupled receptors, group III AGS also regulate $G\alpha_{s}$-coupled receptors. Consequently, AGS from group III are candidate for regulation of hD1R activity since free Gβγ subunit can also stimulate AC activity (AC2, AC4, AC5, AC6 and AC7). However, the main pitfall of this hypothesis is that the mechanisms regulating AGS protein activity are unknown. It is possible that phosphorylation of Ser/Thr residues on group III AGS by PKC increases binding affinity of AGS for Gβγ and therefore

promote AC activity. With the pseudosubstrate site located on IL3 of hD1R acting like a "PKC docking site", PKC could be in close proximity to Gβγ to promote Gβγ-mediated AC potentiation through AGS activation.

## 2.2. Possible mechanism

According to previous results from our lab, the PMA-mediated potentiation of hD1R is blocked by 50% using pharmacological inhibitors (Jackson et al. 2005). PKC inhibitors used (Gö6976, bis-indolylmaleimide I and Gö6983) are ATP-competitive inhibitors and consequently reduce PKC kinase activity (Jackson et al. 2005, Gschwendt *et al.* 1996, Martiny-Baron *et al.* 1993). Potentially, this implies two mechanistic components. The first mechanistic component involves the PKC kinase domain, which is inhibited by pharmacological PKC inhibitors. Given that IL3 of hD1R is necessary but does not require phosphorylation for PMA-mediated hD1R potentiation, this may implicate a role for an intermediary protein controlled by PKC activity. This unknown protein may potentiate hD1R through a direct physical interaction. The second mechanistic component involves either a region located outside the PKC kinase domain or a protein-protein interaction controlled in a PKC-independent but phorbol ester-dependent fashion (e.g. non-PKC phorbol ester receptors). Perhaps, this explains the residual hD1R potentiation upon PMA treatment.

Alternatively, one single mechanism may induce PMA-mediated hD1R potentiation. In fact, the partial inhibition of the PMA-mediated hD1R potentiation observed with bis-indolylmaleimide I and Gö6983 treatment could be simply explained by a partial reduction of the affinity of PKC kinase domain for the putative

pseudosubstrate site on IL3 of hD1R caused by the binding of these drugs on PKC kinase domain. The use of kinase-dead PKCε and PKC inhibitors specifically interfering with translocation of PKC to plasma membrane such as sphingosine would help testing this hypothesis (Bazzi & Nelsestuen 1987).

## 3. Physiological relevance of PKC-mediated regulation of D1R and D5R

Results obtained during my thesis suggest different molecular mechanisms are involved in the opposite regulation of human D1-like receptors by PKC in HEK293 cells. As mentioned in the introduction, D1R and D5R play a role in several signaling pathways in neurons. In the following section, I discuss the potential relevance of these molecular mechanisms in the regulation of D1-like dopaminergic receptors by PKC in vivo and how PKC modulation of D1R and D5R responsiveness may impact brain functions associated with these receptors.

### 3. 1. D1R in striatum for motor control

Results described in this thesis could bring an important contribution to knowledge of the molecular mechanisms involved in basal ganglia and consequently in motor function. First, it is important to describe briefly basal ganglia wiring to appreciate the potential impact of these novel findings on dopaminergic control of motor system.

As stated in the **introduction part (Fig. 8)**, the nigrostriatal pathway is the main neuronal tract involved in motor function. On the glutamatergic (stimulatory) influence of cerebral cortex (**Fig. 2 (1)**), neurons originating from substantia nigra pars compacta (SNpc) release dopamine, leading to activation of D1R and D2R expressed on medium

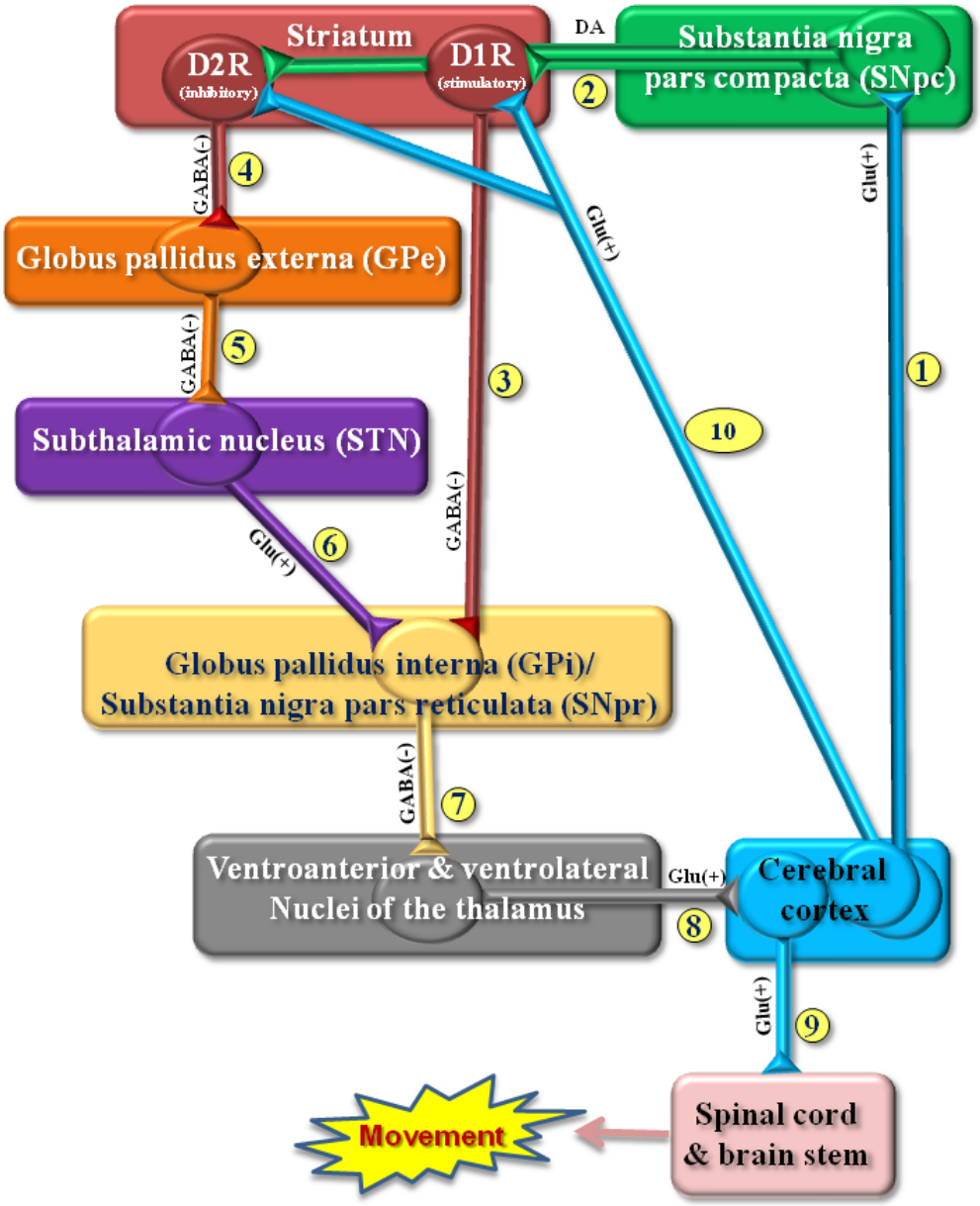


**Figure 2. The basal ganglia wiring.**
Under glutamatergic control (stimulatory) of cerebral cortex **(1)**, neurons from substantia nigra pars compacta (SNpc) release dopamine (DA), leading to activation of D1R and D2R expressed on medium spiny neurons (MSN) in striatum **(2)**. DA-induced D1R activation promotes firing of D1R-expressing MSN resulting in release of the inhibitory neurotransmitter GABA **(3)**. Consequently, neurons located in globus pallidus interna (GPi) and in substantia nigra pars reticulata (SNpr) are inhibited. Inhibition of these neurons can also be induced by an indirect pathway via D2R activation also expressed by other MSN in striatum. Activation of D2R results in inhibition of D2R-expressing MSN, promoting disinhibition of the GABAergic neurons from globus pallidus externa (GPe) **(4)** and consequently inhibition of glutamatergic subthalamic nucleus (STN) neurons **(5)**. Hence, glutamatergic neurons from STN reduce glutamate release **(6)** leading to inhibition of neurons located in GPi and SNpr. Once inhibited, GABAergic neurons from GPi and SNpr disinhibits glutamatergic neurons from ventroanterior and ventrolateral nuclei of the thalamus **(7)** and therefore promote firing of cortical neurons **(8)** generating movement via spinal cord and brain stem stimulation **(9)**.

spiny neurons (MSN) that compose the majority of striatal neuronal cells (**2**). Agonist-induced stimulation of D1R activity then promotes firing of D1R-expressing MSN and release of the inhibitory neurotransmitter GABA and hence inhibits neurons located in globus pallidus interna (GPi) and in substantia nigra pars reticulata (SNpr) (**3**). Neuronal activity in GPi and SNpr can also be inhibited by an indirect pathway through activation of D2R also expressed by distinct MSN in striatum. D2R activation leads to inhibition of D2R-expressing MSN. The inhibition of D2R-expressing MSN cells (less GABA released) promotes disinhibition of the GABAergic neurons located globus pallidus externa (GPe) (**4**) (more GABA released) and consequently inhibition of glutamatergic neurons located in the subthalamic nucleus (STN) (**5**). Because neurons from STN are stimulatory neurons (they release the excitatory neurotransmitter glutamate), inhibition of these cells also inhibits neurons originating from GPi and SNpr (**6**). Inhibition of GABAergic neurons from GPi and SNpr then disinhibits glutamatergic neurons located in the ventroanterior and ventrolateral nuclei of the thalamus (by virtue of reduced GABA release) (**7**) and consequently promote firing of specific cortical neurons (**8**) generating movement through stimulation of spinal cord and brain stem (**9**).

As mentioned in the introduction part, stimulation of mGluR5 has been shown to potentiate dopamine-induced cAMP formation via PKC activation in striatum. Results reported in this thesis using HEK293 cells demonstrate that PKC-mediated potentiation of cAMP formation observed in striatum is achieved through D1R not D5R. As striatum is a site of convergence of both glutamatergic corticostriatal pathway (**10**) and dopaminergic nigrostriatal pathway (**2**), PKC-mediated potentiation of D1R is probably one of the mechanisms leading to striatum stimulation by glutamatergic neurons from

prefrontal cortex. Upon decision to elicit a movement, stimulation of glutamatergic neurons from the prefrontal cortex mediate glutamate release at their synapse (in striatum). This glutamate binds on the $G\alpha_q$-coupled mGluR5 expressed by MSN, leading to PKC activation (**Fig. 3**). In a parallel way, D1R, also expressed at the surface of MSN, is activated by dopamine released by neuron from the SNpc projecting to striatum. D1R activation is potentiated by mGluR5-mediated PKC activation. Importantly, the activity of glutamatergic neurons from the corticostriatal pathway can enhance the firing probability of MSN and therefore promote movement through PKC-mediated D1R potentiation. Consequently, potentiation of D1R signaling by PKC may contribute to the fine tuning required for motor control.

## 3. 2. D1R and D5R in subthalamic nucleus for awareness modulation

As mentioned in the previous section, STN is part of the basal ganglia and therefore has an important role in motor control. However, this nucleus also contributes to the overall level of vigilance. This level of vigilance is dictated by the STN firing mode. When neurons from STN fire in tonic mode (characterized by single spikes) (**in blue on Fig. 4**), the vigilance level is high. The tonic mode occurs when attention and awareness are required. In contrast, during slow-wave sleep or when somnolence or drowsiness occur, neurons from STN are in burst-firing mode characterized by clusters of high frequency spikes (**in red on Fig. 4**) (Steriade *et al.* 1993, Steriade 2001).

Studies on STN suggest that firing mode of this nucleus is controlled by (**1**) dopaminergic input from SNpc and by (**2**) glutamatergic input from thalamic parafascicular nucleus (PFN) (Brown *et al.* 1979, Hassani *et al.* 1997, Francois *et al.* 2000, Cragg *et al.* 2004).

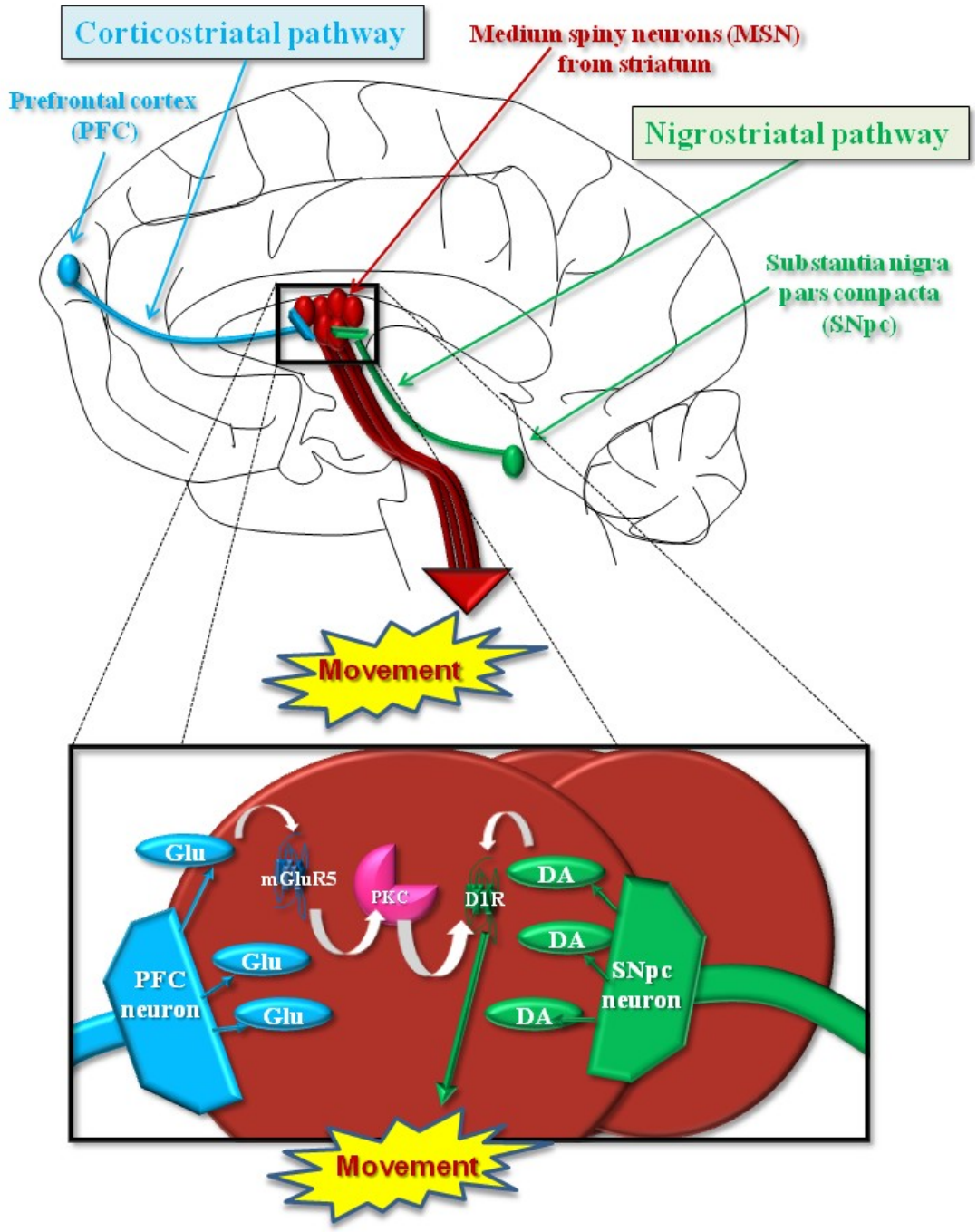


**Figure 3. Potentiation of D1R-expressing striatal neuron activity by glutamatergic corticostriatal pathway via PKC.**

Striatum is a site of convergence of both glutamatergic corticostriatal pathway **(in blue)** and dopaminergic nigrostriatal pathway **(in green)**. Upon decision to elicit a movement, firing of glutamatergic neurons from the prefrontal cortex (PFC) results in glutamate release in striatum. Glutamate binding on the $G\alpha_q$-coupled mGluR5 expressed by medium spiny neurons (MSN) leads to PKC activation. In a parallel way, D1R, also expressed at the surface of MSN, is activated by dopamine released by neuron from the substantia nigra pars compacta (SNpc) projecting to striatum. D1R activation is potentiated by mGluR5-mediated PKC activation. Hence, firing of glutamatergic neurons from the corticostriatal pathway enhances the firing probability of MSN and therefore promote movement through PKC-mediated D1R potentiation. This regulatory mechanism may contribute to the fine tuning required for motor control.

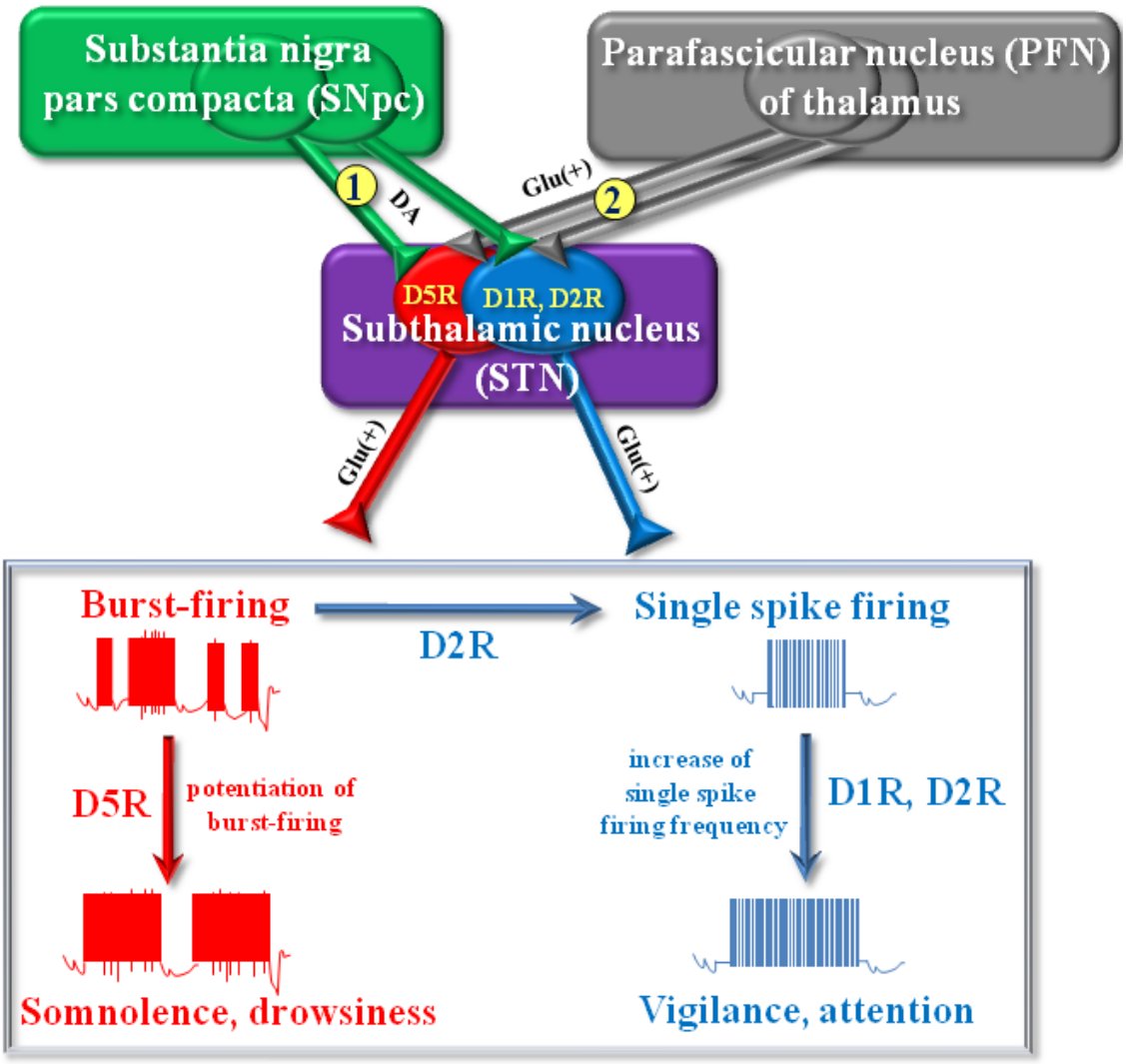


**Figure 4. Role of PKC-mediated D1-like receptor regulation in modulating awareness.**
The subthalamic nucleus (STN) firing mode is controlled by **(1)** dopaminergic input from substantia nigra pars compacta (SNpc) **(in green)** and by **(2)** glutamatergic input of thalamic parafascicular nucleus (PFN) **(in gray)**. At low dopamine concentrations situations (e.g. models of Parkinson's disease), the burst-firing mode in STN is potentiated by virtue of the high agonist-independent activity associated with D5R **(in red)**. This results in generation of clusters of high frequency spikes by STN. Interestingly, this STN firing mode is also observed during slow-wave sleep, somnolence or drowsiness states (i.e. when PFN is almost inactive). In contrast, in presence of dopamine (when neurons from SNpc are active), activation of both D1R and D2R lead to the increase of single spike firing frequency of STN **(in blue)**. Additionally, D2R activation also turns burst-firing into single-spike firing. Single spike firing occurs when awareness, vigilance and attention are required and is associated to PFN high activity. Interestingly, $G\alpha_q$-coupled receptors mGluR1 and mGluR5 are expressed by STN neurons and consequently, glutamate released by neurons from PFN can activate PKC in STN neurons. Here, it is hypothesized that the modulator effect of PFN on STN firing mode can be mediated by a regulation of dopaminergic receptors by PKC. In fact, firing of glutamatergic neurons from PFN would stimulate PKC activity via activation of mGluR1 and mGluR5 leading to simultaneous potentiation of D1R activity and desensitization of D5R constitutive activity. Therefore, glutamatergic inputs from PFN to STN would favor and stabilize the single spike firing mode of STN neurons.

SNpc activity is important for stabilization of single spike firing mode and consequently to maintain a high degree of vigilance. Studies suggest that dopamine-mediated activation of both D1R and D2R increases single spike firing frequency (Baufreton et al. 2005). Additionally, D2R activation turns burst-firing into a tonic mode (single-spike firing). Consequently, both activation of D1R and D2R would stabilize the tonic tone required for vigilance state. Hence, in a dopamine depletion state, the burst mode is prevalent in STN of rodent and primate as well as patients with Parkinson's disease (Bergman *et al.* 1994, Hollerman & Grace 1992, Overton & Greenfield 1995, Hassani *et al.* 1996, Hassani & Feger 1999, Ni *et al.* 2001). In fact, in situations associated with temporary low amount of dopamine produced by SNpc or in chronic dopamine-depleted state (in Parkinson's disease), dopamine receptors located in STN would only signal through their agonist-independent activity. D5R is also expressed in STN and this would favor D5R-mediated signaling given its natural high constitutive activity (Baufreton et al. 2003, Tiberi & Caron 1994). As opposed to D1R and D2R, D5R-induced signaling is associated with potentiation of burst-firing neurons via L-type calcium activation in STN.

As mentioned above, in addition to SNpc, thalamic glutamatergic inputs from PFN also control STN firing mode. In normal state, when enough information is relayed by thalamus, STN has a tonic firing mode (Crossman 1990). In contrast, when no or little information is relayed by the thalamus, STN fires in burst mode. Given that postsynaptic dendrites of STN neurons express both $G\alpha_q$-coupled receptors mGluR1 and mGluR5, glutamatergic inputs to these neurons would lead to PKC activation in these cells (Kuwajima *et al.* 2004). As results reported in this thesis demonstrate that PKC potentiates D1R activity but also dramatically reduces D5R constitutive activity,

glutamatergic inputs to STN would increase the single spike firing frequency and simultaneously forbid the potentiation of burst-firing. Hence, glutamatergic input from thalamus would favor and "stabilize" the single spike firing mode of STN neurons. I hypothesize that this mechanism could be responsible of the positive effect of thalamic activity on the vigilance state.

## 3. 3. D1R and D5R in hippocampus for learning and memory

Like previously mentioned in the **introduction (part 4.2)**, dopaminergic neurons from VTA project to NAc via the mesolimbic pathway. However, these dopaminergic neurons also send projections to hippocampus (**Fig. 5 (1)**) (Verney *et al.* 1985, Gasbarri *et al.* 1994). Indeed, activation of hippocampal D1-like receptors is involved in LTP at CA1 synapses and their activation improves cognitive performance and spatial memory of aged memory-impaired and normal rats (Huang & Kandel 1995, Otmakhova & Lisman 1996, Otmakhova & Lisman 1998, Bach *et al.* 1999, Hersi *et al.* 1995b, Steele *et al.* 1997, Steele *et al.* 1996). Previous studies suggest that these positive effects of D1-like receptor agonists on hippocampal functions are mediated by an increased release of acetylcholine (ACh) by hippocampal cholinergic nerve terminals upon D1-like receptor stimulation (Day & Fibiger 1994, Hersi *et al.* 1995a, Imperato *et al.* 1993). Subsequently, using D5R-deficient mice, Quirion's group identified D5R (not D1R) as the receptor involved in D1-like-mediated release of acetylcholine in hippocampus through an unknown mechanism (**Fig. 5 (2)**) (Hersi et al. 2000, Laplante et al. 2004). Moreover, in absence of dopamine, this group has shown presence of a tonic level of ACh released in

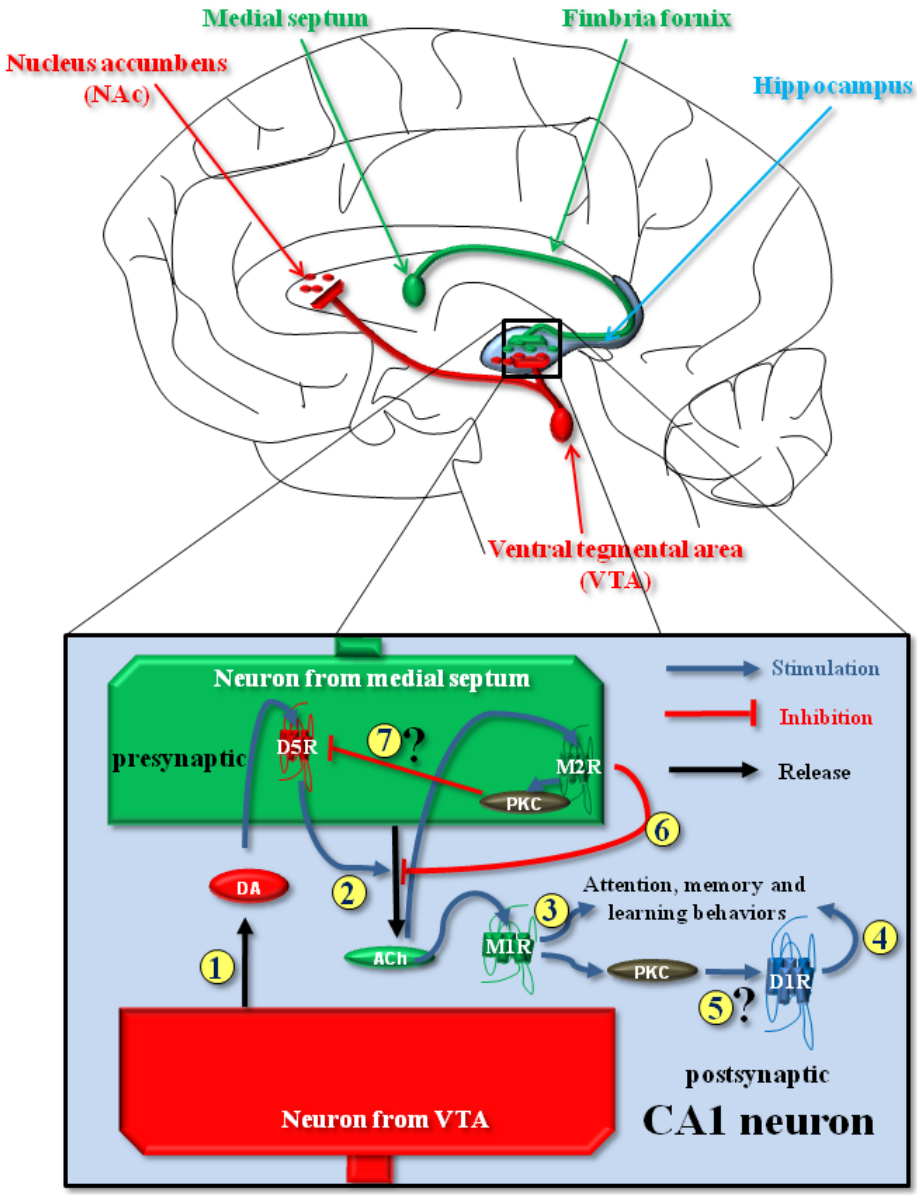


**Figure 5. Role of PKC-mediated D1-like receptor modulation in regulating hippocampus activity.** Hippocampus receives dopaminergic innervation from ventral tegmental area (VTA) that also sends projection to nucleus accumbens **(1)**. Hippocampal D1-like receptors are important for LTP at CA1 synapses, cognitive performance and spatial memory. Stimulation of D5R (but not D1R) is associated to an increase of acetylcholine (ACh) release **(2)** mainly by neurons originating from medial septum and terminating in CA1 region of hippocampus via the fimbria fornix, forming the septohippocampal projections. ACh mediates its important effects on hippocampus mostly through activation of the post-synaptic $G\alpha_{q/11}$-coupled M1 muscarinic ACh receptor (M1R) **(3)**. Given that D1R is also expressed in CA1 neurons and that both M1R and D1R are important for LTP in CA1 as well as learning and memory **(4)**, I hypothesize that the $G\alpha_{q/11}$-coupled M1R mediates at least part of its effect through PKC-mediated D1R potentiation **(5)**. As opposed to M1R, the $G\alpha_i$-coupled M2 muscarinic ACh receptor (M2R) is presynaptic in hippocampus and behaves as an autoreceptor. Therefore, M2R stimulation inhibits ACh release by septohippocampal terminals **(6)**. As M2R stimulation had been described to activate PKC, I hypothesize that M2R-mediated inhibition on ACh release would be at least in part explained by D5R inhibition **(7)**. Hence, in this hypothetic model, modulation of D5R activity by PKC would control the retro-inhibition loop to avoid over-activation of hippocampus by ACh. The main idea of this model is that opposite regulation of D1R and D5R by PKC would also mediate an opposite action on hippocampal activity and consequently contribute to modulation of the level of hippocampal functions.

the hippocampus in wild type but not in D5R-deficient mice. These results highlight the important role of the high D5R constitutive activity and its role in maintenance of tonic ACh release in hippocampus. In fact, ACh is a powerful neurotransmitter required for hippocampal function. The main cholinergic input comes from the septohippocampal projections. Septohippocampal neurons are located in the medial septum and their axons compose the fimbria fornix (**Fig. 5**). Hence, lesions of this cholinergic projection had been associated with important attentional, learning and memory deficits (Fibiger 1991). ACh-mediated effects on hippocampus are mainly produced via activation of the post-synaptic $G\alpha_{q/11}$-coupled M1 muscarinic ACh receptor (M1R) (**Fig. 5 (3)**) (Porter *et al.* 2002, Dutar & Nicoll 1988, Muller & Misgeld 1986, Segal & Fisher 1992). In the introduction (part 4.3.1), it was mentioned that D5R is the more abundant D1-like receptor in hippocampus. However, D1R is also present. Indeed, using D1R knock-out mice, Granado et al. established a crucial role of D1R (but not D5R) in LTP of CA1 hippocampal neuron and spatial learning and memory (**Fig. 5 (4)**) (Granado *et al.* 2008). Given that both M1R and D1R are expressed by CA1 neurons and both are important for LTP in CA1 as well as learning and memory, I hypothesize that the $G\alpha_{q/11}$-coupled M1R mediates at least part of its effect through PKC-mediated D1R potentiation (**Fig. 5 (5)**).

In contrast to M1R, the $G\alpha_{i}$-coupled M2 muscarinic Ach receptor (M2R) is generally located presynaptically in hippocampus and behaves as an autoreceptor and consequently inhibits ACh release by septohippocampal terminals (**Fig. 5 (6)**) (Lapchak *et al.* 1989, Li *et al.* 2007, Quirion *et al.* 1995, Pohorecki *et al.* 1988, Raiteri *et al.* 1984). Therefore, M2R and D5R have opposed actions on ACh release by these terminals. Interestingly, M2R stimulation can also lead to PKC activation through activation of

PI3K (Wang *et al.* 1999, Callaghan *et al.* 2004). Consequently, I hypothesize that M2R-mediated inhibition on ACh release would be at least in part explained by PKC-induced D5R inhibition (**Fig. 5 (7)**) (Wang et al. 1999, Callaghan et al. 2004). Therefore, in this hypothetic model of D5R inhibition by M2R, regulation of D5R activity by PKC would be part of the retro-inhibition loop to avoid over-activation of hippocampus by ACh.

In the hypothetic model of D1R and D5R regulation by PKC in hippocampus, PKC-mediated D1R potentiation would promote learning and memory upon activation of postsynaptic M1R, while in contrast PKC-mediated D5R desensitization through activation of presynaptic M2R would result in a reduction of ACh release and consequently inhibition of hippocampus activity. Therefore, the level of hippocampus activity would be modulated by the opposite regulation of D1R and D5R by PKC.

## 3. 4. D1R evolution and emergence of consciousness?

Sequence alignment of IL3 of D1R from several mammals shows that the region required for PMA-mediated potentiation is almost identical, except for the cluster composed of two consecutive alanine residues. Interestingly, only monkeys and human have these two alanine residues. Hence, non-primate mammals have a serine residue substituted to the first alanine of that cluster. Results obtained with hD1-AANT-SSSS on figure 8 in chapter 4 of this thesis suggest that substitution of both alanine residues by a serine strongly reduces PMA-induced potentiation of the hD1R. This suggests that presence of one serine at that particular position would partially reduce D1R potentiation upon PMA treatment. Consequently, D1R potentiation by PKC in most animals is possibly lower than the one observed for monkeys and humans. Maybe this slight

difference improving D1R potentiation by PKC could be one of the numerous factors underlying the superior cognitive function associated with primates and the related emergence of consciousness.

## 4. Conclusions and future studies

Results reported in this thesis illustrate how two receptors having a high degree of identity, such as hD1R and hD5R, can be regulated in an opposite fashion. To my knowledge, this is a unique model. Despite the fact that β1- and β2-adrenergic receptors are highly conserved, these two GPCRs are both desensitized upon PKC activation. Notably, my results highlight how the presence or absence of specific serine residues on a receptor dictates the outcome of this receptor regulation by a protein kinase like PKC. Most importantly, these small structural changes between D1R and D5R and their opposing regulation by PKC may be very important in the central mechanisms responsible for maintenance of the fine tuning required in normal brain functions such as motor control, vigilance, attention, memory and learning.

The take home message of this thesis is that hD1R potentiation and hD5R desensitization by PKC are mediated by two different mechanisms. While potentiation of hD1R responsiveness is regulated in a receptor phosphorylation-independent and IL3-dependent manner, desensitization of hD5R activity is mediated through phosphorylation of specific serine residues also located in IL3 of hD5R. Future studies need to be done to investigate in more detail the precise molecular mechanisms underlying the receptor phosphorylation-independent hD1R potentiation upon PMA treatment. Notably, as the removal of central region of IL3 of hD1R (21 residues) resulted in a complete loss of

hD1R potentiation upon PMA treatment, it could be useful to refine this deletion to decipher more precisely the motif responsible for this regulation. Additionally, the use of kinase-dead PKCs will be necessary to confirm the hypothesis of a hD1R potentiation mediated by PKC docking to the putative IL3 motif in hD1R and to exclude the requirement of PKC kinase activity for hD1R potentiation by PMA. Moreover, the possible involvement of signaling partners such as GRK, RGS or AGS needs to be investigated using co-immunoprecipitation assays in absence and presence of PMA.

Most importantly, the existence of this opposite regulation of hD1R and hD5R by PKC, and their underlying mechanisms, need to be verified in neuronal cellular models such as primary striatal and hippocampal cultures. Our lab recently reproduced the PMA-mediated potentiation of cAMP production in rat striatal culture. Stimulation of cAMP production obtained with dopamine in the presence of sulpiride (a D2-like receptor antagonist) as well as with SKF81297 (a D1-like receptor agonist) was potentiated by PMA treatment. Similar studies need to be conducted using striatal culture from D1R knockout mice or from mice expressing PD3-hD1R or a mutant form of D1R in which the minimal motif responsible for the PMA-mediated D1R potentiation had been deleted. These experiments would assess if the molecular mechanism underlying PKC-mediated potentiation in striatum is similar to the one observed in our heterologous cellular model (HEK293 cells). Additionally, PKC activation in these experiments could also be done using a mGluR5 specific agonist to confirm that this hD1R regulatory mechanism by PMA treatment also occurs upon a glutamatergic input.

In a similar fashion, the possible role of PKC-mediated hD1R potentiation and hD5R desensitization in stabilization of the single-spike firing mode in STN discussed

previously could be investigated. This could be achieved by recording the firing pattern of neurons from STN upon PMA in presence or absence of dopamine. If PKC activation results in stabilization of the single-spike firing mode, the same experiments could be repeated with STN neurons from mice expressing the equivalent of hD5-SSSS-AAAA, PD3-hD1R or the mutant form of D1R in which the minimal motif responsible for the PMA-mediated D1R potentiation had been deleted. These additional studies using STN neurons from mutant mice could validate if the molecular mechanisms underlying D1R and D5R regulation by PKC in HEK293 cells are also applicable in neurons from STN. The PKC role in regulating D5R activity in hippocampus and ACh release can also be investigated by recording ACh release from neurons originating from medial septum in presence and absence of PMA. Moreover, the possible D1R potentiation by M1R in CA1 neurons through activation of PKC could also be studied by measuring cAMP produced upon stimulation of M1R in presence or absence of PKC inhibitors using CA1 neurons from wild type, D1R knockout and mutant D1R mice. Ultimately, all these recordings of cellular outputs could be correlated with behavioral studies to assess the impact of opposite regulation of D1R and D5R on motor control, attention, learning and memory.

Currently, there are no therapeutic agents selectively acting on D1R or D5R. With growing evidence that D1-like receptors are involved in neuropsychiatric disorders, the development of pharmacologic tools acting on D1R or D5R is important. Identification of the mechanisms involved in the regulation of these two receptors and the precise structural determinant implicated is an important step in the elaboration of more targeted therapeutics. Indeed, large spectrum therapeutics are generally associated with important side effects. Nowadays, with the increasing span life, age-related pathologies such as

Alzheimer's, Parkinson's diseases and dementia are growing in number and consequently there is a necessity to come up with better treatments.

# Chapter 7

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