The dissection of the molecular mechanism underlying the facilitative action of prostaglandin E receptor EP1 on dopamine D1 receptor-induced cAMP production

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Summary of abbreviations

AC	Adenylyl Cyclase
ATP	Adenosine Triphosphate
β2-AR	β2 -Adrenergic receptor
cAMP	Cyclic Adenosine Monophosphate
CaM	Calmodulin
cGMP	Cyclic Guanine Monophosphate
DAG	Diacylglycerol
DARPP-32	Dopamine-and cAMP-regulated phosphoprotein -32 kDa
G protein	Gunanine nucleotide-binding protein
GEF	Guanine nucleotide Exchange Factors
GPCR	G Protein-Coupled Receptor
GRK	G protein coupled Receptor Kinase
IP_3	Inositol 1,4,5-triphosphate
NSAIDs	Non-Steroidal Anti Inflammatory Drugs
PKA	Protein Kinase A
PI3K	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
PGE_2	Prostaglandin E_2

Abstract

Initially, the signal transduction of G-protein-coupled receptor (GPCR) has been widely considered to be a relatively straightforward process. A distinct receptor selectively couples to its cognate G protein to initiate activation of its effector through the G_{α} subunit. Recent studies have shown that $G_{\beta\gamma}$ subunits released from the GPCR regulate effectors in a $G_{\beta\gamma}$ subtype specific manner. Adenylyl cyclase (AC) is one of these effectors of G proteins. Whereas the $G_{\alpha s}$ subunit activates all membrane-bound AC isoforms, the $G_{\beta\gamma}$ subunit can facilitate or suppress $G_{\alpha s}$-mediated AC activity, depending on AC isoform. This property makes AC an ideal site of convergence from signaling pathways from multiple GPCRs. It has been reported that multiple GPCRs form homo/hetero-mers. Notably, simultaneous activation of multiple GPCRs can activate distinct signaling pathway that is not activated by either of each protomer. However, the mechanism underlying such crosstalk between multiple GPCRs remains poorly understood.

It was previously reported that prostaglandin E receptor EP1 facilitates dopamine D1 receptor signaling in striatal slices and promotes behavioral responses induced by D1 receptor agonists. Here using HEK-293T cells expressing D1 and EP1, I have analyzed the mechanism underlying EP1-mediated facilitation of D1 receptor signaling. Fluorescent immunostaining showed that EP1 and D1 receptors are partly colocalized in the cells, and co-precipitation experiments revealed a molecular complex of EP1 and D1 receptors. Treatment of the cells with ONO-DI-004, an EP1-selective agonist, enhanced cAMP production induced by D1 agonists, SKF-81297 and SKF-83822. Although this facilitative effect of EP1 stimulation was not affected by pharmacological blockade of EP1-induced Ca^{2+} increase, it was blocked by overexpression of $G_{t\alpha}$ as a $G_{\beta\gamma}$ scavenger.

Consistently, depletion of AC7, a $G_{\beta\gamma}$-sensitive AC isoform, abolished the facilitative action of EP1 on D1-induced cAMP production. Notably, neither $G_{t\alpha}$ overexpression nor AC7 depletion affected cAMP production induced by D1 stimulation alone. In contrast, depletion of AC6, another AC isoform, reduced cAMP production induced by D1 stimulation alone, but spared its facilitation by EP1 stimulation. Collectively, these data suggest that, through the complex formation with D1, EP1 signaling directs the D1 receptor through $G_{\beta\gamma}$ to be coupled to AC7, an AC isoform distinct from those utilized by D1 receptor alone, in HEK-293T cells. Thus, our finding highlights a novel role for distinct AC isoforms underlying a unique signaling property of GPCR heteromers.

1. Introduction

1.1. G protein-coupled receptors

G protein-coupled receptors (GPCRs) are seven transmembrane receptors that constitute a large family of proteins that mediate various cellular responses to hormones, neurotransmitters and other extracellular stimuli. There are more than 1000 human genes that encode GPCRs, each of them plays specific physiological and pathophysiological roles in various organs in the whole body (Prinster et al., 2005). Pharmacological, biochemical and molecular techniques have characterized, identified and cloned GPCRs over the last 30 years, and it has been established that many bioactive compounds such as naturally found and synthetic therapeutic drugs act through binding to distinct GPCRs. Therefore, GPCRs have been a prominent focus of drug design platforms (Prinster et al., 2005).

Structurally, GPCRs are composed of seven transmembrane α helices, connected via 3 intracellular loops and 3 extracellular loops, which are bound by an extracellular amino-terminus and an intracellular carboxyl-terminus (Wess, 1998). GPCRs can be classified into 3 major groups in regards to their sequence homology (Wess, 1998). The rhodopsin-type receptors constitute the largest family of GPCRs called Class A, which includes receptors for biogenic amines, peptides, protein hormones, bioactive lipids, and nucleotides. These GPCRs possess 20 conserved amino acid residues that are distributed in several extracellular, intracellular and transmembrane domains for protein stability and/or receptor activation. Class B or the secretin/glucagon receptor family includes GPCRs that characteristically have a long extracellular N-terminal domain and many of which are receptors for peptide hormones (Gesty-Palmer and Luttrell, 2004). Class C receptors, such as metabotropic glutamate receptors and $GABA_B$ receptors, represent a

distinct group of GPCRs, which possess a characteristically large extracellular N-terminal domain called the Venus flytrap module involved in ligand recognition. In addition, other minor classes, such as the frizzled and smoothened receptor family, have also been proposed (Schulte, 2010).

As the term GPCR suggests, these cell surface receptors orchestrate their cellular responses through coupling to heterotrimeric guanine nucleotide-binding proteins (G proteins). Heterotrimeric G proteins are composed of 3 subunits, G_{α}, G_{β}, and G_{γ} (Neves et al., 2002). Among these subunits, the G_{α} subunit binds to either GDP or GTP depending on its activation. An inactive form of GPCR forms a complex with the GDP-bound form of the heterotrimeric G protein complex. The GPCR acts as a guanine nucleotide exchange factor (GEF), and the ligand binding is thought to induce a conformational change of the GPCR to facilitate its GEF activity for converting the GDP-bound form of the G protein to its GTP-bound form. This conversion simultaneously dissociates the heterotrimeric G protein complex from the GPCR as well as the G_{α} subunit from the $G_{\beta\gamma}$ heterodimer. The released G_{α} subunit binds to its effector molecules, such as adenylyl cyclase (AC) and phospholipase C (PLC), for transducing its signal (Conklin and Bourne, 1993; Wess, 1998; Gesty-Palmer and Luttrell, 2004). The signal is completed when the GTPase activity of the G_{α} subunit hydrolyzes GTP to GDP. In parallel, the activated GPCR is desensitized by phosphorylation through a G protein-coupled receptor kinase (GRK) followed by β-arrestin recruitment. The GDP-bound form of the G_{α} subunit re-associates with the $G_{\beta\gamma}$ heterodimer and an inactive GPCR (Wess, 1998).

1.2. G protein subtypes and their effectors

There are 16 known mammalian G_α subunit genes and the further existence of additional splice variants increases the amount to 20 varieties (Neves et al., 2002). It has long been believed that a given GPCR preferentially binds to a specific G_α subtype, and that this specific coupling between the GPCR and the G_α subtype is a critical determinant for its response to extracellular stimuli (Neer, 1995; Kimple et al., 2011). G_α subunits can be grouped into 4 classes based on the effectors they activate. The first class is comprised of cholera toxin sensitive G_α subunits, such as $G_{\alpha s}$ and $G_{\alpha olf}$. These G_α subunits bind to and stimulate AC for cAMP production and subsequent protein kinase A (PKA) activation (Neer, 1995; Neves et al., 2002). The second class includes pertussis toxin sensitive G_α subunits, such as $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$ as well as $G_{\alpha o}$ subunits. The activated form of these G_α subunits bind to and inhibit AC, thereby decreasing cAMP production (Gesty-Palmer and Luttrell, 2004). The third class includes $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$ and $G_{\alpha 15}$. These G_α subunits stimulate PLC to produce inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), which in turn increases intracellular Ca^{2+} by IP_3 receptors and activates protein kinase C (PKC), respectively (Berridge et al., 2000; Neves et al., 2002). The fourth group includes $G_{\alpha z}$, $G_{\alpha 12}$ and $G_{\alpha 13}$, which stimulate specific types of Rho GEFs to activate Rho small GTPase, a critical regulator for actin cytoskeleton and cell morphology (Jiang et al., 1998). Whereas most G_α subunits are expressed in various cell types, some G_α subunits, such as $G_{\alpha t}$ and $G_{\alpha gust}$, are expressed primarily in retinal cells and taste buds, respectively (Wess, 1998).

Although the G_α subunit has been a prominent focus of GPCR signaling, $G_{\beta\gamma}$ subunits, once thought merely to act as a negative regulator of GPCR signaling (Neer, 1995), are now well regarded as a contributor to multiple signal transduction pathways. $G_{\beta\gamma}$ subunits can bind to distinct effector molecules for their functions, as first discovered by $G_{\beta\gamma}$-

mediated activation of inwardly rectifying K^+ channels downstream of muscarinic acetylcholine receptor (Logothetis et al., 1987). Later studies revealed that $G_{\beta\gamma}$ subunits directly bind to and regulate some types of voltage dependent Ca^{2+} channels, AC, PLCβ, phosphatidylinositol 3-kinase (PI3K), and GRK (Clapham and Neer 1993). So far, there are 6 subtypes of G_β and 13 subtypes for G_γ, and multiple configurations of $G_{\beta\gamma}$ subunits can exist (Khan et al., 2013). Notably, it has been shown in the heterologous expression system that not all $G_{\beta\gamma}$ subunits regulate effector molecules in the same manner, as exemplified in voltage-gated Ca^{2+} channels, inwardly rectifying K^+ channels, PLC, PI3K and AC (Albert and Robillard, 2002). In addition, GPCRs coupled to the same G_α subunit may display selectivity in the $G_{\beta\gamma}$ subunits utilized for their signaling.

1.3. Adenylyl cyclase

Since AC can be regulated by both G_α subunits and $G_{\beta\gamma}$ subunits, AC may play an important role in detecting the convergence from multiple signaling pathways. AC is an enzyme that catalyzes the conversion of ATP to cAMP. ACs have been categorized by their regulators, such as Ca^{2+} and $G_{\beta\gamma}$ subunits. Among 10 AC isoforms, 9 isoforms are membrane-bound and share 60% homology. All membrane-bound AC isoforms can be activated by $G_{\alpha s}$ subunits. Forskolin can activate all these AC isoforms except AC9, which is only weakly or not at all activated by forskolin. All ACs consist of a short and variable amino-terminus, followed by two repeats of a module composed of 6 transmembrane spans (M1) and (M2) and two cytoplasmic domains termed C1 and C2 (Pierre et al., 2009) The two cytoplasmic domains form a catalytic core with two ATP binding sites for AC activity (Tesmer et al., 1997). The GTP-bound form of $G_{\alpha s}$ subunits binds to a crevice on the outside of C2 and at a second site on C1 and rearranges both domains by inducing a conformational change, promoting the C1-C2 interface to

stimulate AC activity. $G_{\alpha i}$ subunits bind to C1 domain to block the formation of the catalytic core required for AC activity, though $G_{\alpha i}$ subunits can inhibit only specific AC isoforms (see later; Tesmer et al., 1997). Besides these membrane-bound isoforms, there is one soluble AC isoform that is not activated by either $G_{\alpha s}$ or forskolin (Sadana and Dessauer, 2009).

According to major regulators, membrane-bound AC isoforms have been categorized into four groups. Group I of ACs composed of AC1, AC3, and AC8 are stimulated by Ca^{2+} in a calmodulin (CaM) -dependent manner. Group II includes AC2, AC4, and AC7, and these AC isoforms are stimulated by $G_{\beta\gamma}$ in the presence of $G_{\alpha s}$. AC5 and AC6 constitute group III, and are inhibited by Ca^{2+}. AC9 belongs to Group IV as the only isoform which is not at all or only weakly stimulated by forskolin (Sadana and Dessauer 2009; **Table 1.1**). Importantly, the level of the $G_{\alpha i/o}$-mediated inhibition of AC activity varies across AC isoforms. Thus, the strongest inhibition can be observed for group III isoforms. Group I AC isoforms can also be inhibited, but to lesser extents. In contrast, Group II AC isoforms cannot be inhibited by $G_{\alpha i/o}$ subunits.

AC regulatory properties and subcellular location							
Group	**Type**	**$G_{\alpha s}$**	**Ca^{2+}**	**$G_{\beta\gamma}$**	**$G_{\alpha i/o}$**	**Forskolin**	**Location**
1	AC1	↑	↑(CaM)	↓	↓	↑	Lipid raft
	AC3	↑	↑(CaM)	↓	↓	↑	Lipid raft
	AC8	↑	↑(CaM)	↓	↓	↑	Lipid raft
2	AC2	↑		↑		↑	Non-lipid raft
	AC4	↑		↑		↑	Non-lipid raft
	AC7	↑		↑		↑	Non-lipid raft
3	AC5	↑	↓		↓	↑	Lipid raft
	AC6	↑	↓		↓	↑	Lipid raft
4	AC9	↑	↓			Weak or N.A.	Not tested

Table 1.1. Classification of ADCY isoforms by their regulation

It is known that AC isoforms belonging to different groups have different subcellular locations. Thus, AC isoforms in Group I and Group III are localized to lipid rafts, whereas Group II AC isoforms are excluded from lipid rafts (**Table 1.1**). Since cAMP increase is thought to locally occur in the vicinity of AC, spatial separation of receptors and ACs in lipid rafts and non-lipid rafts may allow for signaling through distinct pathways, involving distinct GPCRs and AC isoforms.

1.4. GPCR oligomerization

It is now known that some GPCRs form a complex with other GPCRs. The notion that GPCRs form heteromers was initially demonstrated by the breakthrough findings of dimer formation among the class C GPCRs, such as $GABA_B$ receptors (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999) and taste receptors (Nelson et al., 2001; Zhao et al., 2003; Temussi, 2009). These pioneering works demonstrated the functional relevance of dimer formation by its requirement for receptor expression and signaling. Since then, the field has continued to expand with the additional characterization of dimer formation among class A GPCRs, demonstrated to occur in both endogenous and heterologous systems (Pin et al., 2007). While the purpose of heteromer formation in Class A GPCRs remains diverse, a frequently characterized function is that one protomer induces a change in the efficacy of the signaling of another protomer (Siehler and Milligan, 2011). This is the case for the adenosine A_{2A} receptor and the dopamine D2 receptor heteromer. In rat striatal membranes, the activation of the A_{2A} receptor reduces the ligand binding affinity of D2 receptors (Ferré et al., 1991). Similarly, the heteromer containing the serotonin 2A receptor (2AR) and the metabotropic glutamate receptor 2 (mGluR2) was revealed to contain specific interacting transmembrane domains (González-Maeso et al., 2008). Subsequently, functional

characterization of the mGluR2/2AR heteromer demonstrated that the activation of each protomer negatively regulates the G protein signaling of the other protomer (Fribourg et al., 2011).

A new functional role for heteromer formation in the activation of distinct protomer signaling pathways has been emerging from the characterization of heteromers comprising dopamine receptors (Rashid et al., 2007; So et al., 2005; Kern et al., 2012). For example, the D1-D2 heteromer can evoke G_q-mediated Ca^{2+} increase when both D1 and D2 receptors are concomitantly activated (Rashid et al., 2007; So et al., 2005;). Another study showed the D2 receptor and the ghrelin receptor, GHSR1a, form a heteromer in both an overexpression system as well as in central nervous system neurons (Kern et al., 2012). In the D2-GHSR1a heteromer, activation of D2 receptors induces a $G_{\beta\gamma}$ subunit-dependent increase in intracellular Ca^{2+} in neuroblastoma cells, only if the GHSR1a is also expressed (Kern et al., 2012). Collectively, these studies portray a novel purpose for heteromer formation, whereby the coupling of a given GPCR to a downstream effector is altered by the presence of another GPCR. However, the mechanisms for these occurrences remains elusive.

1.5. Dopamine receptors, signaling pathways and functions

Dopamine is a neuromodulator implicated in various neural functions including motor control and learning, emotion, motivation, working memory and attention (Beaulieu and Gainetdinov 2011; Missale et al., 1998). After it is released to the extracellular space, dopamine exerts its functions through binding to its cognate GPCRs (Andersen and Jansen, 1990b; Sibley and Monsma, 1992). There are two types of dopamine receptors called D1 and D2 that were originally classified according to their opposing actions on

cAMP. Later molecular cloning has identified five dopamine receptors, named D1, D2, D3, D4 and D5. Since activation of D1 and D5 receptors stimulates cAMP increase, these receptors are called D1-like receptors. D1-like receptors share a high degree of homology in their transmembrane domains and have very long carboxyl termini (Tiberi et al., 1991). In contrast, D2, D3, and D4 receptors that reduce the level of cAMP are called D2-like receptors and have shorter intracellular C-termini than D1-like receptors.

The D1-like dopamine receptors activate $G_{\alpha s/olf}$ subunits to stimulate cAMP production and subsequent activation of PKA. Then, PKA phosphorylates various ion channels and pumps as well as transcription factors such as cAMP response element binding protein (CREB) to mediate short and long-lasting actions of D1-like receptors (Svenningsson et al., 2004). The 32-kDa dopamine and cAMP-regulated phosphoprotein (DARPP-32) is also a PKA substrate that is abundantly expressed in striatal projection neurons (Svenningsson et al., 2004). Upon activation of dopamine D1 receptors, PKA phosphorylates DARPP-32 at its threonine 34 residue. This phosphorylated form of DARPP-32 inhibits the phosphatase activity of protein phosphatase-1 (PP-1). Thus, DARPP-32 acts as a positive regulator for PKA signaling downstream of D1 receptors (Hemmings et al., 1984). In contrast, D2-like receptors are coupled to $G_{\alpha i/o}$ subunits that inhibit AC, cAMP production and PKA activity along with DARPP-32 phosphorylation at its Thr 34 residue (Andersen et al., 1990a; Missale 1998; Kebabian and Greengard, 1971). Importantly, dopamine receptor signaling has since been shown to couple to other G_{α} subunits and effectors besides the originally characterized pathways mentioned above.

Dopamine receptors are highly expressed in the brain, and each receptor shows distinct distributions. The striatum shows the highest expression of dopamine receptors in the

brain. Over 90% of striatal neurons in rodents are GABAergic projection neurons called medium spiny neurons (MSN), (Gerfen, 2004). D1 receptors and D2 receptors are expressed in mostly separate populations of MSNs, the dynorphin-positive MSNs constituting the striatonigral pathway and the enkephalin-positive MSNs constituting the striatopallidal pathway, respectively. D2 receptors are also found at the presynaptic terminals of dopaminergic projections, and act as autoreceptors that negatively regulate dopamine release (Beaulieu and Gainetdinov, 2011). Dopamine receptors in the striatum including the nucleus accumbens have been implicated in locomotor activity. Pharmacological analyses showed cooperative actions of D1-like receptors and D2-like receptors in locomotor activity. Another important function of dopamine in the nucleus accumbens is its involvement in reward and reinforcement (Missale et al., 1998).

Other dopaminoceptive areas, such as the prefrontal cortex, the hippocampus and the amygdala, show expression of dopamine receptors. Both D1 and D2 receptors in the prefrontal cortex are critical for working memory functions as well as behavioral flexibility (Xu et al., 2009; Beaulieu and Gainetdinov, 2011). Recently, dopamine signaling in the prefrontal cortex has also been shown to suppress behavioral changes induced by repeated social defeat stress, such as social avoidance (Tanaka et al., 2012). As many of commercially available antipsychotics inhibit dopamine D2 receptors (Seeman et al., 1975), dopamine has been implicated in the pathophysiology of schizophrenia and mood disorders. Collectively, dopamine and dopamine receptors have many physiological and pathophysiological functions and thus finding new methods to modulate their activity is highly desired.

1.6. Prostanoid receptors and their signal transduction

It has been shown that biogenic amines, peptides, bioactive lipids, nucleotides, and hormones can modulate dopamine receptor signaling. One of these molecules is prostaglandin (PG) E_2. PGE_2 is a bioactive lipid derived from arachidonic acid by sequential actions of cyclooxygenases and PGE synthase (Narumiya and Furuyashiki, 2011; **Figure 1.1**). PGE_2 binds to its four cognate GPCRs, EP1, EP2, EP3 and EP4 for its functions. EP1 is mainly coupled to intracellular Ca^{2+} increase and induces smooth muscle contraction. EP2 and EP4 receptors are both mainly coupled to $G_{\alpha s}$ and stimulate cAMP production and relax smooth muscles. EP3 receptors are mainly coupled to inhibitory cAMP response through $G_{\alpha i/o}$ (**Figure 1.1.**), but alternative splicing isoforms of EP3 can be coupled to other signaling pathways such as stimulatory cAMP response (Hirata and Narumiya, 2011).

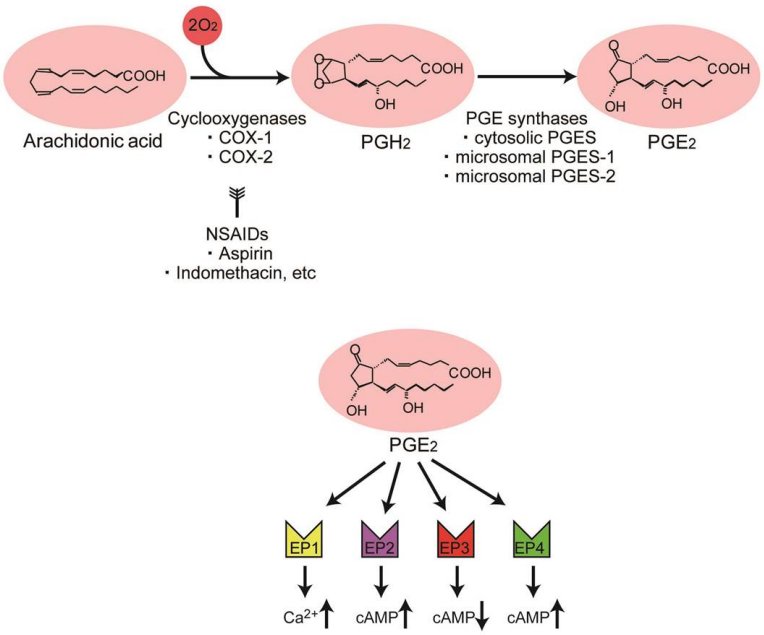

Figure 1.1. PGE_2 synthetic pathway and receptor subtypes

PGE_2 and its receptors exert a wide range of physiological and pathophysiological actions on the body, ranging from inflammation and immune response as well as cardiovascular and bone homeostasis. Given the action of non-steroidal anti-inflammatory drugs (NSAIDs) to block PGE_2 synthesis, PGE_2 in the brain has been implicated in sickness-associated symptoms, such as fever, glucocorticoid release, anorexia and pain. Systemic symptoms of acute inflammation, such as fever, can be induced using the bacterial endotoxin lipopolysaccharide (LPS) and proinflammatory cytokines like interleukin (IL)-1β. Prostanoid receptor deficient mice were examined under these conditions and only EP3 deficient mice displayed reduced febrile responses (Ushikubi et al., 1998; Lazarus et al., 2007). EP3 receptors expressed in the GABAergic neurons of the hypothalamus were shown to be the site of action for PGE_2-EP3 actions in fever generation (Nakamura et al., 2002). LPS also activates the hypothalamic pituitary adrenal (HPA) axis. HPA activation measured by adrenocorticotropic hormone release from the pituitary has been shown to be impaired in EP1 and EP3 deficient mice through distinct mechanisms (Matsuoka et al., 2003).

A previous study using knockout mice serendipitously found that PGE_2-EP1 signaling regulates dopamine-related behaviors without apparent inflammation (Matsuoka et al. 2005). Later studies have identified two different actions of EP1 on the dopaminergic system (Kitaoka et al., 2007; Tanaka et al. 2009; Tanaka et al. 2012). First, EP1 suppresses dopaminergic activity through augmenting inhibitory synaptic inputs to dopamine neurons. Consistent with this action, EP1-deficient mice showed behavioral abnormalities due to hyperdopaminergic activity, such as impulsive behaviors under acute exposure to environmental and social stressors and the lack of behavioral depression induced by repeated stress. Second, EP1 augments dopamine receptor signaling in striatal

neurons. This EP1 action was originally suggested by a behavioral finding that EP1 deficient mice showed reduced hyperlocomotion induced by cocaine and D1 receptor agonists (Kitaoka et al., 2007). Consistently, EP1 and D1 receptors are co-expressed in striatal neurons, and PGE_2-EP1 signaling augments D1-induced phosphorylation of DARPP-32 at its Thr34 residue in striatal slices.

Surprisingly, EP1 is also expressed with D2 in striatal neurons, and genetic deletion of EP1 impairs the action of D2 agonists in reducing PKA-mediated phosphorylation of DARPP-32 in striatal slices as well as in inducing catalepsy in mice. These findings suggest that EP1 activation increases cAMP production in concert with D1, but facilitates D2-mediated decrease in cAMP production. However, the mechanism underlying these apparently opposing EP1 actions remains unclear. To address this issue, I hypothesize that either D1 or D2 receptors may form a complex with EP1 receptors to allow for distinct types of regulation by EP1.

1.7. Aims of thesis

The aims of this thesis is (1) to evaluate the *in vivo* characterized role for EP1 receptors as a regulator of dopamine signaling using the *in vitro* heterologous expression system of HEK-293T cells, and (2) to determine the molecular mechanism of EP1 receptor mediated effects on dopamine receptor signaling. Here, I demonstrate a newly identified heteromer composed of D1 and EP1 receptors. In this heteromer, the activation of EP1 facilitates D1 receptor signaling, promoting its maximal cAMP response. I further demonstrate that EP1 activation directs the D1 receptor through $G_{\beta\gamma}$ subunits, thereby coupling it to AC7, an AC isoform distinct from those preferentially utilized in D1 alone signaling.

Materials and Methods

Molecular cloning and plasmids

For recombinant DNA expression in mammalian cells, I made an expression vector containing the mouse dopamine D1 receptor with several modifications; at its N-terminus the signal sequence (SS) derived from influenza hemagglutinin (HA) was added to promote its membrane insertion (Guan et al., 1992) followed by the FLAG peptide, to identify the construct in subsequent analyses. First, the open reading frame for enhanced green fluorescent protein (EGFP) was removed by digesting the back-bone vector, pEGFP-C1 (Clontech, Mountain view, CA), with AgeI and BsrGI. Ligation with insert oligo DNA, containing the AgeI site, the Kozak sequence, SS, the FLAG peptide, and the BsrGI site (5' – ACC GGT CCA CCA TGA AGA CGA TCA TCG CCC TGA GCT ACA TCT TCT GCC TGG TAT TCG CCG ACT ACA AGG ACG ATG ATG ACG CCT GTA CA – 3'), and the digested pEGFP-C1 produced a new plasmid, named pSS-FLAG-C1. The D1 receptor cDNA from pBluescript KS(+) containing the open reading frame of mouse D1 receptor (RIKEN, Saitama, Japan) was modified from the second amino acid by PCR attachment of the restriction sited for BsrGI and PstI at its N and C termini, respectively. The primers used are as follows, the forward primer contains the BsrGI site and the N-terminal portion of D1 receptor (5' – TAA TGT ACA GCT CCT AAC ACT TCT ACC ATG G – 3'). The reverse primer contains the C-terminal portion of D1 receptor and the PstI site (5' – CGT TTC TGC AGA ACC CAA TAT TCA GGT TGA ATG CTG – 3'). The PCR product and its destination vector, pSS-FLAG-C1, were digested with BsrGI and PstI, ligated and termed pSS-FLAG-mD1.

To overexpress the human β2-adrenergic receptor (ADRB2), I obtained cDNA encoding ADRB2 attached with BsrGI and HindIII sites from total RNA of HEK-293 cells by RT-

PCR. The following primers were used; the forward primer containing the BsrGI site and the N-terminal portion of ADRB2 sequence (5' – ATC TAG TGT ACA GGG CAA CCC GGG AAC GGCA – 3'). The reverse primer containing the C-terminal portion of ADRB2 sequence and the HindIII site (5' – AGT ATT AAG CTT TTA CAG CAG TGA GTC ATT TGT ACT ACA – 3'). This PCR product along with the destination vector, pSS-FLAG-C1, was digested with BsrGI and HindIII, ligated and termed pSS-FLAG-hADBR2.

To overexpress the mouse EP1 receptor, I added the SS and the HA peptide to its N-terminus by the following procedure. Digestion of pEGFP-C1 with NheI and AgeI resulted in a fragment that was fused to oligo DNA containing the NheI site, the Kozak sequence, the HA peptide sequence, and the AgeI site (5' – GCT AGC CCA CCA TGA AGA CGA TCA TCG CCC TGA GCT ACA TCT TCT GCC TGG TAT TCG TAT CCT TAC GAC GTT CCG GAC TAC GCA ACC GGT – 3'). The resultant plasmid was termed pSS-HA-EGFP-C1. Then, the open reading frame of EP1 from the plasmid for EP1 expression (Watabe et al., 1993) was attached by PCR from the second amino acid to AgeI and KpnI at its N and C termini, respectively, using the two following primers; the forward primer containing the AgeI site and the N-terminal portion of the EP1 sequence (5' – ATC GAA CCG GTA GCC CCT GCG GGC TTA A – 3') and the reverse primer containing the C-terminal portion of the EP1 sequence and the KpnI site (5' – GCT CAC CAT GGA GGC ACA GTC GAG GCT G – 3'). This PCR product and its destination vector, pSS-HA-EGFP-C1, was digested with AgeI and Acc65I, ligated and termed pSS-HA-mEP1.

Plasmids for recombinant DNA expression of the αl subunit of transducin ($G_{t\alpha}$), the β1

subunit of G protein (G_β), and the γ2 subunit of G protein (G_γ, transcript variant 1) under the cytomegalovirus promoter were purchased from OriGene Technologies (Rockville, MD). The recombinant DNA expression vector for the mouse EP2 receptor was obtained by inserting the open reading frame of EP2 into the EcoRI site of pCMS-EGFP (Clontech, Mountain View, CA) (Matsuoka et al., 2003).

Sequences were confirmed for all open reading frames of the above plasmids by conventional DNA sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA).

Maintenance of cell culture and transfection

Human embryonic kidney (HEK) 293T cells (ATCC, Manassas, VA) were cultured on Type I collagen-coated dishes (Iwaki Glass, Tokyo, Japan), except for immunofluorescence experiments where cells were cultured directly on coverslips. The cells were maintained in Dulbecco's Modified Eagle's Medium (D-MEM, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (PAA, Etobicoke, Ontario) in an atmosphere maintained with 5% CO_2 at 37^{o}C.

Transfection of plasmid DNA to HEK-293T cells with Effectene was carried out for all procedures (QIAGEN, Alameda, CA), except for immunoprecipitation (**Figure 3.6. A**), calcium measurement (**Figure 3.8. A**), radioligand binding assay (**Figure 3.7.**), and subcellular fractionation (**Figure 3.13.**) in which FuGENE HD was used instead (Promega, Madison, WI). Following DNA transfection, cells were maintained for 24-48 hrs before use in each experiment. For siRNA transfection, Lipofectamine RNAiMAX (Life Technologies, Carlsbad CA) was used and cells were cultured for 48 hrs before use.

For siRNA experiments targeting human AC isoforms (AC5, AC6 and AC7), pre-validated mixture of multiple siRNAs targeting the indicated isoforms (MISSION esiRNA) were purchased from Sigma Aldrich (St. Louis, MO). For negative control siRNA, stealth RNAi siRNA negative control Med GC (Life Technologies) was used in all siRNA experiments. To evaluate the knockdown efficiency using these siRNAs quantitative RT-PCR was carried out (**Figure 3.11. A**).

Pharmacological treatment

The following sources were used to obtain the drugs used in this study: ONO-DI-004 and ONO-AE1-259 (are both kind gifts from ONO Pharmaceuticals, Osaka, Japan); Forskolin (Tocris Bioscience, Bristol, UK); (-)-Isoproterenol and SKF-81297 (Sigma-Aldrich, St. Louis, MO); SKF-83822 (a kind gift from National Institute of Mental Health, Bethesda, MD).

Descriptions for the condition of various pharmacological treatments in this study are as follows. After cells are washed in Leibovitz's L-15 medium (Life Technologies) pre-warmed to 37˚C without serum, the appropriate agonists or their vehicles diluted in L-15 medium are applied to the cells and then incubated at 37^{o}C for 5 min. For intracellular Ca^{2+} blockade (**Figure 3.8**), cells were incubated in L-15 medium containing 10 μM BAPTA-AM (Life Technologies, Carlsbad, CA) or its vehicle (DMSO) for 30 min at 37^{o}C prior to agonist stimulation. For the other method of Ca^{2+} blockade, the cells were incubated in L-15 medium containing 1 mM EGTA and 100 nM thapsigargin (Life Technologies) for 30 min at 37^{o}C. To block G_i signaling (**Figure 3.10**), cells were incubated in serum-free D-MEM supplemented with pertussis toxin (0.2 μg/ml; List Biological Labs, Campbell, CA) or its vehicle in a humidified incubator at 37^{o}C with 5%

CO_2 for overnight.

cAMP experiments

Cells plated on collagen I-coated 24 well dishes were used 24-36 hrs following transfection for all cAMP assays. Following removal of the L-15 medium containing agonists or vehicle, cells were immediately lysed with 0.1 M HCl and the plates were rotated for 20 min at room temperature (RT). Cell lysis was checked under a microscope and the supernatants were collected to 1.5 ml tubes on ice. The cAMP containing supernatants were diluted five times with the EIA buffer provided by the cAMP EIA kit (Cayman, Ann Arbor, MI) and cAMP measurement was carried out according to the manufacturer's protocol. cAMP values are normalized to the maximal level of cAMP induced by forskolin (10 μM), except for the experiments with AC siRNAs (**Figure** 3.11 and **Figure** 3.12). Since forskolin-induced cAMP response is affected differently by distinct AC isoforms, either the absolute concentration of cAMP in supernatants (**Figure 3.11.**) or those normalized to the cAMP level induced by 500 nM SKF-83822 (**Figure 3.12.**) was used for data analyses in these experiments.

Calcium measurement

Intracellular calcium concentration was measured using the Fluo-4-AM Direct Calcium Assay Kit (Life Technologies) according to the manufacturer's protocol. Briefly, cells that had been grown on collagen I coated black 96 well plates were incubated in calcium assay buffer (Hank's Balanced Salt Solution containing 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.1% BSA and 2.5 mM Probenecid) containing Fluo-4-AM for 30 min at 37^{o}C. The fluorescent signals were measured with a fluorescent microplate reader (FlexStation 3, Molecular Devices, Sunnyvale, CA) at excitation of 494 nm and emission of 516 nm.

Immunofluorescence

Confocal microscopy was employed to examine the immunofluorescence of EP1 and D1 receptor expressing cells. The protocol for immunostaining was carried out as previously described (Matsuoka et al., 2003). In brief, cells were plated and maintained on coverslips of 12 mm in diameter (Thermo Fisher Scientific, Waltham, MA, USA). Following several washes in Dulbecco's modified phosphate buffered saline (D-PBS, Nissui Pharmaceuticals, Tokyo, Japan), cells were fixed with D-PBS containing 4% paraformaldehyde (Polysciences, Warrington, PA) for 30 min at RT. Next the cells were permeabilized in blocking buffer (D-PBS containing 2% goat serum, 1% bovine serum albumin, 0.01% Triton X-100 and 0.05% Tween-20) for 60 min at RT. First antibodies, namely rat anti-HA antibody (1:1,000 dilution; 3F10, Roche Diagnostics, Indianapolis, IN) and mouse anti-FLAG M2 antibody (1:1,000 dilution; Sigma-Aldrich, St. Louis, MO), were diluted in blocking buffer, and cells were incubated for overnight at 4˚C with both of these antibodies. The next day cells were washed for 5 min each, three times, in D-PBS containing 1% Tween-20. Cells were incubated with appropriate secondary antibodies conjugated with Alexa488 or Alexa555 (Life Technologies) at 1:200 dilution in blocking buffer for 2 hrs at RT. Finally, the cells attached onto cover slips were briefly washed in D-PBS and mounted with Prolong Gold Antifade Reagent (Life Technologies). Fluorescent images were acquired by TCS-SP5 confocal microscopy (Leica, Microsystems, Nussloch, Germany) and processed using Image J software (NIH Betheseda, MD) or Photoshop (Adobe, San Jose, CA) for illustrative purposes only.

Immunoprecipitation

Cells plated onto collagen-I coated 6 well plates were maintained for 24-48 hrs after transfection. Then the cells were collected, re-suspended, and solubilized in

immunoprecipitation buffer (50 mM Tris-HCl pH 6.8, 150 mM NaCl, 1 mM EDTA, and 1% Fos-Choline-14 (n-Tetradecylphosphocholine)) with protease inhibitors (Complete Mini, EDTA- free; Roche Diagnostics, Indianapolis, IN) for 20 min at 4˚C, followed by brief sonication on ice. Lysates were centrifuged at 12,000 x g for 10 min at 4˚C and FLAG-tagged D1 receptors (**Figure 3.6. B**), were precipitated by applying lysates to anti-FLAG M2 antibody-conjugated beads (Sigma-Aldrich) according to the manufacturer's protocol. Then, precipitated proteins were eluted in 2 x SDS sample buffer (125mM Tris HCl pH 6.8, 20% Glycerol, 4%SDS, 0.02% BPB, 50 mM dithiothreitol (DTT)). EP1 receptor immunoprecipitation (**Figure 3B**) was carried out in cell lysates that were pre-cleared at 4˚C for 10 min using a 50% slurry of Protein A/G beads (Cytosignal, Irvine, CA). Beads were pelleted by 1 min centrifugation, 1000 x g, and the protein concentration of the pre-cleared lysate was determined by the BCA method (Thermo Fisher Scientific, Rockford, IL). The pre-cleared cell lysate of 1 ml containing 500 µg protein was incubated overnight with rabbit anti-EP1 antibody (0.5 mg/ml; Cayman Chemical, Ann Arbor, MI). The immune complex was precipitated with the EP1 antibody by the Protein A/G Magnetic IP kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Precipitated proteins were eluted in the sample buffer provided by the kit supplemented with 50 mM DTT, just prior to use. The precipitated samples were incubated at 4˚C for 40 min, then at RT for 10 min, and the beads were collected by magnetic separation prior to Western blotting.

Western blotting

Protein samples dissolved in SDS sample buffer or 6 x Laemmili buffer (sucrose density experiments) were loaded onto a 10% polyacrylamide gel (Atto, Tokyo, Japan) and were immediately submitted to SDS-PAGE. The proteins were transferred from the gel onto a

0.45-μm polyvinylidene fluoride membrane (Millipore, Milford, MA) by the semi-dry transfer method (Atto). Following transfer, non-specific binding was blocked by incubating the blot for 30 min at RT in Tris-buffered saline (TBS; 50 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing 3% skim milk (Difco). The membranes were incubated overnight at 4˚C in a hybridization bag with the specified primary antibody diluted in 3% skim milk/TBS blocking buffer. Antibodies used are at the following dilutions; rabbit anti-EP1 antibody (1:100 dilution; Cayman Chemical), rat anti-D1 antibody (1:1,000 dilution; D2944, Sigma-Aldrich), mouse anti-HA antibody (1:1,000 dilution; clone HA.11; Covance, Princeton, NJ), rabbit anti-ACV/VI antibody (1:200 dilution; Santa Cruz Biotechnology, Dallas, TX), rabbit anti-Flotillin-1 antibody (1:1,000 dilution; Cell Signaling, Danvers, MA) and mouse anti-GAPDH antibody (1:3,000 dilution; clone 6C5, Ambion, Carlsbad, CA). The next day, following three washes in TBS for 10 minutes each, the membrane was incubated with an horseradish peroxidase-conjugated secondary antibody corresponding to the IgG of the appropriate species (1:5,000 dilution; GE Healthcare Biosciences, Pittsburgh, PA) in 3% skim milk/TBS for one hour at RT. After three washes in TBS for 10 minutes each, the membrane was subjected to detection with ECL Plus or ECL Prime (GE Healthcare Biosciences). Images of Western blots were captured following exposures with standard X-ray film or with the ChemiDOC XRS chemiluminescence imager (Bio Rad).

Radioligand binding assay

Saturation binding analyses was performed for D1 receptors. The indicated agonists were added to the cells expressing EP1 and D1 receptors for five minutes. Then, the cells were used to isolate membrane fractions for subsequent use used in binding assays. Briefly, a cell scraper was used to collect the cells. Brief centrifugation was used to pellet the cells and the cell pellet was re-suspended in binding assay buffer (5 mM Tris-HCl, 100 mM

NaCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4 and protease inhibitors). Next, cells were homogenized by passage through a 1 ml syringe with a 26 gauge needle on ice 5 times. The homogenates were briefly sonicated and centrifuged at 3,000 x rpm for 5 minutes at 4˚C. Finally, the supernatants were ultracentrifuged at 43,000 x rpm for 60 minutes at 4˚C. The supernatant was discarded and the membrane-containing pellet was re-suspended in binding assay buffer. The BCA assay (Pierce) was used to measure the protein concentration of the re-suspended membrane fraction. For saturation binding analyses, the scintillation proximity assay (SPA) method was used. Then 10 μg membranes, 0.5 mg of wheat germ agglutinin (WGA) coated scintillation beads (Perkin Elmer, Wellesley, MA), and the indicated amounts of [^{3}H]-SCH-23390, a radiolabeled D1 receptor antagonist (Perkin Elmer), were diluted in binding assay buffer in a 96-well opaque Opti-Plate (Perkin Elmer). The reactions were incubated for one hour at 25˚C, and then scintillation signals were measured with the TopCount liquid scintillation reader (Perkin Elmer, Wellesley, MA). Non-specific binding was assessed by addition of excess unlabeled D1 ligands, either SCH-23390 (Sigma Aldrich, St. Louis, MO) or Butaclamol (Sigma Aldrich).

Quantitative RT-PCR

Following transfection, total RNA was extracted from HEK-293T cells using TRIzol (LifeTechnologies) according to the manufacturer's protocol. Next, cDNA was generated from the obtained total RNA with reverse transcriptase polymerase chain reaction (RT-PCR) using PrimeScript Reverse Transcriptase (TaKaRa Bio, Otsu, Shiga, Japan). The subsequent cDNA was mixed with SYBR Premix ExTaq kit (TaKaRa Bio) and appropriate primers, and quantitative PCR was performed with Thermocycler C1000

(BioRad, Hercules, CA). The sequences of the primers used in this study are commercially available (OriGene).

Subcellular fractionation

To separate lipid rafts from non-lipid rafts, I carried out subcellular fractionation using sucrose density gradients, as previously described (Ostrom and Liu, 2007). In brief, I collected the transfected cells from three confluent 10 cm dishes in 500 mM sodium bicarbonate buffer, pH 11 in the presence of protease inhibitors. Next, keeping the samples on ice, I homogenized the cells with a Dounce homogenizer followed by three repetitions of brief sonication with 1 minute rest in between each repetition. Then, I mixed 2 ml of homogenates with 2 ml of 90% sucrose in MES-buffered saline (MBS; 25 mM MES, 150 mM NaCl, pH 6.5) for a final sucrose concentration of 45%. This homogenate was overlaid with 4 ml of 35% sucrose followed by 4 ml of 5% sucrose, and was centrifuged at 37,000 rpm in a SW41 Ti rotor at 4^{o}C for 20 hours. After centrifugation, 1 ml fractions were collected from the top to the bottom, resulting in 12 fractions in total. Protein concentration was measured using BCA assay or microBCA assay (Pierce). 6 x sample buffer solution containing reducing reagents (09499-14, Nacalai Tesque, Kyoto, Japan) was added to solubilize the fractions which were subsequently boiled at 95˚C for 5 minutes. The samples were spun-down before taking 18 μl of each fraction for loading onto 10% SDS-PAGE, and then subjected to Western blotting, as described above.

Statistical analyses

GraphPad Prism 5 was used for statistical analyses (GraphPad Software, La Jolla, CA). Unpaired *t*-tests were performed for pairwise comparisons. One-way analysis of variance

and two-way analysis of variance were performed for comparisons across three groups or larger and for two-factor analyses, respectively. Analyses were followed by post hoc multiple comparison tests (Newman-Keuls or Bonferroni). For saturation binding experiments, scatchard plot analyses were performed and transforms were plotted to linear regression curve fit. Statistical significance was considered as *P* values less than 0.05. The data in this study are shown as means ± S.E.M.

Results

Activation of EP1 enhances cAMP production induced by D1 receptors in HEK-293T cells

Through overexpression of both D1 and EP1 receptors in human embryonic kidney (HEK) 293T cells, I addressed the mechanism of how EP1 receptor activation enhances dopamine D1 receptor signaling in striatal neurons (Kitaoka et al., 2007). Importantly, HEK-293T cells do not endogenously express either D1 or EP1 receptors. First, to examine the effect of EP1 on cAMP production induced by D1 receptor stimulation, an EP1-specific agonist, ONO-DI-004, was used. First, a dose-dependent increase in cAMP production was observed when cells were treated with SKF-81297, a D1 receptor agonist, at 30, 100 and 500 nM for 5 minutes (**Figure 3.1 A**). cAMP production induced by SKF-81297 was then significantly enhanced in a dose-dependent manner by the concomitant application of ONO-DI-004, an EP1 receptor-specific agonist, at 1 and 10 μM (**Figure 3.1 A**). This result suggests that, in a cell-autonomous manner, EP1 receptor mediates facilitation of D1 receptor signaling. Notably, EP1 receptor activation alone cannot induce cAMP production, as no significant increase in the level of cAMP was produced by treatment with ONO-DI-004, in the absence of SKF-81297 (**Figure 3.1 A**).

Among D1 receptor agonists, two have been shown to exert distinct signaling pathways for the D1 receptor: SKF-81297 increases not only cAMP production but also phosphoinositide hydrolysis through binding to the D1-D2 heterodimer, whereas SKF-83822 only stimulates cAMP production through the D1 homodimer (Verma et al., 2010). Therefore, whether EP1 activation could enhance cAMP production induced by SKF-83822 was next examined. In cells expressing EP1 and D1 receptors, treatment with SKF-83822 at 100 and 500 nM for 5 minutes produced an increase in cAMP levels (**Figure 3.1**

B). These cAMP responses are considered to be saturated because the cAMP responses at these two concentrations are similar. Interestingly, the saturated cAMP responses were further enhanced by simultaneous treatment with EP1 agonist, ONO-DI-004 (**Figure 3.1 B**). These results suggest that the maximal level of D1 receptor signaling is increased upon EP1 receptor stimulation in HEK-293T cells.

Next, cells expressing either D1 or EP1 receptors alone were analyzed to validate the specificity of SKF-83822 and ONO-DI-004. Treatment with the EP1 agonist, ONO-DI-004 at 10 μM failed to affect D1-induced cAMP response in D1 receptor alone expressing HEK-293T cells (**Figure 3.1 C**). In parallel, treatment with the D1 receptor agonist, SKF-83822, at 100 or 500 nM lacked an effect on cAMP production in cells that express EP1 receptors alone (**Figure 3.1 D**). Taken together, in these experimental conditions, the effects of ONO-DI-004 and SKF-83822 are specific to EP1 and D1 receptors, respectively.

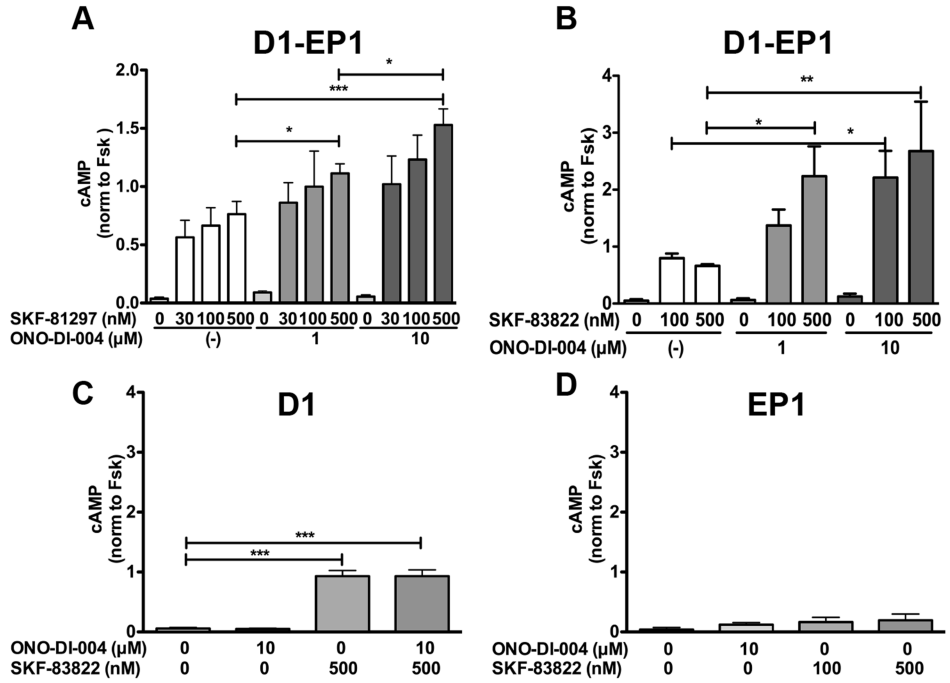

Figure 3.1. (Ehrlich et al., 2013). In HEK-293T cells, EP1 receptor activation enhances D1 receptor induced cAMP production. (A, B) EP1 receptor activation mediated enhancement of D1 receptor-induced cAMP response. HEK-293T cells overexpressing D1 and EP1 receptors ("D1-EP1") were treated for 5 minutes with either a D1 agonist (SKF-81297 in (A) and SKF-83822 in (B)) in the presence or absence of an EP1 agonist (ONO-DI-004) as indicated. Intracellular cAMP levels were determined using a cAMP enzyme immunoassay and normalized to the cAMP level induced by 10 μM forskolin (Fsk). n = 5-6 for each column in (A) and n = 4-5 for each column in (B). (C) In D1 receptor alone expressing cells ONO-DI-004 lacks an effect on D1-induced cAMP response. As indicated, either SKF-83822, ONO-DI-004 or both agonists were added to the HEK-293T cells overexpressing D1 receptors alone ("D1"). cAMP levels were determined as described above. n = 6 for each column. (D) Cells which do not overexpress D1 receptors lack an effect of SKF-83822 and ONO-DI-004 on the cAMP level. As indicated, SKF-83822, ONO-DI-004, or both agonist treatments were applied to HEK-293T cells overexpressing EP1 receptors alone ("EP1"). cAMP levels were determined as above. n = 3 for each column. * P < 0.05, ** P < 0.01, *** P < 0.001 for Newman-Keuls multiple comparison tests following one-way analysis of variance.

EP1 receptor actions are specifically coupled to D1 receptor signaling

Whether EP1 activation enhances any $G_{\alpha s}$-induced cAMP response regardless of the type of stimulated GPCR from which $G_{\alpha s}$ is activated was examined next. For this purpose, two GPCRs derived from distinct families but which share the same cognate G protein as D1 receptors, $G_{\alpha s}$, was employed. The action of EP1 on cAMP production induced by EP2, another subtype of prostaglandin E receptors, was examined first. Maximal increase

in cAMP production was induced by addition of ONO-AE1-259, an EP2 receptor-specific agonist, at 0.5 and 1 μM in HEK-293T cells overexpressing both EP1 and EP2 receptors (**Figure 3.2. A**). Simultaneous application of EP1 receptor agonist, ONO-DI-004, at 10 μM failed to induce an effect on EP2 receptor mediated cAMP responses (**Figure 3.2. A**). Next, the action of EP1 on the β2-adrenergic receptor (ADRB2) induced cAMP response was examined. The ADRB2 agonist, isoproterenol, at 100 and 500 nM dose-dependently increases cAMP production in HEK-293T cells that overexpress both EP1 and ADRB2 receptors (**Figure 3.2. B**). The concomitant application of EP1 agonist, ONO-DI-004, at 10 μM did not augment the ADRB2 induced cAMP responses (**Figure 3.2. B**). Therefore, rather than facilitating $G_{\alpha s}$-stimulated cAMP production in general, activation of the EP1 receptor specifically augments D1 receptor signaling.

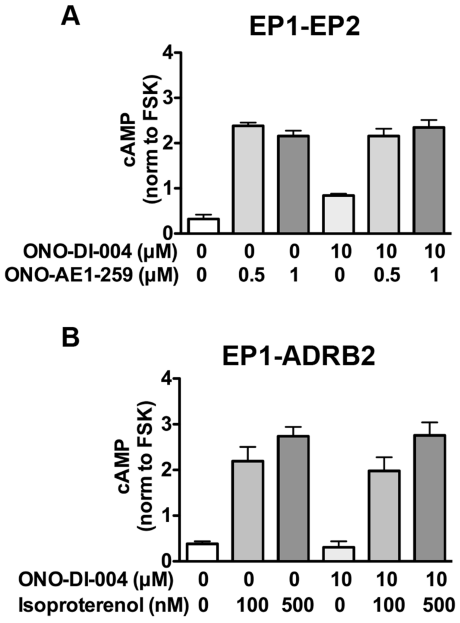

Figure 3.2. (Ehrlich et al., 2013). cAMP response induced by EP2 or ADRB2 receptors were not affected by EP1 receptor activation in HEK-293T cells. (A) The EP2 receptor-induced cAMP response lacks an effect of EP1 receptor activation. HEK-293T cells overexpressing EP1 and EP2 ("EP1-EP2") receptors were treated with ONO-DI-004, an EP1 agonist, or ONO-AE1-259, an EP2 agonist, or both as indicated. The cAMP levels were determined and analyzed as described in the legend of Figure 1. n = 3-4 for each column. (B) cAMP response induced by ADRB2 lacks an effect of EP1 receptor activation. HEK-293T cells overexpressing EP1 and ADRB2 ("EP1-ADRB2") were treated with either ONO-DI-004 or Isoproterenol, an agonist for β-adrenergic receptors, or both as indicated. n = 3-5 for each column. Note that treatment with ONO-DI-004 did not exert an effect on cAMP responses induced by either EP2 or ADRB2 receptors.

Heteromer formation by EP1 and D1 receptors in HEK-293T cells

I suspected that EP1 receptors form a heteromer with D1 receptors, given that the stimulatory action of EP1 is specific to D1 receptors. I used anti-hemagglutinin (HA) antibody and anti-FLAG antibody for detection of HA-tagged EP1 and FLAG-tagged D1, respectively, to examine whether these receptors co-localize in HEK-293T cells by immunofluorescent staining. First, to confirm that anti-HA antibody and anti-FLAG antibody selectively recognize HA-tagged EP1 and FLAG-tagged D1, respectively, I examined the cells that express either HA-tagged EP1 or FLAG-tagged D1 (**Figure 3.3.**).

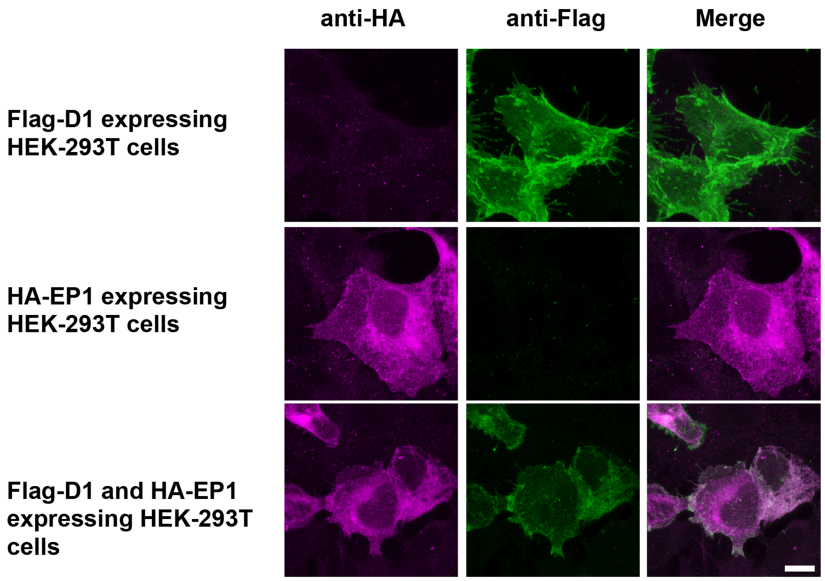

Figure 3.3. (Ehrlich et al., 2013). Immunostaining for validation of the specificity for antibodies detecting FLAG-tagged D1 and HA-tagged EP1 receptors in HEK-293T cells. Fluorescent immunostaining was carried out with anti-FLAG antibody and anti-HA antibody in HEK-293T cells overexpressing either FLAG-tagged D1 or HA-tagged EP1, or the two combined. Signals with anti-FLAG antibody (green) and those with anti-HA antibody (magenta) are shown. Shown at the bottom, is the same image of cells expressing both FLAG-tagged D1 and HA-tagged EP1 as used in Figure 3.4. Scale bar, 10 μm.

Whereas D1 receptor signals were localized mostly at the cell periphery (**Figure 3.4.** top) EP1 receptor signals were observed both at the periphery and around the nucleus in the cells that are overexpressing both EP1 and D1. A considerable proportion of these signals co-localized, as observed by the punctate signals of EP1 and D1 receptors along the edge of the cell at a higher magnification (**Figure 3.4.** bottom). These data also suggest that each of these receptors are present either alone or in complex with each other, as not all D1 signals co-localized with EP1 signals, and vice versa.

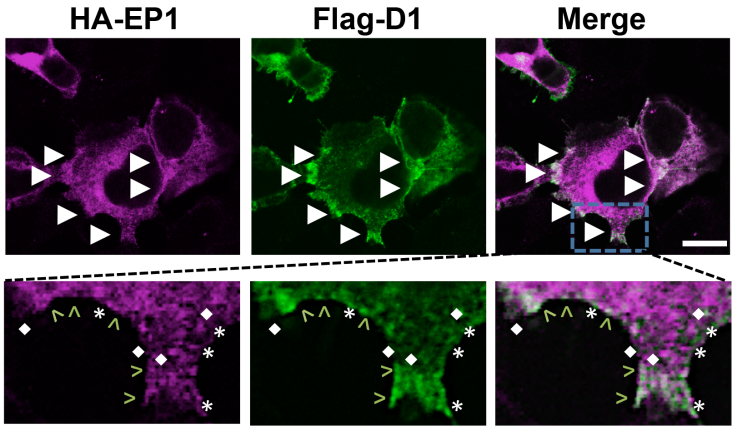

Figure 3.4 (Ehrlich et al., 2013). In HEK-293T cells, EP1 and D1 receptors colocalize and form a heteromeric complex. (A) Fluorescent immunostaining using anti-HA antibody (magenta) and anti-FLAG antibody (green) to examine the localization of HA-tagged EP1 receptors and FLAG-tagged D1 receptors, respectively, EP1 and D1 receptor expressing HEK-293T cells. Representative images are shown at the top, and a dotted square indicates the area that is magnified and shown at the bottom. Arrowheads indicate colocalization of heteromeric EP1 and D1 receptors. White diamonds indicate signals for EP1 that are not colocalized with D1 receptors. Asterisks indicate signals for D1 receptors that are not colocalized with EP1 receptors. Scale bar, 10 μm for top images and 5 μm for bottom images.

Next I examined whether these receptors can be coprecipitated to directly analyze the complex formation of EP1 and D1 receptors. Although signals for EP1 or those for D1 receptors were detected selectively in cell lysates expressing the corresponding receptors, multiple bands were present (**Figure 3.5.**). I therefore precipitated EP1 receptors with anti-EP1 antibody that recognizes the C terminus and detected it with anti-HA antibody that recognizes the N terminus to identify specific bands corresponding to the intact HA-

tagged EP1 (**Figure 3.5, A and B**). Similarly, I precipitated D1 receptor with anti-FLAG antibody that recognizes the N terminus and detected it with anti-D1 antibody that recognizes the C terminus to identify specific bands corresponding to intact FLAG-tagged D1 receptors (**Figure 3.5, C and D**). The bands identified by this method were then used in my subsequent analyses.

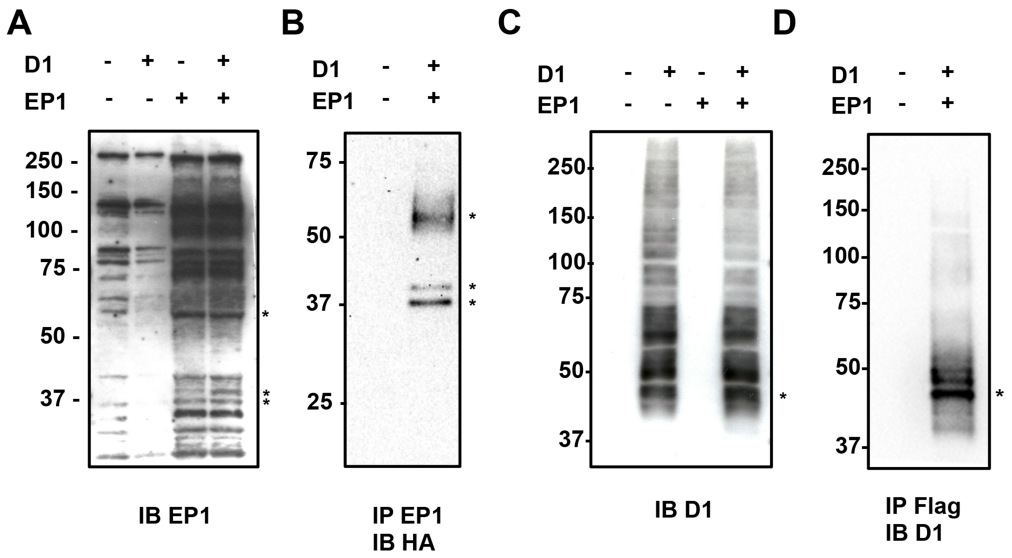

Figure 3.5. (Ehrlich et al., 2013). Western blotting to detect HA-tagged EP1 and FLAG-tagged D1 receptor signals. Whole cell lysates were prepared from HEK-293T cells expressing either HA-tagged EP1 or FLAG-tagged D1, or both, as indicated. In (A) and (C), Western blotting was carried out for the lysates (IB) with anti-EP1 antibody and anti-D1 antibody, respectively. In (B) and (D), proteins were immunoprecipitated (IP) with either anti-EP1 antibody or anti-FLAG antibody to identify which band(s) contains intact HA-tagged EP1 or FLAG-tagged D1 receptors, respectively, and were subjected to Western blotting with anti-HA antibody or anti-D1 antibody. HA and FLAG tags are located at the N termini of EP1 and D1, whereas anti-EP1 antibody and anti-D1 antibody recognize the C termini of these receptors. Not all EP1 and D1 receptor bands that were detected in the whole cell lysates (B and D) were precipitated with anti-HA antibody and anti-FLAG antibody, respectively (A and C). The intact receptors detected in precipitated samples in (B) and (D) are also the corresponding bands in whole cell lysates in (A) and (C), as highlighted by asterisks.

EP1 receptor specific antibodies immunoprecipitated D1 receptors, only when both EP1 and D1 receptors are overexpressed in HEK-293T cells (**Figure 3.6. A**). Conversely, FLAG antibodies recognizing the D1 receptor immunoprecipitated EP1 receptors only in the presence of both EP1 and D1 receptors (**Figure 3.6. B**). Taken together, in HEK-293T cells, EP1 and D1 receptors form a heteromeric complex.

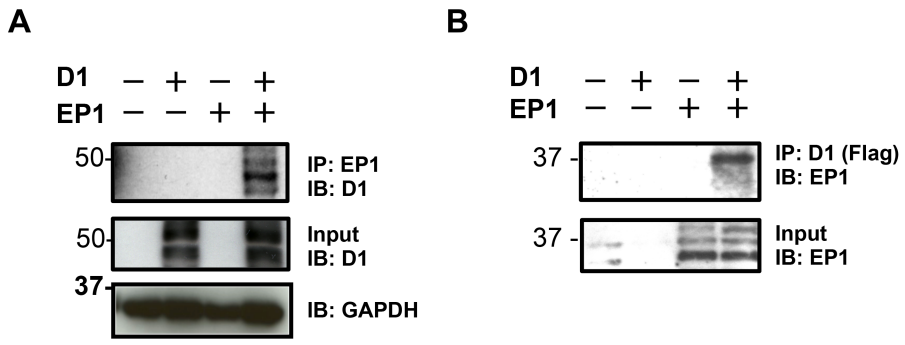

Figure 3.6. (Ehrlich et al., 2013). EP1 and D1 receptors coprecipitate. Immunoprecipitation (IP) was performed with cell lysates from HEK-293T cells that overexpressed either D1 or EP1 receptors or both as indicated. (A), EP1 receptors were precipitated with anti-EP1 receptor antibody, and the precipitated proteins (top) and total cell lysates (middle) were subjected to immunoblotting (IB) with anti-D1 receptor antibody. As an internal control for immunoblotting, GAPDH was used (bottom). In (B), D1 receptors were precipitated with anti-FLAG antibody, and the precipitated proteins (top) and total cell lysates (bottom) were subjected to immunoblotting with anti-EP1 antibody. At the left side of each blot, size markers can be found. Representative images from 3 independent experiments are shown. Note that in both (A) and (B) EP1 and D1 receptors were coprecipitated.

EP1 receptor-induced Ca^{2+} increase does not affect the facilitative action of EP1 on D1 receptor signaling

I next performed saturation binding analyses using [^{3}H]-SCH-23390, a radiolabeled ligand for the D1 receptor, since it has been demonstrated that stimulation of one protomer may alter the ligand binding profile of the other protomer (Ferré et al., 1991). However, the maximum binding capacity (B_{max}) and the dissociation constant (K_d) of D1 receptors was not significantly affected by EP1 stimulation (**Figure 3.7.**).

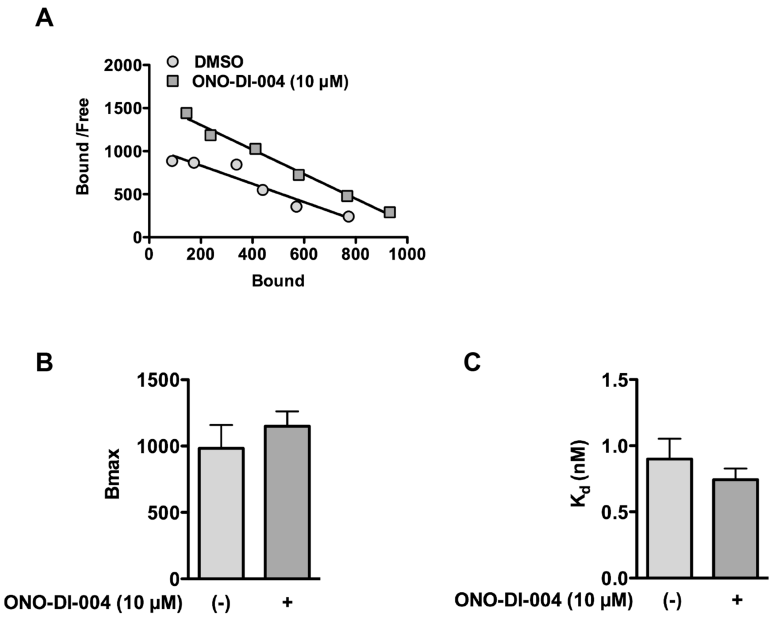

Figure 3.7. (Ehrlich et al., 2013). EP1 receptor activation failed to significantly affect the ligand binding of D1 receptors. Ligand binding analyses were carried out following incubation with EP1 receptor agonist, ONO-DI-004 (10 μM) or its vehicle (DMSO) for 5 minutes at 37˚C in intact HEK-293T cells expressing EP1 and D1 receptors. Subsequently, these cell lysates were used to prepare membrane fractions which were then subjected to saturation ligand binding analyses with [^{3}H]-SCH-23390, a radio-labeled ligand for the D1 receptor. The maximal binding capacity (Bmax) and the dissociation constant (Kd) derived from the scatchard plot shown in (A) are shown in (B) and (C), respectively. Note that EP1 stimulation did not alter either Bmax or Kd of D1 receptors. n = 3 for each condition.

Next, I examined whether the facilitative action of EP1 on D1 receptor signaling involves EP1 receptor induced intracellular signaling. In various cell types, the EP1 receptor is primarily coupled to intracellular Ca^{2+} increase (Hirata and Narumiya, 2011). Since several isoforms of adenylyl cyclase (AC), such as AC1, AC3 and AC8, can be potentiated by intracellular Ca^{2+} increase (Siehler and Milligan, 2011, Sunahara et al., 1996), I next examined whether EP1 receptor activation enhancement of D1 receptor signaling involves intracellular Ca^{2+}. To this end, using the two following methods intracellular Ca^{2+} was depleted. First, as measured by Fluo-4-AM, a cell-permeable fluorescent Ca^{2+} indicator, intracellular Ca^{2+} increase induced by ONO-DI-004 at 10 μM was completely blocked in the cells pretreated with 1,2-Bis(2-aminophenoxy)ethane-

N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester), BAPTA-AM, a cell permeable Ca^{2+} chelator, at 10 μM for 30 minutes (**Figure 3.8. A**). However, the facilitative action of EP1 on D1-induced cAMP increase was not affected by depletion of Ca^{2+} (**Figure 3.8. B**). A 30 minute bath application of the cells in a cocktail composed of EGTA at 1 mM and thapsigargin at 100 nM was also used to examine the effect of Ca^{2+} depletion on EP1 receptor mediated enhancement of D1 receptor signaling. Whereas this treatment induced a complete blockade of the intracellular Ca^{2+} increase induced by ONO-DI-004 (**Figure 3.8. C**), the facilitative effect of EP1 on D1-induced cAMP production was also unaffected by this method of Ca^{2+} depletion (**Figure 3.8. D**). Taken together, the effect of EP1 in facilitating D1 receptor signaling does not require EP1 receptor induced intracellular Ca^{2+} signaling.

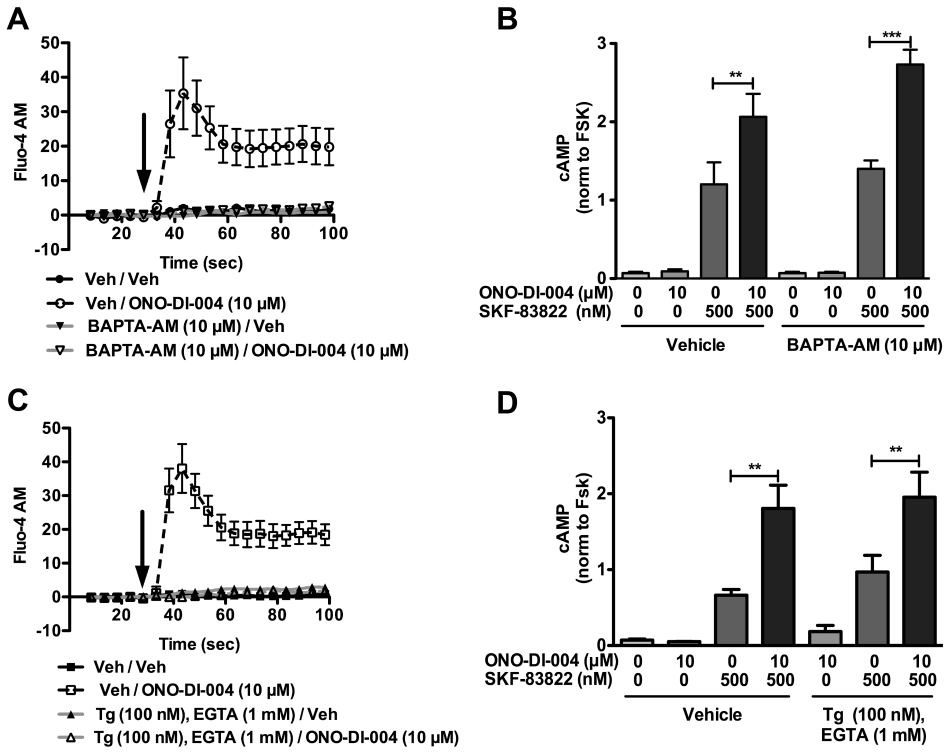

Figure 3.8. (Ehrlich et al., 2013). The facilitative effect of EP1 on D1-induced cAMP response, does not require EP1 receptor induced intracellular Ca^{2+} increase. (A) BAPTA-AM pretreatment induces a blockade of EP1-induced Ca^{2+} increase. HEK-293T cells overexpressing EP1 and D1 receptors were pretreated with or without BAPTA-AM, a cell-permeable Ca^{2+} chelator, and were loaded with Fluo-4-AM, a cell-permeable Ca^{2+} indicator, to measure intracellular Ca^{2+} mobilization induced by ONO-DI-004, an EP1 receptor agonist. BAPTA-AM pretreatment blocked the Ca^{2+} increase by bath application of ONO-DI-004 (arrow). (B) The facilitative action of EP1 on D1-induced cAMP response is unaffected by depletion of Ca^{2+} with BAPTA-AM. I treated the cells overexpressing EP1 and D1 receptors for 5 minutes with either SKF-83822 or ONO-DI-004 or both as indicated in the presence or absence of BAPTA-AM pretreatment. Following which the intracellular cAMP content was determined and analyzed using an enzyme immunoassay. The action of ONO-DI-004 in enhancing cAMP response induced by SKF-83822 was not blocked by pretreatment of the cells with BAPTA-AM. In each column, n = 4. (C) A cocktail of thapsigargin and EGTA also blocked EP1-induced Ca^{2+} increase. HEK-293T cells overexpressing EP1 and D1 receptors were pretreated with a cocktail of thapsigargin (100 nM) and EGTA (1 mM) or its vehicle. As described in (A) intracellular Ca^{2+} mobilization induced by ONO-DI-004 was measured and thapsigargin and EGTA blocked this Ca^{2+} increase. (D) The facilitative action of EP1 on D1-induced cAMP response is unaffected by pretreatment with thapsigargin and EGTA. In each column, n = 3-5. ** P < 0.01; *** P < 0.001 for Newman-Keuls multiple comparison tests following one-way ANOVA.

The EP1 facilitative action on D1-induced cAMP response involves $G_{\beta\gamma}$ subunits

In addition to AC isoforms that can be stimulated by intracellular Ca^{2+}, in concert with $G_{\alpha s}$ subunits, $G_{\beta\gamma}$ subunits can stimulate AC isoforms such as AC2, AC4 and AC7 (Siehler and Milligan 2011, Sunahara et al., 1996). I next overexpressed the G_{α} subunit of transducin ($G_{t\alpha 1}$) as a $G_{\beta\gamma}$ scavenger to evaluate the involvement of $G_{\beta\gamma}$ subunits in EP1-mediated facilitation of D1 signaling. In these experiments, to exclude a confounding effect of stimulating cGMP phosphodiesterase, a primary downstream effector of $G_{t\alpha}$ (Tang and Gilman, 1991), the cells were pretreated with 3-isobutyl-1-methylxanthine (IBMX). I first examined the effect of pretreating the cells with IBMX on the EP1 action in D1 receptor signaling. D1 stimulation by the addition of SKF-83822 at 1, 10 and 500 nM induced cAMP response in a dose-dependent manner in the presence of IBMX (**Figure 3.9. A**). This cAMP response by SKF-83822 at both concentrations of 10 and 500 nM was significantly increased by simultaneous treatment with ONO-DI-004, an EP1 agonist, at 10 μM (**Figure 3.9. A**). Thus, in the absence or presence of IBMX, EP1 activation facilitates D1 receptor signaling. Next, the effect of $G_{\beta\gamma}$ depletion on the EP1 facilitative action of D1 receptor signaling was examined. SKF-83822 at 1, 10 and 500 nM induced cAMP increase in a dose-dependent manner in HEK-293T cells that overexpressed $G_{t\alpha}$ in the presence of IBMX. However, D1-induced cAMP responses at any tested concentrations of SKF-83822 lacked the effect induced by concurrent activation of EP1 with ONO-DI-004 at 10 μM (**Figure 3.9. B and C**). This finding suggests that the facilitative effect of EP1 on D1 receptor signaling involves $G_{\beta\gamma}$ subunits.

A combination of the $G_{\beta 1}$ subunit and the $G_{\gamma 2}$ subunit is known to activate a $G_{\beta\gamma}$-sensitive AC isoform in vitro (Diel et al., 2006). I therefore overexpressed these $G_{\beta\gamma}$ subunits to examine whether the presence of free $G_{\beta\gamma}$ subunits is sufficient to enhance D1 receptor signaling. However, the SKF-83822-induced cAMP response was rather reduced by

overexpression of $G_{\beta 1\gamma 2}$ subunits (**Figure 3.9. D**). Thus, the facilitative effect of EP1 on D1-induced cAMP response could not be mimicked by overexpressing $G_{\beta 1\gamma 2}$ subunits.

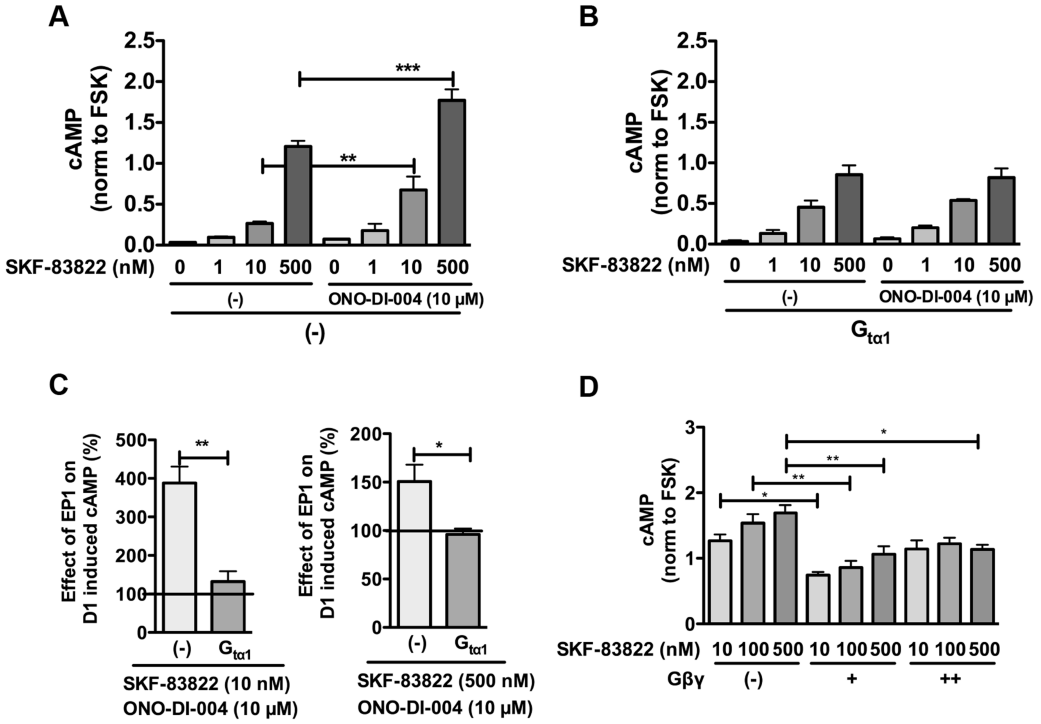

Figure. 3.9. (Ehrlich et al., 2013). The facilitative effect of EP1 on D1-induced cAMP response involves $G_{\beta\gamma}$ subunits. (A, B) Facilitation of D1-induced cAMP response by EP1 receptor activation is examined with or without overexpression of $G_{t\alpha 1}$, a $G_{\beta\gamma}$ scavenger. HEK-293T cells overexpressing D1 and EP1 receptors without $G_{t\alpha 1}$ (A) or with $G_{t\alpha 1}$ (B), were pretreated for 10 minutes with IBMX at 1 mM, followed by treatment for 5 minutes with either ONO-DI-004 or SKF-83822 or both as indicated, in the continued presence of IBMX. The cAMP levels were determined and analyzed using a cAMP enzyme immunoassay. In the presence of IBMX (A), treatment with ONO-DI-004 enhanced cAMP response induced by SKF-83822. However, in the presence of $G_{t\alpha 1}$ overexpression (B) this effect of ONO-DI-004 was not observed. For each column in (A) n = 3-5, and for each column in (B) n = 4. (C) With or without $G_{t\alpha 1}$ overexpression, the rate of increase by EP1 activation in D1-induced cAMP response is shown. The cAMP values for SKF-83822 with ONO-DI-004 treatment, which are shown in (A) and (B), were normalized to those induced by SKF-83822 at corresponding concentrations without ONO-DI-004. (D) The effect on D1-induced cAMP response by $G_{\beta\gamma}$ overexpression is examined. HEK-293T cells, treated with SKF-83822 as indicated, overexpressed EP1 and D1 receptors with or without (-) $G_{\beta\gamma}$ overexpression at lower (+) and higher (++) amounts and the cAMP levels were determined and analyzed by enzyme immunoassay. In each column n = 3. * P < 0.05, ** P < 0.01, *** P < 0.001 for Newman-Keuls multiple comparison tests following one-way analysis of variance , except for unpaired t-tests in (C), in which * P < 0.05 and ** P < 0.01.

Since $G_{\alpha i}$ signaling has been frequently associated with the potentiating actions of $G_{\beta\gamma}$ subunits on AC isoforms (Federman et al., 1992), I next examined whether the effect of EP1 on D1 signaling involves $G_{\alpha i}$ subunits. However, the effect of ONO-DI-004 at 10 μM on D1-induced cAMP increase was not affected by pretreatment with pertussis toxin, a blocker for $G_{\alpha i}$ signaling (**Figure 3.10.**).

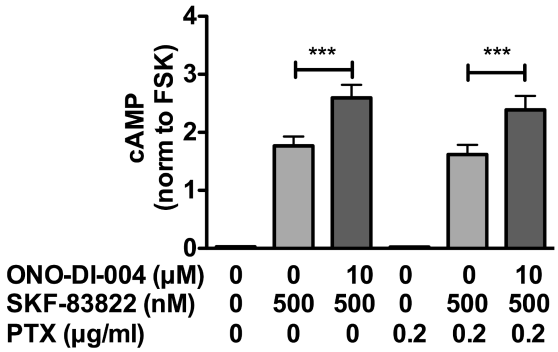

Figure 3.10 (Ehrlich et al., 2013). The facilitating effect of EP1 on D1-induced cAMP response does not involve $G_{\alpha i}$ signaling. Overnight incubation with pertussis toxin, PTX (0.2 µg/ml), to inhibit $G_{\alpha i}$ signaling or its vehicle, in HEK-293T cells overexpressing EP1 and D1 receptors. Susequently, the cells were treated for 5 minutes with either ONO-DI-004 or SKF-83822 or both as indicated. The facilitating effect of EP1 on D1-induced cAMP response was unaffected by pretreatment with pertussis toxin. For Newman-Keuls multiple comparison tests following a one-way analysis of variance ***P < 0.001.

The facilitative effect of EP1 on D1-induced cAMP response is mediated by AC7, a $G_{\beta\gamma}$-stimulated AC isoform

As described above, $G_{\beta\gamma}$ subunits are involved in the action of EP1 on D1 signaling. Therefore I examined whether a $G_{\beta\gamma}$-stimulated AC isoform could mediate the facilitative effect of EP1 on D1-induced cAMP response. It was reported that AC7, an isoform that can be stimulated by $G_{\beta\gamma}$ subunits, is expressed in HEK-293 cells (Atwood et al., 2011), from which HEK-293T cells have been derived. Consequently, I analyzed the involvement of AC7 as well as that of two other isoforms highly expressed in HEK-293 cells, AC5 and AC6.

To begin, the mRNA expression of these AC isoforms in HEK-293T cells was confirmed. In two independent experiments, knockdown of these AC isoforms reduced mRNA levels to approximately 20% of their mRNA levels with control siRNA (20.1% and 21.6% for AC5 knockdown, 19.2% and 24.1% for AC6 knockdown, 20.0% and 29.5% for AC7 knockdown) (**Figure 3.11. A**). Next, AC isoforms involved in D1-induced cAMP response without EP1 receptor activation were examined. cAMP increase induced by SKF-83822 at 500 nM was partially, but significantly, reduced by AC6 knockdown (**Figure 3.11. B**). In contrast, SKF-83822-induced cAMP increase was not significantly affected by knockdown of either AC5 or AC7 (**Figure 3.11. B**). This result suggests that, without simultaneous EP1 receptor activation, specific AC isoforms including, but not limited to, AC6 couple to D1 receptors.

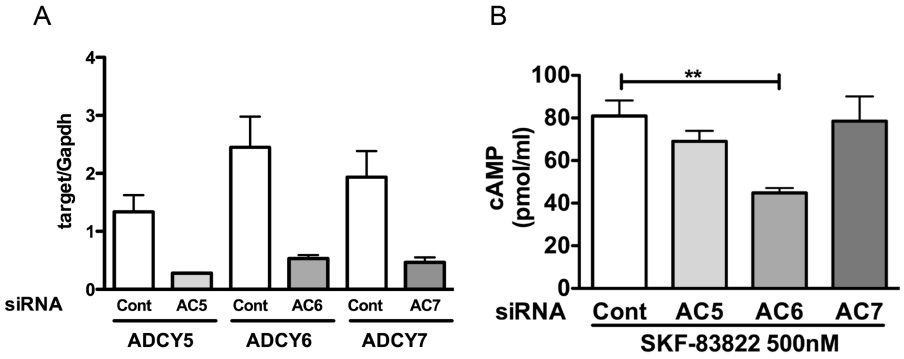

Figure 3.11. (Ehrlich et al., 2013). cAMP production by D1 stimulation alone involves adenylyl cyclase isoform, AC6. siRNAs targeting respective AC isoforms (AC5, AC6 and AC7) or control scramble siRNA (Cont) were transfected to HEK-293T cells overexpressing EP1 and D1 receptors for 48 hours. (A) To evaluate knockdown efficiency of AC isoforms qPCR was performed. Knockdown efficiency of approximately 20% was achieved for the indicated AC isoforms. (B) Cells were treated with 500 nM SKF-83822 for 5 minutes after knocking-down the indicated AC isoforms. The cAMP levels were determined by enzyme immunoassay as described in the legend of Figure 1. Note that a reduction in D1 receptor mediated cAMP response was observed for knockdown of AC6, but not for other AC isoforms. For each column, n = 4-5. **P < 0.01 for Newman-Keuls multiple comparison tests following one-way analysis of variance.

The AC isoform involved in the facilitative effect of EP1 on D1-induced cAMP response was next examined by employing knockdown of the respective AC isoforms. Simultaneous treatment with ONO-DI-004 at 10 μM significantly enhances cAMP response induced by SKF-83822 in the cells transfected with control siRNA (**Figure 3.12. A**). With or without EP1 activation, the D1-induced cAMP response was not affected by AC5 knockdown (**Figure 3.12. B**). The facilitative effect of ONO-DI-004 on D1 induced cAMP response was not affected by knockdown of AC6 that suppresses cAMP response induced by D1 stimulation alone (**Figure 3.12. C**). Surprisingly, the facilitative action of ONO-DI-004 on D1 receptor induced cAMP response was abolished by knockdown of AC7 (**Figure 3.12. D and E**), an isoform that does not affect the cAMP response induced by D1 stimulation alone (**Figure 3.11.**).

These results suggest that, whereas distinct AC isoforms including AC6 mediate cAMP production induced by D1 stimulation alone, EP1 activation enhances D1-induced cAMP production through AC7, a $G_{\beta\gamma}$-sensitive AC isoform.

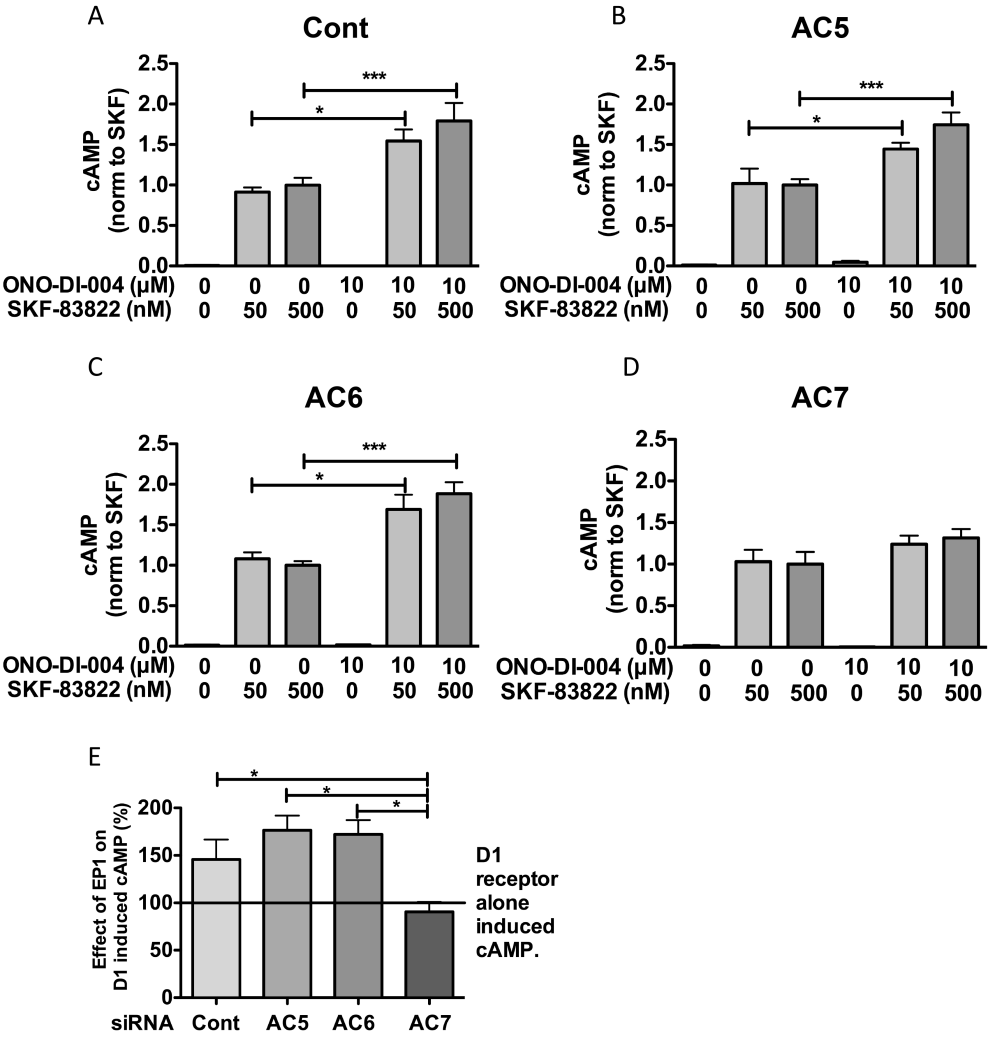

Figure 3.12. (Ehrlich et al., 2013). AC7, a $G_{\beta\gamma}$-sensitive isoform, is required for EP1 receptor mediated enhancement of D1-induced cAMP response. (A-D) Treatment with either ONO-DI-004 or SKF-83822 or both as indicated, in HEK-293T cells overexpressing EP1 and D1 receptors 48 hours after transfection with siRNAs targeting the respective AC isoforms (AC5, AC6 and AC7) or control scramble siRNA (Cont). The cAMP levels were determined as described in the legend of Figure 1 by enzyme immunoassay. In these experiments the cAMP levels were normalized to the values induced by 500 nM SKF-83822 alone, since the knockdown of different AC isoforms considerably varied the forskolin-induced cAMP response. For each column, n = 3-5. (E) The rate of increase by EP1 receptor activation on D1-induced cAMP responses across knockdown of the respective AC isoforms is shown. To calculate the effect of EP1 on D1 induced cAMP, the cAMP levels induced by SKF-83822 with ONO-DI-004 treatment, which are shown in (B-D), were normalized to those without ONO-DI-004. Note that the facilitating effect of ONO-DI-004 on D1-induced cAMP response was only abolished by AC7 knockdown. For Newman-Keuls multiple comparison tests following one-way analysis of variance * P < 0.05, *** P < 0.001.

Analysis of the effect of EP1 expression on D1 receptor subcellular distribution

Collectively, these results suggest that distinct AC isoforms are employed for cAMP production by the D1 receptor alone and the D1 receptor in complex with EP1. As it is known that AC6 and AC7 are localized in lipid rafts and non-lipid rafts respectively (Cooper and Crossthwaite, 2006), my findings raise the possibility that these two populations of D1 receptors are localized in different membrane domains and/or undergo distinct spatial regulation to be coupled to different AC isoforms. Therefore, I next analyzed the localization of D1 receptors in the absence or presence of EP1 receptors using subcellular fractionation by sucrose density gradients. In these experiments, in agreement with previous reports (Yu et al., 2004; Ostrom et al., 2002), D1 receptors and AC5/6 were mostly localized in lipid raft domains. In the cells coexpressing D1 and EP1 receptors, I failed to detect an alteration in the distribution of D1 receptors (**Figure 3.13**). Importantly, flotillin-1, a marker for lipid rafts, was almost exclusively found in the lipid raft fraction 5 along with AC5/6, whereas D1 receptors were distributed much more broadly, extending into non-lipid raft fractions (**Figure 3.13**), where EP1was also found (data not shown).

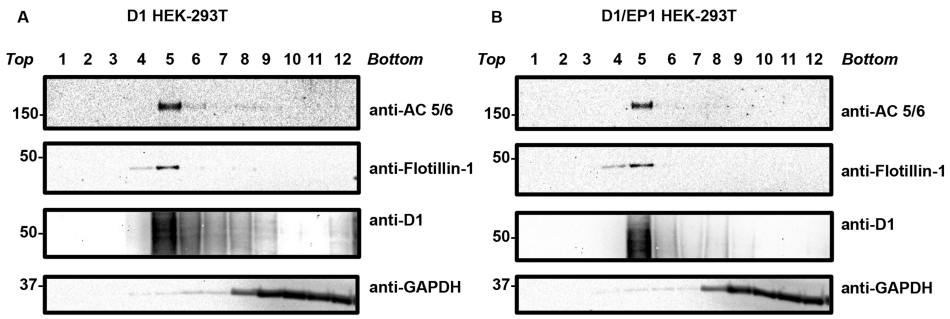

Figure 3.13. (Ehrlich et al., 2013). D1 receptor subcellular distribution is not altered by simultaneous EP1 expression. Subcellular fractionation using the sucrose density gradient method was carried out in whole cell lysates prepared from HEK-293T cells expressing D1 receptors alone (A) or with EP1 receptors (B). Following 20 hour ultracentrifugation twelve fractions were subjected to Western blotting. Flotillin-1 and GAPDH were used to designate lipid raft-containing fractions (fractions 4 to 6) and non-lipid raft fractions (fractions 9 to 12), respectively. Most observed D1 signals were in lipid raft-containing fractions. However, a minor species of D1 receptors also existed in non-lipid raft fractions. This data suggests that D1 receptor complexes have a heterogenous subcellular distribution in contrast with AC5/6 that distinctly localized in lipid raft fractions.

To test whether EP1 receptors regulate D1 receptors in non-lipid raft domains, I analyzed the effect of methyl-β-cyclodextrin (mβC), an agent that depletes cholesterol and disrupts lipid rafts, on the action of EP1 on D1-induced cAMP production. Consistent with a major localization of D1 receptors in lipid rafts, treatment of the cells with mβC at 1% for 30 min before stimulation augments cAMP production induced by D1 agonists alone, suggesting a role for lipid rafts in negatively regulating D1 receptor signaling. In contrast, concurrent EP1 stimulation increased D1-induced cAMP production by the same extent in the absence or presence of mβC (data not shown). These findings are consistent with the notion that EP1 regulates D1 receptor signaling in mβC-insensitive non-lipid raft domains.

Discussion

In an earlier report, it was demonstrated that in the mouse striatum PGE receptor subtype EP1 enhances signaling of dopamine D1 receptors and promotes D1-induced behaviors such as hyperlocomotion (Kitaoka et al., 2007). In the present study, the mechanism underlying this facilitative action of EP1 on D1 receptor signaling has been examined using HEK-293T cells. In a manner independent of EP1-induced Ca^{2+} increase, EP1 receptor activation facilitates cAMP production induced by D1 receptor agonists. This EP1 action on D1 receptor signaling is mediated through $G_{\beta\gamma}$ subunits and AC7, a $G_{\beta\gamma}$ subunit-sensitive AC isoform. Strikingly, the D1 receptor alone-induced cAMP response is mediated by other AC isoforms such as AC6, but not AC7. Thus, this study demonstrates that cAMP production induced by D1 receptors alone or D1 receptors facilitated by EP1 receptor activation is mediated through distinct AC isoforms (**Figure 3.14**).

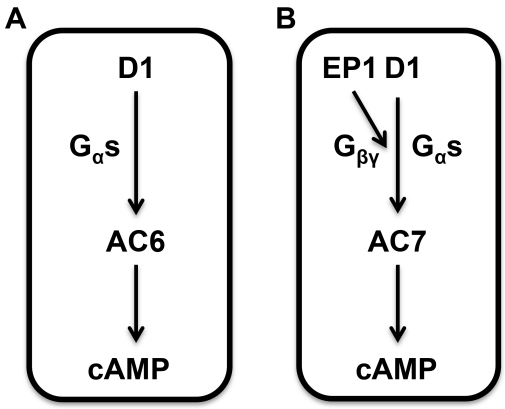

Figure 3.14. (Ehrlich et al., 2013). The proposed mechanism for the EP1 receptor facilitative action on D1 receptor signaling. (A) Activation of D1 receptor alone induces cAMP responses preferentially through AC6. (B) Simultaneous stimulation of EP1 receptors in the EP1-D1 heteromer acts on a distinct AC isoform, AC7, through $G_{\beta\gamma}$ subunits. Importantly, $G_{\alpha s}$ subunits released from activated D1 receptors in the EP1-D1 heteromer are required for this action.

4.1 A role for $G_{\beta\gamma}$-AC7 in EP1-mediated facilitation of D1-induced cAMP response

The EP1 receptor mediated action on D1 receptor signaling through $G_{\beta\gamma}$ subunits is supported by two findings. First, the facilitative action of EP1 on D1-induced cAMP production is blocked by overexpression of the $G_{t\alpha}$ subunit as a $G_{\beta\gamma}$ scavenger (**Figure 3.9. A and B**). Second, this EP1 action was also abolished by knockdown of AC7, a $G_{\beta\gamma}$ subunit-stimulated AC isoform (**Figure 3.12**). Previous studies have shown a combination of $G_{\beta 1}$ and $G_{\gamma 2}$ subunits can activate a $G_{\beta\gamma}$-sensitive AC isoform in vitro when this AC isoform is overexpressed (Diel et al., 2006). However, in my condition with endogenous AC levels, overexpression of $G_{\beta 1}$ and $G_{\gamma 2}$ subunits failed to increase D1-induced cAMP response (**Figure 3.9. D**) and rather suppressed D1-induced cAMP response. This inhibitory effect of $G_{\beta\gamma}$ subunit overexpression might counteract with a facilitative action of $G_{\beta\gamma}$, if it exists. The action of these $G_{\beta\gamma}$ subunits has been shown to be conditional upon the AC isoform to be coupled. For example, the overexpression of these $G_{\beta\gamma}$ subunits facilitated D1-induced cAMP response with overexpression of AC7, a $G_{\beta\gamma}$-sensitive AC isoform, but not with overexpression of AC5 (Yoshimura et al., 1996). Therefore, the coupling of D1 receptors to the $G_{\beta\gamma}$-sensitive AC isoform, over the others, could be facilitated by EP1 receptor activation.

Whereas, in principle, both $G_{\alpha s}$ and $G_{\beta\gamma}$ subunits could be derived from D1 receptor activation, only in the presence of EP1 activation is AC7 involved in D1-induced cAMP production. It has been shown, by studies on the inhibition of voltage-gated calcium channels and activation of G protein-coupled inwardly rectifying potassium channels, that not all $G_{\beta\gamma}$ dimers can regulate a given effector molecule (Albert and Robillard, 2002). Furthermore, $G_{\beta\gamma}$ subunits composed of specific G_{β} and G_{γ} isoforms have been

demonstrated to be required for several GPCRs and their respective signaling pathways (Albert and Robillard, 2002). Therefore, the facilitative action of EP1 receptors on D1 receptor induced-AC7-cAMP signaling could involve only the $G_{\beta\gamma}$ isoforms that are activated by EP1 receptors, but not those by D1 receptors, though this possibility remains to be tested.

4.2 Distinct signaling machinery of the D1-EP1 heteromer from the D1 receptor alone

I have shown here that EP1 and D1 receptors form a complex, as evidenced by their coprecipitation from HEK-293T cells (**Figure 3.6 A**). The fact that EP1 stimulation specifically facilitates D1 receptor signaling, but not those of other GPCRs tested, EP2 or ADRB2, (**Figure 3.2**) is consistent with the existence of the D1-EP1 complex. Intriguingly, the existence of both the D1-EP1 complex and the D1 receptor that is not associated with EP1 is supported by my observation that not all D1 receptors are colocalized with EP1 (**Figure 3.2 A**). It is attractive to postulate that AC6 and AC7 selectively bind to the D1 receptor alone and the D1-EP1 complex, respectively, given that the AC isoforms are used differently for cAMP production by D1 stimulation alone and its facilitation by EP1 stimulation, as demonstrated in this study. This prospect lends support to the concept that, in comparison to the cognate signaling profile of a GPCR, a GPCR heteromer often acts as a distinct entity (Ferré et al., 2009).

However, it is not sufficient to say that the EP1 receptor facilitative action on D1 receptor signaling is only caused by the complex formation of EP1 and D1 receptors. For example, in airway smooth muscle cells, EP1 receptor activation suppresses cAMP response induced by ADRB2 which forms a heteromer with EP1 (McGraw et al., 2006), although I

failed to observe this EP1 action on ADRB2 signaling in HEK-293T cells (**Figure 3.2 B**). Although EP1 can form a complex with both of these receptors, the function of EP1 activation is distinct, so that EP1 can exert opposite actions on cAMP production induced by D1 and ADRB2. Since $G_{\beta\gamma}$ subunits can either stimulate or inhibit AC activity, depending on the type of AC isoforms, EP1 could regulate cAMP production induced by D1 and ADRB2 by utilizing distinct AC isoforms. It has been shown for cAMP production induced by several GPCRs that a specific AC isoform is utilized. For example, in mouse smooth muscle cells and HEK-293 cells, AC2 and AC6 mediate cAMP increase induced by EP2 receptors and ADRB2, respectively (Bogard et al., 2012). Consistently, ADRB2 and AC6 were localized in lipid rafts, from which EP2, AC2 are excluded as shown by subcellular fractionation (Bogard et al., 2012; Crossthwaite et al., 2005). Likewise, D1 receptors that are coupled to AC6 without EP1 and those that are coupled to AC7 with EP1 could localize to different membrane domains, lipid raft domains and non-lipid raft domains, respectively. Subcellular fractionation and cyclodextrin experiments in the present study have suggested that EP1 receptors facilitate D1 receptor signaling in non-lipid raft domains. Consistent with this finding, AC7 that is coupled to D1 in the presence of EP1 is known to be localized to non-lipid raft domains (Cooper and Crossthwaite, 2006). It was reported that AC can form a signaling complex with GPCRs and $G_{\beta\gamma}$ subunits during their biosynthesis in the endoplasmic reticulum in the heterologous system (Dupré et al., 2009). Thus whether a D1-AC6 complex and a D1-EP1-AC7 complex are preassembled during their biosynthesis, or alternatively, these complexes are dynamically assembled upon GPCR activation, should be investigated in the future.

It has been suggested that, whereas EP1 activation increases cAMP production induced by D1 stimulation, EP1 facilitation of D2 receptor signaling mediates a decrease in cAMP production (Kitaoka et al., 2007). The presence of AC isoforms with distinct regulations by $G_{\beta\gamma}$ subunits could account for the apparently opposing actions of EP1. Thus, whereas AC7 mediates the facilitative effect of $G_{\beta\gamma}$ subunits in cAMP production induced by D1 in the complex with EP1, another AC isoform that is inhibited by $G_{\beta\gamma}$ subunits could be involved in the action of EP1 on D2 signaling. However, this possibility remains to be analyzed in future investigations.

4.3 Physiological implications for the D1-EP1-AC7 complex.

The D1-EP1-AC7 pathway identified in the present study appears to be of physiological relevance, given that EP1 receptor activation is critical for augmentation of D1 receptor signaling in striatal slices and hyperlocomotion induced by D1 receptors (Kitaoka et al., 2007). Thus, my study indicates that at least two kinds of D1 receptors, the one in the D1-EP1 heteromer and the one that is not associated with EP1, are expressed in striatal neurons, and the former kind of D1 receptors is activated proportionally according to the PGE_2 content in the brain. It was recently shown that exposure to stressors, such as social defeat, increases the PGE_2 content in the mouse brain (Tanaka et al., 2012). Thus, the D1-EP1 heteromer identified here may provide the mechanism for stress exposure to modulate D1 receptor signaling via the PGE_2-EP1 receptor signaling pathway. Consistent with this notion, previous studies show that repeated stress enhances cocaine-induced conditioned place preference (Krishnan et al., 2007), which requires dopamine receptor signaling in the nucleus accumbens. Furthermore, EP1 is critical for repeated stress-induced behavioral changes such as social avoidance. However, a role for EP1-D1 interaction in the nucleus accumbens in repeated stress warrants future investigation.

The functional importance of the diversity of AC isoforms is evidenced by recent studies using mice deficient in AC isoforms. AC isoform-specific functions have been the most characterized for AC1 and AC8. Mice lacking either of AC1 or AC8 are impaired in long-term memory for passive avoidance and contextual and spatial memory, as well as long-term synaptic plasticity in the hippocampus and the cerebellum (Villacres et al., 1995; Wang et al., 2003). In the striatum, the most dominant isoform is AC5. Genetic deletion of AC5 greatly reduced cAMP production induced by D1 and adenosine A2A receptors and abolished D2-induced suppression of cAMP production in striatal slices (Visel et al., 2006; Lee et al., 2002; Iwamoto et al., 2003).

Notably, AC5-deficient mice that were impaired in D1-induced cAMP response still showed increased hyperlocomotion induced by D1 receptor agonists (Iwamoto et al., 2003). Whereas the signaling pathway underlying D1-induced hyperlocomotion in the absence of AC5 has not been elucidated, it is possible that another AC isoform coupled to D1 receptors could play a role. Knockout mice lacking either of Group II AC isoform, AC2, AC4 or AC7, have not been well characterized (Sadana and Dessauer, 2009). Since AC7 homozygous knockout mice are embryonically lethal (Hines et al., 2006), it has hindered analyses on behavioral functions of AC7. However, it is known that overexpression of AC7 induces the phosphorylation of DARPP-32 at its Thr-34 residues in striatal neurons (Donohue et al., 2005). Furthermore, AC7 overexpression increases, whereas heterozygous deletion of AC7 decreases, depression-like behaviors as measured by the forced swim test and the tail suspension test in female mice (Hines et al., 2006). These findings illustrate the importance of AC7 in emotional behaviors. Whether EP1 or D1 receptors are involved in these AC7 functions remains for future investigations. The findings in my study indicate that the D1-EP1 complex in the brain offers an excellent

platform to elucidate the distinct physiological roles of the AC isoforms in stress induced emotional behaviors.

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