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Prostaglandin E receptor EP1 forms a complex with dopamine D1 receptor, and directs D1-induced cAMP production to adenylyl cyclase 7 through mobilizing $G_{\beta\gamma}$ subunits in HEK-293T cells.

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Running title: *EP1 directs D1 signaling to AC7 through $G_{\beta\gamma}$ subunits.*

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AC, adenylyl cyclase; ADRB2, β2-adrenergic receptor; PGE_2, Prostaglandin E_2;

ABSTRACT

The mechanism underlying the crosstalk between multiple G-protein-coupled receptors (GPCRs) remains poorly understood. We 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, we 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.

Introduction

G-protein-coupled receptors (GPCRs) are one of the primary targets for therapeutics in clinical use (Prinster et al., 2005; Milligan, 2009; Siehler and Milligan, 2011). They often form a heteromeric complex, but the mechanism underlying the crosstalk between multiple GPCRs remains poorly understood. Classically, it was assumed that each type of GPCR should be invariably coupled to a specific G protein and its effectors (Milligan, 2009). Given this assumption, the crosstalk of multiple GPCRs has been viewed as the sum of their signaling pathways (Panetta and Greenwood, 2008). The discovery of GPCR heteromers and the understanding of their actions and functions have greatly challenged this classical view of GPCR signaling (Prinster et al., 2005; Milligan, 2009; Fuxe et al., 2010). The concept of GPCR heteromers was originally substantiated by the discovery of dimer formation of 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), which is required for proper expression and function of these receptors. Later experiments have suggested that class A GPCRs, many of which are neurotransmitter receptors, can also form heteromers in the heterologous system and in native tissue (Pin et al., 2007). In many cases, one protomer of these GPCR heteromers can modulate the signaling efficacy of the other protomer (Siehler and Milligan, 2011). For example, the adenosine A_{2A} receptor forms a heteromeric complex with the dopamine D2 receptor, and the activation of the former receptor reduces the ligand binding of the latter receptor (Ferré et al., 1991). In another example, the serotonin 2A receptor forms a complex with the metabotropic glutamate receptor 2 through a specific transmembrane domain (González-Maeso et al., 2008). The

activation of each protomer of this heteromer negatively regulates G protein signaling of the other protomer (Fribourg et al., 2011).

Recent studies on newly identified heteromers of dopamine receptors have expanded a role for the GPCR heteromer in activating distinct signaling pathways from each protomer (Rashid et al., 2007; So et al., 2005; Kern et al., 2012). Dopamine receptors are divided into two families, D1-like receptors and D2-like receptors, each of which is primarily coupled to G_s-mediated cAMP increase and G_i-mediated cAMP decrease, respectively (Missale et al., 1998). However, the activation of the D1 receptor in the heteromer with the D2 receptor can evoke G_q-mediated Ca^{2+} increase only if the D2 receptor is simultaneously activated (Rashid et al., 2007; So et al., 2005). In another example, the D2 receptor forms a heteromer with the ghrelin receptor GHSR1a in the heterologous system and in CNS neurons (Kern et al., 2012). It was reported that stimulation of the D2 receptor activates a $G_{\beta\gamma}$ subunit-dependent rise in intracellular Ca^{2+} in neuroblastoma cells, only when GHSR1a is co-expressed (Kern et al., 2012). These examples illustrate a novel mode of signaling crosstalk in which the coupling of a certain GPCR to a downstream effector is altered by the presence of another GPCR. However, the molecular mechanism underlying such crosstalk remains elusive.

It is known that dopamine receptor signaling is modulated by several neurotransmitters and neuromodulators (Svenningsson et al., 2004, Nishi et al., 2011). Prostaglandin E_2 (PGE_2), a bioactive lipid derived from arachidonic acid, is one of these substances (Furuyashiki and Narumiya, 2011), and exerts its functions through one of four GPCRs named EP1, EP2, EP3 and EP4 (Hirata and Narumiya, 2011). We previously reported that EP1 and D1 receptors are co-expressed in striatal projection neurons, and that PGE_2-EP1 signaling augments D1-induced phosphorylation of DARPP-32 at its

Thr34 residue in striatal slices and promotes hyperlocomotion induced by D1 agonists (Kitaoka et al., 2007). However, the mechanism underlying this EP1 action remains unknown.

In this study, we analyzed the mechanism underlying the facilitative effect of EP1 on D1 receptor signaling in HEK-293T cells expressing EP1 and D1. We have found that EP1 and D1 receptors form a complex in these cells, and that EP1 activation facilitates D1-induced cAMP production through $G_{\beta\gamma}$ subunits and adenylyl cyclase 7 (AC7), a $G_{\beta\gamma}$-sensitive AC isoform (Sadana and Dessauer, 2009). Our findings also suggest that distinct AC isoforms mediate cAMP production by D1 receptors alone and that induced by D1 receptors in the D1-EP1 complex.

Materials and Methods

Plasmids. Plasmids for mammalian expression of the αl subunit of transducin ($G_{t\alpha}$), the β1 subunit of G protein (G_{β}), the γ2 subunit of G protein (G_{γ}, transcript variant 1) under the CMV promoter were obtained from OriGene Technologies (Rockville, MD). A plasmid for mammalian expression of the mouse EP2 receptor under the CMV promoter 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).

A plasmid for mammalian expression of mouse dopamine D1 receptor fused at its N terminus to the signal sequence (SS) derived from influenza hemagglutinin (Guan et al., 1992) and the Flag peptide was generated as follows. First, to remove the open reading frame of EGFP in pEGFP-C1 (Clontech, Mountain view, CA), pEGFP-C1 was digested with AgeI and BsrGI. The remaining portion of pEGFP-C1 was fused with the 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'). The resultant plasmid is named pSS-Flag-C1. Then, the open reading frame of the D1 receptor from the second amino acid attached with BsrGI and PstI at its N and C termini, respectively, was generated from pBluescript KS(+) containing the open reading frame of mouse D1 receptor (RIKEN, Saitama, Japan) by conventional PCR using the two following primers. 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'). Finally, this PCR fragment was digested with BsrGI and PstI, and ligated to the fragment of

pSS-Flag-C1 after BsrGI and PstI digestion.

A plasmid for mammalian expression of the human β2-adrenergic receptor (ADRB2) fused at its N terminus to SS and the Flag peptide was generated as follows. First, we obtained cDNA encoding ADRB2 attached with BsrGI and HindIII sites at its N and C termini, respectively, from total RNA of HEK-293 cells by RT-PCR using the two following primers. The forward primer contains 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 contains 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 fragment was digested with BsrGI and HindIII, and ligated to the fragment of pSS-Flag-C1 after BsrGI and HindIII digestion.

A plasmid for mammalian expression of mouse EP1 receptor fused at its N terminus to SS and the HA peptide was generated as follows. First, pEGFP-C1 was digested with NheI and AgeI, and the resultant fragment was fused to the 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'). This plasmid is named pSS-HA-EGFP-C1. Then, the open reading frame of EP1 from the second amino acid attached with AgeI and KpnI at its N and C termini, respectively, was generated from the plasmid for EP1 expression (Watabe et al., 1993) by conventional PCR using the two following primers. The forward primer contains the AgeI site and the N-terminal portion of the EP1 sequence (5' – ATC GAA CCG GTA GCC CCT GCG GGC TTA A – 3'). The reverse primer contains the C-terminal portion of EP1 sequence and the KpnI site (5' – GCT CAC CAT GGA GGC ACA GTC GAG GCT G – 3').

Finally, this PCR fragment was digested with AgeI and Acc65I, and ligated to the fragment of pSS-HA-EGFP-C1 after AgeI and Acc65I digestion.

The sequences of the open reading frames of the resultant plasmids were confirmed by conventional DNA sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA).

Cell culture and transfection. HEK-293T cells (ATCC, Manassas, VA) were maintained on Type I collagen-coated dishes (Iwaki Glass, Tokyo, Japan) in Dulbecco's Modified Eagle's Medium (D-MEM, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (PAA, Etobicoke, Ontario) in a humidified atmosphere with 5% CO_2 at 37^{o}C. For cAMP assay, cells were plated on collagen I-coated 24-well dishes. For immunofluorescence, cells were plated on round coverslips of 12 mm in diameter (Thermo Fisher Scientific, Waltham, MA, USA). Plasmids were transfected to HEK-293T cells with Effectene (QIAGEN, Alameda, CA), except for immunoprecipitation (Fig. 3B), calcium measurement (Fig. 4A), radioligand binding assay (Supplemental Fig.3), and Subcellular fractionation (Supplemental Fig.4) in which FuGENE HD was used instead (Promega, Madison, WI). After DNA transfection, cells were maintained for 24-48 hr before each experiment. siRNAs were transfected to HEK-293T cells for 48 hr with Lipofectamine RNAiMAX (Life Technologies, Carlsbad CA). A pre-validated mixture of multiple siRNAs targeting human AC5, AC6 or AC7 (MISSION esiRNA) was obtained from Sigma Aldrich (St. Louis, MO). Stealth RNAi siRNA negative control Med GC was used as a negative control for siRNA experiments (Life Technologies). The specificity and efficacy of the knockdown using these siRNAs in our experimental conditions were validated using quantitative RT-PCR (see the Results section).

Pharmacological stimulation. Drugs used in this study were obtained from the following sources: 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); ONO-DI-004 and ONO-AE1-259 (kind gifts from ONO Pharmaceuticals, Osaka, Japan). After cells were washed in Leibovitz's L-15 medium (Life Technologies) without serum, pre-warmed L-15 medium containing appropriate agonists or their vehicles were applied to the cells at 37^{o}C in the air for 5 min. To block intracellular Ca^{2+} increase (Fig. 4), 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 before stimulation. As an alternative method for Ca^{2+} depletion, 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 (Fig. 6), HEK-293T cells expressing EP1 and D1 receptors are incubated in serum-free D-MEM with pertussis toxin (0.2 μg/ml; List Biological Labs, Campbell, CA) for overnight in a humidified incubator at 37^{o}C with 5% CO_2.

cAMP measurement. After L-15 medium containing agonists or vehicle was removed, cells were immediately lysed with 0.1 M HCl for 20 min at room temperature (RT). cAMP levels in supernatants were measured using cAMP EIA kit (Cayman, Ann Arbor, MI) according to the manufacturer's protocol. Supernatants were stored on ice if assayed on the same day, or kept at -80^{o}C for longer storage. cAMP values were normalized to the maximal level of cAMP induced by forskolin (10 μM), except in the experiments with AC knockdown (Fig. 7 and Fig. 8). In these experiments, since knockdown of respective AC isoforms affected forskolin-induced cAMP response to different extents, either the absolute concentration of cAMP in supernatants (Fig. 7) or

those normalized to the cAMP level induced by 500 nM SKF-83822 (Fig. 8) was used for data analyses.

Fluorescent immunostaining. Fluorescent immunostaining was performed as previously described (Matsuoka et al., 2003). Briefly, cells were fixed with Dulbecco's modified phosphate buffered saline (D-PBS, Nissui Pharmaceuticals, Tokyo, Japan) containing 4% paraformaldehyde (Polysciences, Warrington, PA) for 30 min at RT. After permeabilization 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, the cells were incubated with both rat anti-HA peptide 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) in blocking buffer. After washing in D-PBS containing 1% Tween-20, the cells were incubated with appropriate secondary antibodies conjugated with Alexa488 or Alexa555 (Life Technologies) at 1:200 dilution in the blocking buffer for 2 hr at RT. Finally, cover slips were 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. At 24-48 hr after transfection, cells were re-suspended and solubilized in the 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. Following centrifugation at 12,000 x g for 10 min at 4˚C, Flag-tagged D1 receptors (Figure 3C) were precipitated with 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)). For immunoprecipitation of EP1 receptors (Fig. 3B), cell lysates were pre-cleared at 4˚C for 10 min using Protein A/G beads (Cytosignal, Irvine, CA) in a 50% slurry. Beads were pelleted, 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 with the EP1 antibody was precipitated 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 with the addition of 50 mM DTT and incubated at 4˚C for 40 min, then at RT for 10 min, prior to Western blotting.

Western blotting. Western blotting was performed as previously described (Kitaoka et al., 2007). Briefly, protein samples dissolved in the SDS sample buffer were subjected to SDS-PAGE with a 10% polyacrylamide gel (Atto, Tokyo, Japan), followed by semi-dry transfer (Atto) onto a 0.45-μm PVDF membrane (Millipore, Milford, MA). After blocking non-specific binding with Tris-buffered saline (TBS; 50 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing 3% skim milk (Difco), the membrane was incubated overnight at 4˚C with either rabbit anti-EP1 antibody (1:100 dilution; Cayman Chemical), rat anti-D1 antibody (1:1,000 dilution; D2944, Sigma-Aldrich), or mouse anti-GAPDH antibody (1:3,000 dilution; clone 6C5, Ambion, Carlsbad, CA). After several washes in TBS, the membrane was incubated with HRP-conjugated secondary antibody for IgG of appropriate species (1:5,000 dilution; GE Healthcare Biosciences, Pittsburgh, PA) in

TBS containing 3% skim milk for one hour at RT. After several washes in TBS, the membrane was subjected to detection with ECL Plus or ECL Prime (GE Healthcare Biosciences).

Calcium measurement. Intracellular calcium concentration was measured with the Fluo-4-AM Direct Calcium Assay Kit (Life Technologies) according to the manufacturer's protocol. Briefly, cells were incubated in the 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.

Quantitative RT-PCR. After 48-hr transfection, total RNA was extracted from HEK-293T cells using TRIzol (LifeTechnologies) according to the manufacturer's protocol. cDNA was generated from the resultant total RNA using PrimeScript Reverse Transcriptase (TaKaRa Bio, Otsu, Shiga, Japan). The resultant 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).

Statistical analyses. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, La Jolla, CA). For pairwise comparisons, unpaired *t*-tests were performed. For comparisons across three groups or larger and for two-factor analyses, one-way ANOVA and two-way ANOVA, respectively, followed by post hoc multiple comparison tests (Newman-Keuls or Bonferroni) were performed. *P* values less than 0.05 were considered to be statistically significant. Data are shown as means ± S.E.M.

Results

Stimulation of EP1 enhances cAMP production induced by D1 receptors in HEK-293T cells. To address the mechanism of how EP1 activation enhances dopamine D1 receptor signaling in striatal neurons (Kitaoka et al., 2007), we overexpressed both D1 receptors and EP1 receptors in HEK-293T cells, in which neither of these receptors are endogenously expressed, and examined the effect of ONO-DI-004, an EP1-specific agonist, on cAMP production induced by D1 stimulation. Treatment of the cells with SKF-81297, a D1 receptor agonist, at 30, 100 and 500 nM for 5 min increased cAMP production in a dose-dependent manner (Fig. 1A). Simultaneous application of ONO-DI-004, an EP1-specific agonist, at 1 and 10 μM significantly enhanced cAMP production induced by SKF-81297 in a dose-dependent manner (Fig. 1A). This result suggests that EP1 receptor activation enhances D1 receptor signaling in a cell-autonomous manner. Notably, treatment with ONO-DI-004 in the absence of SKF-81297 produced no significant increase in the cAMP level (Fig. 1A), suggesting that EP1 activation alone cannot induce cAMP production.

It was reported that two classes of D1 agonists have different actions: SKF-81297 increases not only cAMP production but also phosphoinositide hydrolysis through the heterodimer of D1 and D2 receptors, whereas SKF-83822 only stimulates cAMP production through the homodimer of D1 receptors (Verma et al., 2010). Therefore, we examined whether EP1 activation could also enhance cAMP production induced by SKF-83822. Treatment with SKF-83822 at 100 and 500 nM for 5 min increased cAMP production (Fig. 1B). Since the cAMP responses at these two concentrations were similar, these cAMP responses are considered to be saturated. Simultaneous treatment with ONO-DI-004 further enhanced these saturated cAMP responses (Fig. 1B). These results

suggest that EP1 stimulation enhances the maximal level of D1 receptor signaling in HEK-293T cells.

To confirm the specificity of ONO-DI-004 and SKF-83822 on EP1 and D1 receptors, respectively, we examined the effects of these drugs in HEK-293T cells that expressed either D1 or EP1 receptors alone. Thus, in HEK-293T cells that overexpressed D1 receptors alone, treatment with ONO-DI-004 at 10 μM failed to affect D1-induced cAMP response (Fig. 1C). In HEK-293T cells that overexpressed EP1 receptors alone, SKF-83822 did not increase cAMP levels (Fig. 1D). Thus, the effects of ONO-DI-004 and SKF-83822 are specific to EP1 and D1 receptors, respectively, in our experimental conditions.

EP1 activation is specifically coupled to D1 receptor signaling. We next examined whether EP1 activation could enhance G_s-induced cAMP production regardless of the type of stimulated GPCRs. For this purpose, we employed two GPCRs as examples. First, we examined the action of EP1 on cAMP production induced by EP2, another subtype of prostaglandin E receptor coupled to G_s. In HEK-293T cells that expressed both EP1 and EP2 receptors, ONO-AE1-259, an EP2-specific agonist, at 0.5 and 1 μM maximally increased cAMP production (Fig. 2A). These EP2-mediated cAMP responses were not augmented by simultaneous application of ONO-DI-004 at 10 μM (Fig. 2A). As another example, we also examined the action of EP1 on cAMP response induced by the β2-adrenergic receptor (ADRB2). In HEK-293T cells that expressed both EP1 and ADRB2 receptors, treatment with isoproterenol at 100 and 500 nM increased cAMP production (Fig. 2B). These cAMP responses were not augmented by ONO-DI-004, either (Fig. 2B). Therefore, the EP1 receptor specifically facilitates D1 receptor signaling, rather than G_s-stimulated cAMP production in general.

EP1 and D1 receptors form a heteromeric complex in HEK-293T cells. Given that the stimulatory action of EP1 is specific to D1 receptors, we suspected that EP1 receptors form a heteromeric complex with D1 receptors. First, we examined whether these receptors are co-localized in HEK-293T cells by immunofluorescent staining. We used anti-HA antibody and anti-Flag antibody to detect HA-tagged EP1 and Flag-tagged D1, respectively. Using cells expressing either HA-tagged EP1 or Flag-tagged D1, we confirmed that anti-HA antibody and anti-Flag antibody selectively recognize HA-tagged EP1 and Flag-tagged D1, respectively (Supplemental Fig. 1). In the cells overexpressing both EP1 and D1, EP1 signals were observed both at the periphery and around the nucleus of the cell, whereas D1 signals were localized mostly at the cell periphery (Fig. 3A, top). At a higher magnification, punctate signals for EP1 and D1 receptors were observed along the edge of the cell, and a considerable proportion of these signals were co-localized (Fig. 3A, bottom). However, not all D1 signals were colocalized with EP1 signals, and vice versa, suggesting that each of these receptors is present either alone or in complex with each other.

To directly analyze the complex formation of EP1 and D1 receptors, we examined whether these receptors could be co-precipitated. Although signals for EP1 or those for D1 were detected selectively in cell lysates expressing corresponding receptors, multiple bands were observed (Supplemental Fig. 2). To identify specific bands corresponding to intact HA-tagged EP1, we precipitated it with anti-EP1 antibody that recognizes the C terminus and detected it with anti-HA antibody that recognizes the N terminus. Similarly, to identify specific bands corresponding to intact Flag-tagged D1, we precipitated it with anti-Flag antibody that recognizes the N terminus and detected it with anti-D1 antibody that recognizes the C terminus. We decided to limit our analyses to the bands identified

by this method. Immunoprecipitation from cell lysates of HEK-293T cells with EP1 antibodies precipitated D1 receptors only when both EP1 and D1 receptors were overexpressed (Fig. 3B). Conversely, immunoprecipitation from the same cell lysates with antibodies that recognized the Flag tag fused to the D1 receptor precipitated EP1 receptors only in the presence of both EP1 and D1 receptors (Fig. 3C). These results suggest a complex formation of EP1 and D1 receptors in HEK-293T cells.

EP1-induced Ca^{2+} increase is not required for the facilitative action of EP1 on D1 receptor signaling. Since it is known that stimulation of one protomer may alter a ligand binding profile of the other protomer (Ferré et al., 1991), we performed the saturation binding analyses using [^{3}H]-SCH-23390, a radiolabeled ligand for the D1 receptor. However, EP1 stimulation did not affect either the maximum binding capacity (B_{max}) or the dissociation constant (K_d) of D1 receptors (Supplemental Fig. 3). Then we examined an involvement of intracellular signaling for the facilitative action of EP1 on D1 receptor signaling. EP1 receptor is primarily coupled to intracellular Ca^{2+} increase in various cell types (Hirata and Narumiya, 2011). Since intracellular Ca^{2+} increase can potentiate the activity of several isoforms of adenylyl cyclase (AC), such as AC1, AC3 and AC8 (Siehler and Milligan, 2011, Sunahara et al., 1996), we examined whether EP1 activation could enhance D1 receptor signaling through intracellular Ca^{2+}. To this end, we depleted intracellular Ca^{2+} using the two following methods. First, pretreatment with BAPTA-AM, a cell permeable Ca^{2+} chelator, at 10 μM for 30 min completely blocked intracellular Ca^{2+} increase induced by ONO-DI-004 at 10 μM, as measured by Fluo-4-AM, a cell-permeable, fluorescent Ca^{2+} indicator (Fig. 4A). However, this Ca^{2+} depletion did not block the facilitative effect of EP1 on D1-induced cAMP increase (Fig. 4B). We also examined the effect of another method of Ca^{2+} depletion by bath application

of EGTA at 1 mM and thapsigargin at 100 nM for 30 min. Whereas Ca^{2+} depletion with this method also blocked intracellular Ca^{2+} increase induced by ONO-DI-004 (Fig. 4C), this manipulation also failed to disrupt the facilitative effect of EP1 on D1-induced cAMP production (Fig. 4D). Taken together, EP1-mediatd Ca^{2+} increase is not required for the effect of EP1 in facilitating D1 receptor signaling.

$G_{\beta\gamma}$ subunits are involved in the action of EP1 in facilitating D1-induced cAMP response. Besides AC isoforms that can be stimulated by intracellular Ca^{2+}, other AC isoforms, such as AC2, AC4 and AC7, can be stimulated by $G_{\beta\gamma}$ subunits in concert with $G_{\alpha s}$ subunits (Siehler and Milligan 2011, Sunahara et al., 1996). To evaluate the involvement of $G_{\beta\gamma}$ subunits in EP1-mediated facilitation of D1 signaling, we depleted $G_{\beta\gamma}$ subunits by overexpression of the G_{α} subunit of transducin ($G_{t\alpha 1}$) as a $G_{\beta\gamma}$ scavenger. In this experiment, the cells are pretreated with IBMX to exclude a confounding effect of stimulating cGMP phosphodiesterase, a primary downstream effector of $G_{t\alpha}$ (Tang and Gilman, 1991). First, we examined whether pretreatment with IBMX might affect the EP1 action on D1 receptor signaling. After pretreatment with IBMX, D1 stimulation by the addition of SKF-83822 at 1, 10 and 500 nM induced cAMP response in a dose-dependent manner (Fig. 5A). Simultaneous treatment with ONO-DI-004, an EP1 agonist, at 10 μM significantly increased this cAMP response by SKF-83822 at both concentrations of 10 and 500 nM (Fig. 5A). Thus, EP1 activation facilitates D1 receptor signaling in the absence or presence of IBMX. Then we examined the effect of $G_{\beta\gamma}$ depletion on the EP1 action on D1 signaling. In HEK-293T cells that overexpressed $G_{t\alpha}$ in the presence of IBMX, SKF-83822 at 1, 10 and 500 nM induced cAMP increase in a dose-dependent manner. However, concurrent activation of EP1 by ONO-DI-004 at 10 μM failed to augment these D1-induced cAMP responses at any tested concentrations of

SKF-83822 (Fig. 5B and 5C). This finding suggests that $G_{\beta\gamma i}$subunits are involved in the facilitative effect of EP1 on D1 receptor signaling.

To examine whether the presence of free $G_{\beta\gamma}$ subunits is sufficient to enhance D1 receptor signaling, we overexpressed the $G_{\beta 1}$ subunit and the $G_{\gamma 2}$ subunit, a combination of which is known to activate a $G_{\beta\gamma}$-sensitive AC isoform in vitro (Diel et al., 2006). However, overexpression of $G_{\beta 1\gamma 2}$ subunits rather reduced SKF-83822-induced cAMP response (Fig. 5D). Thus, overexpression of $G_{\beta 1\gamma 2}$ subunits is not sufficient to mimic the facilitative effect of EP1 on D1-induced cAMP production.

Since actions of $G_{\beta\gamma}$ subunits have been frequently associated with $G_{\alpha i}$ signaling (Federman et al., 1992), we next examined a possible involvement of $G_{\alpha i}$ subunits in the effect of EP1 on D1 signaling. However, pretreatment with pertussis toxin, a blocker for $G_{\alpha i}$ signaling, failed to affect the effect of ONO-DI-004 at 10 μM on D1-induced cAMP increase (Fig. 6).

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

We first confirmed the mRNA expression of these AC isoforms in HEK-293T cells. Knockdown of these AC isoforms by transfection with siRNA targeting respective AC isoforms reduced mRNA levels of corresponding AC isoforms to approximately 20% of

their mRNA levels with control siRNA in two independent experiments (20.1% and 21.6% for AC5 knockdown, 19.2% and 24.1% for AC6 knockdown, 20.0% and 29.5% for AC7 knockdown). We first examined whether any of these AC isoforms are involved in D1-induced cAMP response without EP1 activation. Knockdown of AC6 significantly, but partially, reduced cAMP increase induced by SKF-83822 at 500 nM (Fig. 7). In contrast, knockdown of either AC5 or AC7 did not significantly affect SKF-83822-induced cAMP increase (Fig. 7). This result suggests that D1 activation without simultaneous EP1 activation is coupled to specific AC isoforms including AC6.

Using knockdown of respective AC isoforms, we next examined the AC isoform involved in the facilitative effect of EP1 on D1-induced cAMP response. In the cells transfected with control siRNA, simultaneous treatment with ONO-DI-004 at 10 μM significantly enhanced cAMP response induced by SKF-83822 (Fig. 8A). Knockdown of AC5 did not affect D1-induced cAMP response with or without EP1 activation (Fig. 8B). Knockdown of AC6 that had suppressed cAMP response induced by D1 stimulation alone (Fig. 7) failed to affect the facilitative effect of ONO-DI-004 on this cAMP response (Fig. 8C). Surprisingly, knockdown of AC7 that had not affected cAMP response induced by D1 stimulation alone (Fig. 7) abolished the facilitative effect of ONO-DI-004 on this cAMP response (Fig. 8D and 8E).

Although partial knockdown in our condition might have failed to detect an AC isoform that plays a minor role, these results suggest that EP1 activation enhances D1-induced cAMP production through AC7, a $G_{\beta\gamma}$-sensitive AC isoform, whereas distinct AC isoforms including AC6 mediate cAMP production induced by D1 stimulation alone.

Discussion

We previously reported that PGE receptor subtype EP1 enhances signaling of dopamine D1 receptors in striatal slices and promotes D1-induced hyperlocomotion (Kitaoka et al., 2007). In the present study, we examined the mechanism underlying this facilitative effect of EP1 on D1 receptor signaling using HEK-293T cells. Stimulation of EP1 facilitates cAMP production induced by D1 receptor agonists in a manner independent of EP1-induced Ca^{2+} increase. Our findings rather suggest that this EP1 action is mediated through $G_{\beta\gamma}$ subunits and AC7, a $G_{\beta\gamma}$ subunit-sensitive AC isoform. Strikingly, cAMP production induced by D1 receptors alone is mediated by other AC isoforms including AC6, but not AC7. Therefore, the present study demonstrates that distinct AC isoforms mediate cAMP production induced by D1 receptors alone and the facilitative action of EP1 on D1-induced cAMP production (Fig. 9).

A role for $G_{\beta\gamma}$-AC7 in EP1-mediated facilitation of D1-induced cAMP production. The involvement of $G_{\beta\gamma}$ subunits in the EP1 action on D1 signaling is supported by two findings. First, overexpression of the $G_{t\alpha}$ subunit as a $G_{\beta\gamma}$ scavenger blocked the facilitative action of EP1 on D1-induced cAMP production (Fig. 5A and B). Second, knockdown of AC7, a $G_{\beta\gamma}$-stimulated AC isoform, also abolished this EP1 action (Fig. 8). Although a combination of $G_{\beta1}$ and $G_{\gamma2}$ subunits is known to activate a $G_{\beta\gamma}$-sensitive AC isoform in vitro (Diel et al., 2006), overexpression of $G_{\beta1}$ and $G_{\gamma2}$ subunits failed to increase D1-induced cAMP response (Fig. 5D). One possibility is that AC activity might be already saturated by GPCR stimulation alone, since it was reported that overexpression of AC6, but not of $G_{\alpha s}$, proportionally increases cAMP response induced by β-adrenergic receptor in rat cardiac myocytes (Gao et al., 1998). However, $G_{\beta\gamma}$ overexpression still failed to facilitate cAMP response to SKF-83822 at a

non-saturating dose of this drug (10 nM). Since $G_{\beta\gamma}$ overexpression rather suppressed D1-induced cAMP response in our study, this inhibition could mask a facilitative action of $G_{\beta\gamma}$, if it exists. It is known that the action of these $G_{\beta\gamma}$ subunits is conditional upon the AC isoform to be coupled. Yoshimura et al., (1996) reported that 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. Therefore, EP1 activation could facilitate the coupling of D1 receptors to the $G_{\beta\gamma}$-sensitive AC isoform over the others.

Whereas D1 activation alone could activate both $G_{\alpha s}$ and $G_{\beta\gamma}$ subunits in theory, AC7 is involved in D1-induced cAMP production only in the presence of EP1 activation. It is known that not all $G_{\beta\gamma}$ dimers can regulate a given effector molecule, as exemplified by inhibition of voltage-gated calcium channels and activation of G protein-coupled inwardly rectifying potassium channels (Albert and Robillard, 2002). Furthermore, several GPCRs have been shown to utilize specific $G_{\beta\gamma}$ isoforms for their respective signaling pathways (Albert and Robillard, 2002). Therefore, only $G_{\beta\gamma}$ isoforms that are activated by EP1 receptors in complex with D1 receptors, but not those by D1 receptors alone, could facilitate the activity of AC7, though this possibility remains to be tested.

The D1-EP1 complex as signaling machinery distinct from the D1 receptor alone. Co-precipitation experiments in this study showed that EP1 and D1 receptors form a complex in HEK-293T cells (Fig. 3B and 3C). Such a molecular complex could provide an environment in which $G_{\beta\gamma}$ subunits are activated to a sufficient local concentration to stimulate AC7 activity. The formation of the D1-EP1 complex is also consistent with the fact that EP1 stimulation specifically facilitates D1 receptor signaling, but not those of other GPCRs tested, EP2 or ADRB2 (Fig. 2). Our observation that not all D1 receptors

are colocalized with EP1 (Fig. 3A) suggests the existence of both the D1-EP1 complex and the D1 receptor that is not associated with EP1. Since AC6 and AC7 are used differently for cAMP production by D1 stimulation alone and its facilitation by EP1 stimulation, respectively, it is plausible that these AC isoforms selectively bind to the D1 receptor alone and the D1-EP1 complex, respectively. This notion lends support to the concept that a GPCR heteromer acts as a distinct entity from its respective protomer (Ferré et al., 2009).

However, complex formation of EP1 and D1 receptors is not sufficient to cause the facilitative action of EP1 on D1 signaling. For example, EP1 activation suppresses cAMP response induced by ADRB2, which forms a heteromer with EP1, in airway smooth muscle cells (McGraw et al., 2006), although we failed to observe this EP1 action on ADRB2 signaling in HEK-293T cells (Fig. 2B). Thus, whereas EP1 forms a complex with either D1 or ADRB2, EP1 activation 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, heteromeric EP1 receptors could direct distinct AC isoforms to regulate cAMP production induced by D1 and ADRB2. It has been shown that several GPCRs utilize a specific AC isoform for cAMP production. For example, in mouse smooth muscle cells and HEK-293 cells, AC2 and AC6 mediate cAMP increase induced by EP2 and ADRB2, respectively (Bogard et al., 2012). Consistent with specific GPCR-AC coupling as such, subcellular fractionation showed that ADRB2 and AC6 are localized in lipid rafts, from which EP2, AC2 and AC7 are excluded (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 be localized in different membrane domains, lipid raft domains and non-lipid raft domains,

respectively. In subcellular fractionation using sucrose density gradients, we have found that D1 receptors and AC5/6 are mainly localized in lipid raft domains, as previously reported (Yu et al., 2004; Ostrom et al., 2002), but that this subcellular distribution of D1 was not apparently altered by simultaneous expression of EP1 (Supplemental Fig. 4). Whereas AC5/6 is distributed similarly as flotillin-1, a marker for lipid rafts, D1 receptors are distributed much more broadly, suggesting a large heterogeneity of D1 receptor complexes. It was reported that AC can form a signaling complex with a GPCR and a $G_{\beta\gamma}$ subunit during their biosynthesis in the ER in the heterologous system (Dupré et al., 2009). Whether the D1-AC6 complex and the D1-EP1-AC7 complex are formed separately during their biosynthesis, or alternatively, whether these complexes are dynamically assembled upon GPCR activation, warrants future investigation.

Physiological implications for the D1-EP1 complex. Since EP1 activation is critical for augmentation of D1 receptor signaling in striatal slices and hyperlocomotion induced by D1 receptors, the D1/EP1-$G_{\beta\gamma}$-AC pathway identified in the present study appears to be of physiological relevance. Thus, our study indicates that striatal neurons express two kinds of D1 receptors, the one in the D1-EP1 heteromer and the one that is not associated with EP1, the former of which is activated proportionally according to the PGE_2 content in the brain. Since exposure to stressors, such as social defeat, increases the PGE_2 content in the mouse brain (Tanaka et al., 2012), the D1-EP1 complex may provide the mechanism for stress exposure to modulate D1 receptor signaling through PGE_2-EP1 signaling.

However, whether the same molecules identified in the present study mediate the D1-EP1 crosstalk *in vivo* remains to be tested. For example, whereas AC6, but neither AC5 nor AC7, mediates cAMP production induced by D1 activation in HEK-293T cells,

cAMP production induced by either forskolin or D1 agonists was greatly reduced in the striatum of AC5-deficient mice (Lee et al., 2002). Therefore, HEK-293T cells used in this study may lack a component that makes D1 receptors preferentially coupled to AC5 as seen in striatal neurons. It has been shown that an AC isoform that is not critical for cAMP production by D1 receptors alone can still play a role in dopamine-related behaviors. For example, genetic deletion of the Ca^{2+}/calmodulin-stimulated AC isoforms, AC1 and AC8, abolishes locomotor sensitization induced by chronic cocaine treatment (DiRocco et al., 2009). In contrast, little is known about physiological functions of the $G_{\beta\gamma}$-sensitive AC isoforms, such as AC2, AC4 and AC7, in the brain. Given the isoform-specific AC functions identified in our study, it is important to choose appropriate stimuli or contexts to reveal the physiological functions of the $G_{\beta\gamma}$-sensitive AC isoforms. According to our findings, the D1-EP1 complex offers an excellent platform to analyze functions of these AC isoforms in the brain.

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Authorship Contributions

Participated in research design: Ehrlich, Furuyashiki, Kitaoka, Narumiya

Conducted experiments: Ehrlich

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Footnotes

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Figure Legends

Fig. 1. EP1 receptor activation enhances cAMP production induced by D1 receptors in HEK-293T cells. (A, B) The effect of EP1 activation on D1-induced cAMP response. HEK-293T cells overexpressing EP1 and D1 receptors ("D1-EP1") were treated for 5 min with either a D1 agonist (SKF-81297 in (A) and SKF-83822 in (B)) or an EP1 agonist (ONO-DI-004) or both as indicated. Intracellular cAMP levels were determined using the cAMP EIA 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) The lack of the effect of ONO-DI-004 on D1-induced cAMP response in the cells without EP1 overexpression. HEK-293T cells overexpressing D1 receptors alone ("D1") were treated with either SKF-83822 or ONO-DI-004 or both as indicated. cAMP levels were determined as described above. n = 6 for each column. (D) The lack of the effect of SKF-83822 and ONO-DI-004 in the cells without D1 overexpression. HEK-293T cells overexpressing EP1 receptors alone ("EP1") were treated with SKF-83822 or ONO-DI-004 or both as indicated. 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 ANOVA.

Fig. 2. EP1 activation does not affect cAMP response induced by EP2 or ADRB2 in HEK-293T cells. (A) The lack of an effect of EP1 activation on EP2-induced cAMP response. HEK-293T cells overexpressing EP1 and EP2 ("EP1-EP2") were treated with either 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) The lack of an effect of EP1 activation on cAMP

response induced by ADRB2. 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 affect cAMP response induced by either EP2 or ADRB2.

Fig. 3. EP1 and D1 receptors are colocalized and form a complex in HEK-293T cells. (A) Localization of EP1 and D1 receptors overexpressed in HEK-293T cells. HA-tagged EP1 receptors and Flag-tagged D1 receptors were overexpressed in HEK-293T cells, and were immunostained using anti-HA antibody (magenta) and anti-Flag antibody (green), respectively. Representative images are shown at the top, and an area indicated by a dotted square is magnified and shown at the bottom. Colocalization of EP1 and D1 receptors is indicated by arrowheads. Signals for EP1 that are not colocalized with D1 receptors are indicated by white diamonds. Signals for D1 that are not colocalized with EP1 receptors are indicated by asterisks. Scale bar, 10 μm for top images and 5 μm for bottom images. (B, C) Co-precipitation of EP1 and D1 receptors. Cell lysates prepared from HEK-293T cells that overexpressed either D1 or EP1 receptors or both were subjected to immunoprecipitation (IP). In (B), EP1 receptors were precipitated with anti-EP1 antibody, and the precipitated proteins (top) and total cell lysates (middle) were subjected to immunoblotting (IB) with anti-D1 antibody. GAPDH was used as an internal control for immunoblotting (bottom). In (C), 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. Size markers are shown at the left side of each blot. Representative images from 3 independent experiments are shown. Note that EP1 and D1 receptors are co-precipitated in both (B) and (C).

Fig. 4. Intracellular Ca^{2+} increase is not required for the facilitating effect of EP1 on D1-induced cAMP response. (A) The blockade of EP1-induced Ca^{2+} increase with BAPTA-AM. Fluo-4-AM, a cell-permeable Ca^{2+} indicator, was loaded onto HEK-293T cells overexpressing EP1 and D1 receptors without or with BAPTA-AM, a cell-permeable Ca^{2+} chelator, and intracellular Ca^{2+} mobilization induced by ONO-DI-004, an EP1 agonist, was measured. Bath application of ONO-DI-004 (arrow) induced intracellular Ca^{2+} increase, and pretreatment with BAPTA-AM blocked this Ca^{2+} increase. Each trace shows averages of three to five independent measurements at respective time points. (B) The lack of an effect of BAPTA-AM on the facilitating effect of EP1 on D1-induced cAMP response. HEK-293T cells overexpressing EP1 and D1 receptors were loaded with BAPTA-AM, and then were treated for 5 min with either SKF-83822 or ONO-DI-004 or both as indicated. The cAMP levels were determined and analyzed as described in the legend of Figure 1. Pretreatment with BAPTA-AM did not abolish the action of ONO-DI-004 in enhancing cAMP response induced by SKF-83822. $n = 4$ in each column. (C) The blockade of EP1-induced Ca^{2+} increase with a cocktail of thapsigargin and EGTA. HEK-293T cells overexpressing EP1 and D1 receptors were pretreated with a cocktail of thapsigargin (100 nM) and EGTA (1 mM) or with its vehicle. Intracellular Ca^{2+} mobilization induced by ONO-DI-004 was measured as described in (A). Pretreatment with thapsigargin and EGTA blocked Ca^{2+} increase by ONO-DI-004. Each trace shows averages of three to five independent measurements at respective time points. (D) The lack of an effect of thapsigargin and EGTA on the facilitating effect of EP1 on D1-induced cAMP response. Pretreatment with thapsigargin and EGTA did not abolish the action of ONO-DI-004 in enhancing cAMP response induced by SKF-83822.

n = 3-5 for each column. ** *P* < 0.01; *** *P* < 0.001 for Newman-Keuls multiple comparison tests following one-way ANOVA.

Fig. 5. $G_{\beta\gamma}$ subunits are involved in the facilitating effect of EP1 on D1-induced cAMP response. (A, B) D1-induced cAMP response and its facilitation by EP1 activation without or with overexpression of $G_{t\alpha1}$, a $G_{\beta\gamma}$ scavenger. HEK-293T cells overexpressing D1 and EP1 receptors without $G_{t\alpha1}$ (A) or with $G_{t\alpha1}$ (B) were pretreated with IBMX for 10 min, and were treated for 5 min with either ONO-DI-004 or SKF-83822 or both as indicated. The cAMP levels were determined and analyzed as described in the legend of Figure 1. Treatment with ONO-DI-004 enhanced cAMP response induced by SKF-83822 in the presence of IBMX (A). This effect of ONO-DI-004 was not observed in the presence of $G_{t\alpha1}$ overexpression (B). *n* = 3-5 for each column in (A) and *n* = 4 for each column in (B). (C) The rate of increase by EP1 activation in D1-induced cAMP response without or with $G_{t\alpha1}$ overexpression. The cAMP levels induced by 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 of $G_{\beta\gamma}$ overexpression on D1-induced cAMP response. HEK-293T cells that overexpressed EP1 and D1 receptors without (-) or with $G_{\beta\gamma}$ overexpression at lower (+) and higher (++) amounts were treated with SKF-83822 as indicated, and the cAMP levels were determined and analyzed as in Figure 1. *n* = 3 for each column. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 for Newman-Keuls multiple comparison tests following one-way ANOVA, except in (C), in which * *P* < 0.05 and ** *P* < 0.01 for unpaired *t*-tests.

Fig. 6. $G_{\alpha i}$ signaling is not involved in the facilitating effect of EP1 on D1-induced cAMP response. HEK-293T cells overexpressing EP1 and D1 receptors were pretreated overnight with pertussis toxin (0.2 μg/ml), an inhibitor for $G_{\alpha i}$ signaling, or its vehicle, and treated with either ONO-DI-004 or SKF-83822 or both as indicated. Pretreatment with pertussis toxin did not affect the facilitating effect of EP1 on D1-induced cAMP response. ***$P < 0.001$ for Newman-Keuls multiple comparison tests following one-way ANOVA.

Fig. 7. AC6 is involved in cAMP production by D1 stimulation alone. HEK-293T cells overexpressing EP1 and D1 receptors were transfected with siRNAs targeting respective AC isoforms (AC5, AC6 and AC7) or control scramble siRNA (Cont) for 48 hr, and were treated with SKF-83822 for 5 min. The cAMP levels were determined as described in the legend of Figure 1. Note that knockdown of AC6, but not that of the others, significantly reduced D1-induced cAMP response. n = 4-5 for each column. **$P < 0.01$ for Newman-Keuls multiple comparison tests following one-way ANOVA.

Fig. 8. EP1 activation enhances D1-induced cAMP response through AC7, a $G_{\beta\gamma}$-sensitive isoform. (A-D) HEK-293T cells overexpressing EP1 and D1 receptors were transfected with siRNAs targeting respective AC isoforms (AC5, AC6 and AC7) or control scramble siRNA (Cont) for 48 hr, and were treated with either ONO-DI-004 or SKF-83822 or both as indicated. The cAMP levels were determined as described in the legend of Figure 1. Since forskolin-induced cAMP response was considerably varied with knockdown of different AC isoforms, the cAMP levels were normalized to the values induced by 500 nM SKF-83822 alone in this experiment. n = 3-5 for each column.

(E) The rate of increase by EP1 activation on D1-induced cAMP response with knockdown of respective AC isoforms. 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 only AC7 knockdown abolished the facilitating effect of ONO-DI-004 on D1-induced cAMP response. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for Newman-Keuls multiple comparison tests following one-way ANOVA.

Fig. 9. The proposed mechanism of action of EP1 on D1 receptor signaling. (A) Stimulation of D1 receptors alone induces cAMP response preferentially through AC6. (B) Activation of EP1 receptors in the EP1-D1 heterodimer stimulates AC7 through $G_{\beta\gamma}$ subunits. This action requires $G_{\alpha s}$ subunits released from activated D1 receptors in the EP1-D1 heterodimer.

Figure 1.

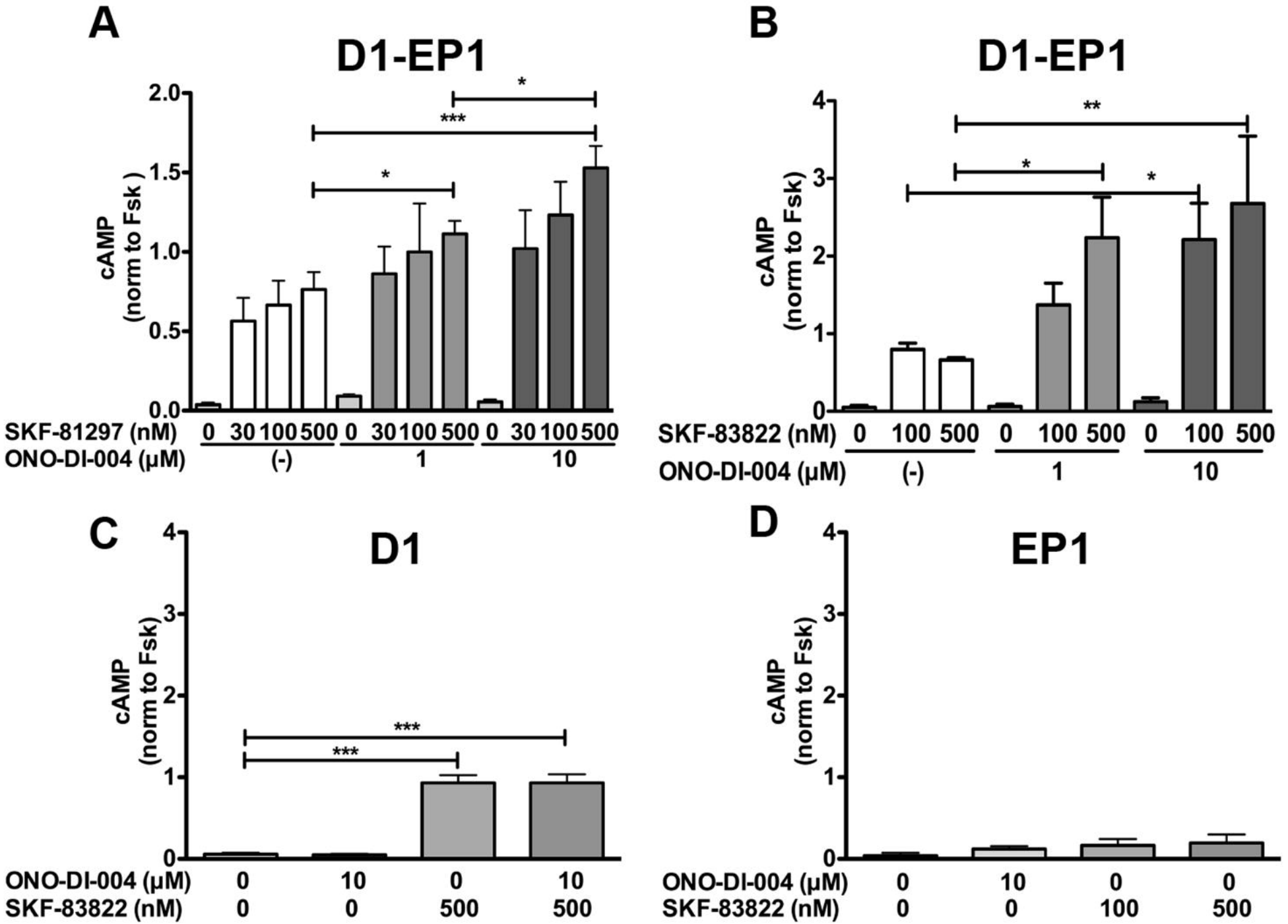

A
D1-EP1
cAMP (norm to Fsk)
2.0
1.5
1.0
0.5
0.0
*
***
*
SKF-81297 (nM) 0 30 100 500 0 30 100 500 0 30 100 500
ONO-DI-004 (μM) (-) 1 10
B
D1-EP1
cAMP (norm to Fsk)
4
3
2
1
0
**
*
*
SKF-83822 (nM) 0 100 500 0 100 500 0 100 500
ONO-DI-004 (μM) (-) 1 10
C
D1
cAMP (norm to Fsk)
4
3
2
1
0
***
***
ONO-DI-004 (μM) 0 10 0 10
SKF-83822 (nM) 0 0 500 500
D
EP1
cAMP (norm to Fsk)
4
3
2
1
0
ONO-DI-004 (μM) 0 10 0 0
SKF-83822 (nM) 0 0 100 500

Figure 2.

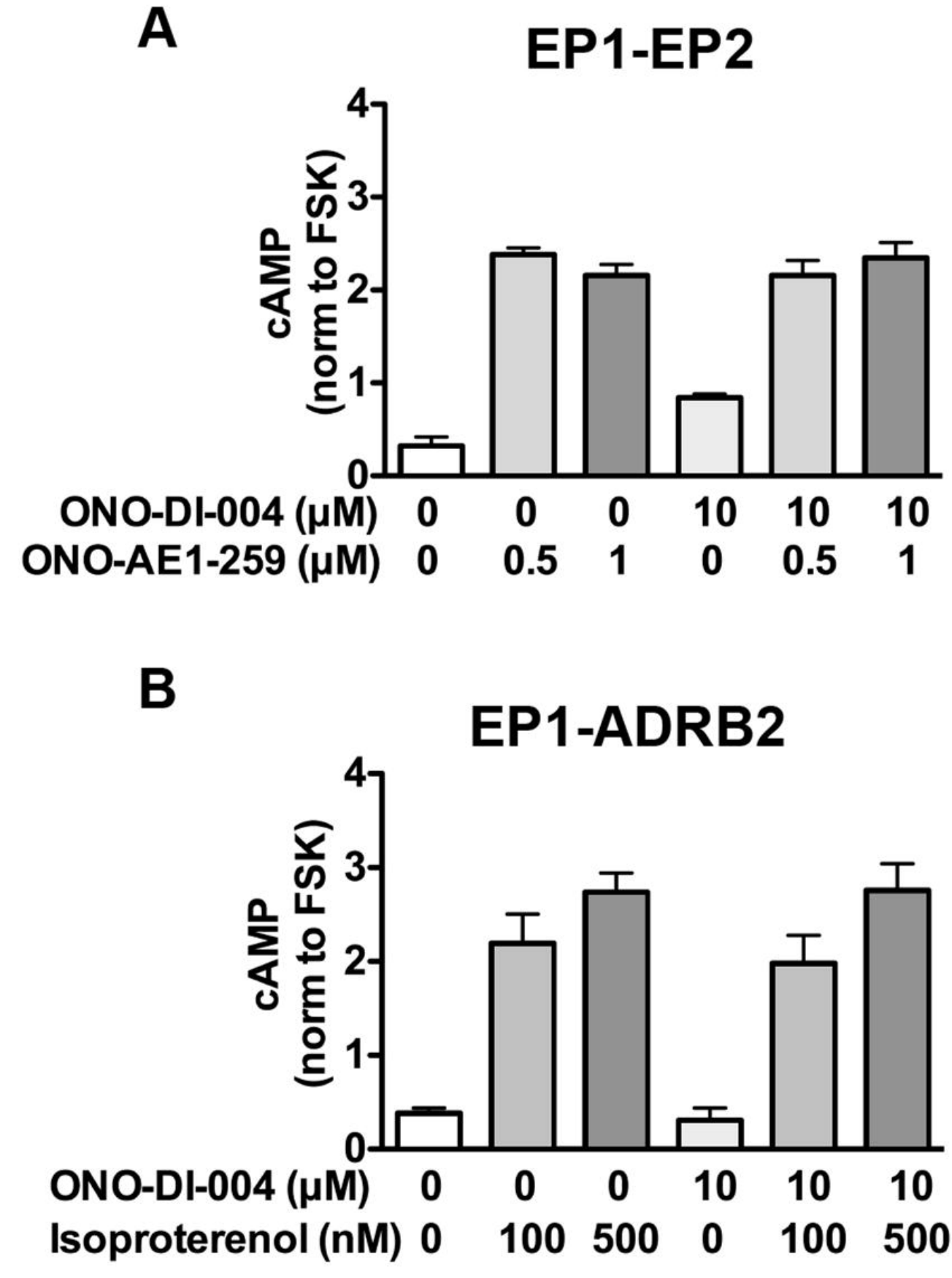

A
EP1-EP2
cAMP
(norm to FSK)
4
3
2
1
0
ONO-DI-004 (μM) 0 0 0 10 10 10
ONO-AE1-259 (μM) 0 0.5 1 0 0.5 1
B
EP1-ADRB2
cAMP
(norm to FSK)
4
3
2
1
0
ONO-DI-004 (μM) 0 0 0 10 10 10
Isoproterenol (nM) 0 100 500 0 100 500

Figure 3.

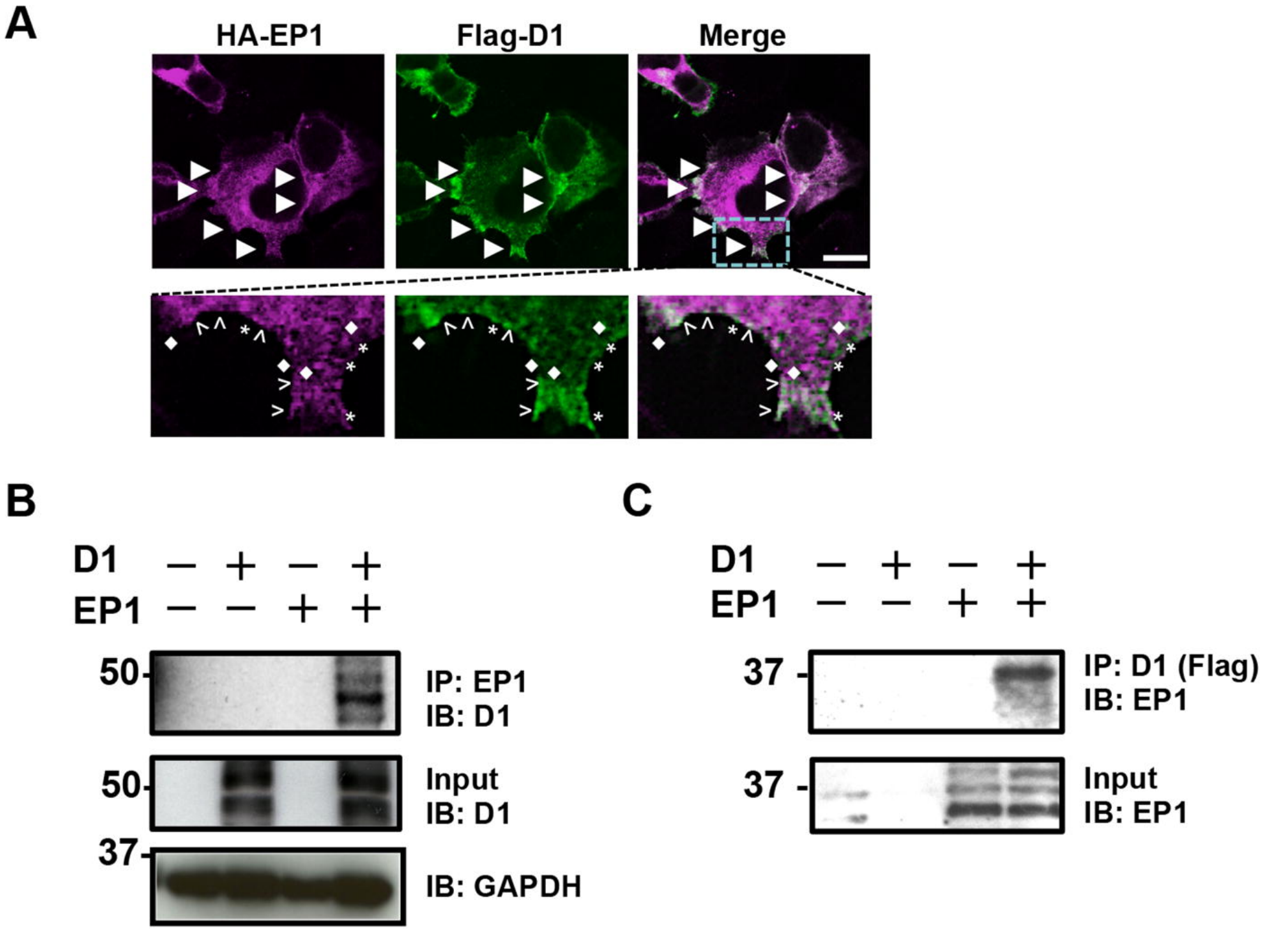

A
HA-EP1
Flag-D1
Merge
B
D1 – + – +
EP1 – – + +
50
IP: EP1
IB: D1
50
Input
IB: D1
37
IB: GAPDH
C
D1 – + – +
EP1 – – + +
37
IP: D1 (Flag)
IB: EP1
37
Input
IB: EP1

Figure 4.

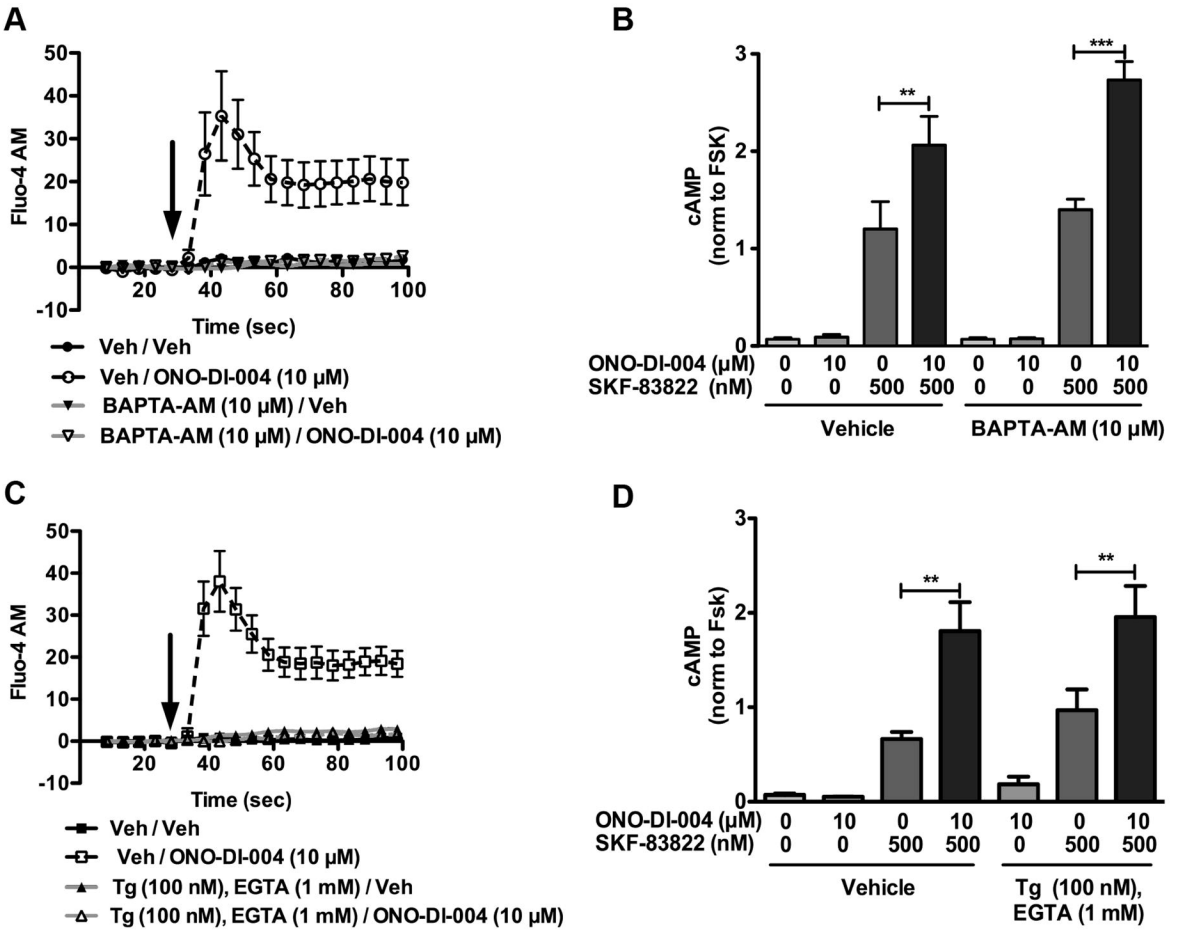

A
Fluo-4 AM
Time (sec)
Veh / Veh
Veh / ONO-DI-004 (10 μM)
BAPTA-AM (10 μM) / Veh
BAPTA-AM (10 μM) / ONO-DI-004 (10 μM)
B
cAMP (norm to FSK)
ONO-DI-004 (μM)
SKF-83822 (nM)
Vehicle
BAPTA-AM (10 μM)
C
Fluo-4 AM
Time (sec)
Veh / Veh
Veh / ONO-DI-004 (10 μM)
Tg (100 nM), EGTA (1 mM) / Veh
Tg (100 nM), EGTA (1 mM) / ONO-DI-004 (10 μM)
D
cAMP (norm to Fsk)
ONO-DI-004 (μM)
SKF-83822 (nM)
Vehicle
Tg (100 nM), EGTA (1 mM)

Figure 5.

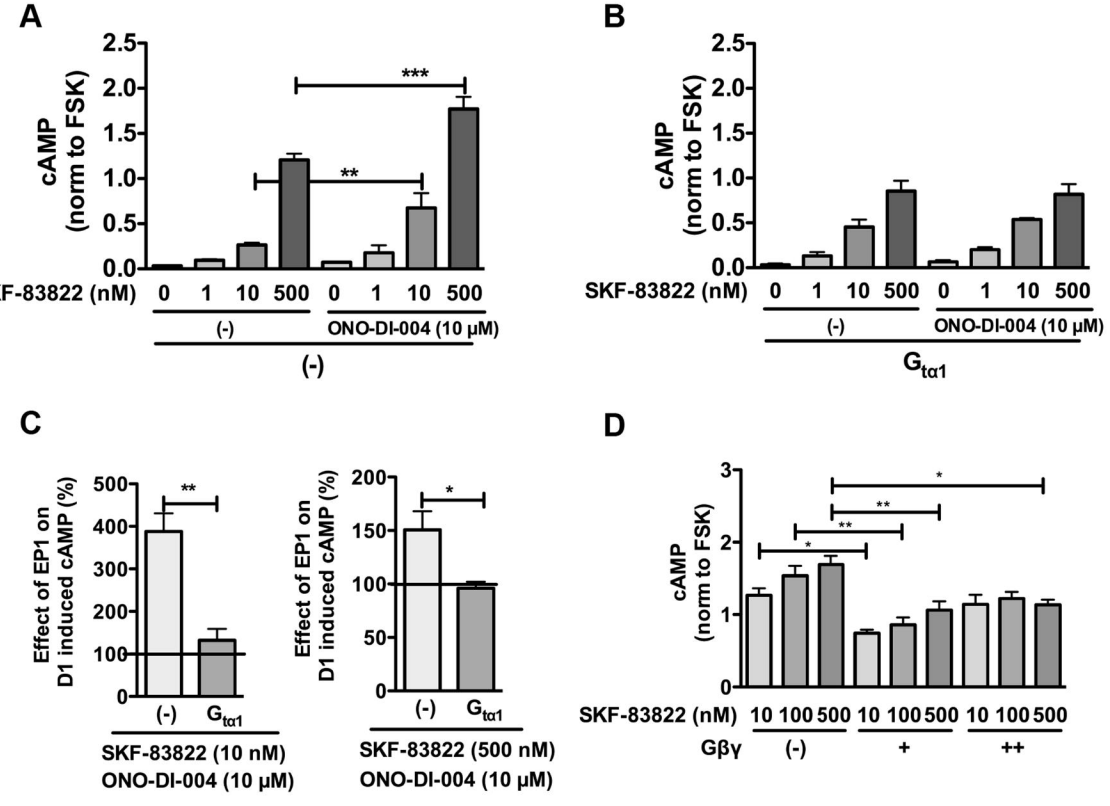
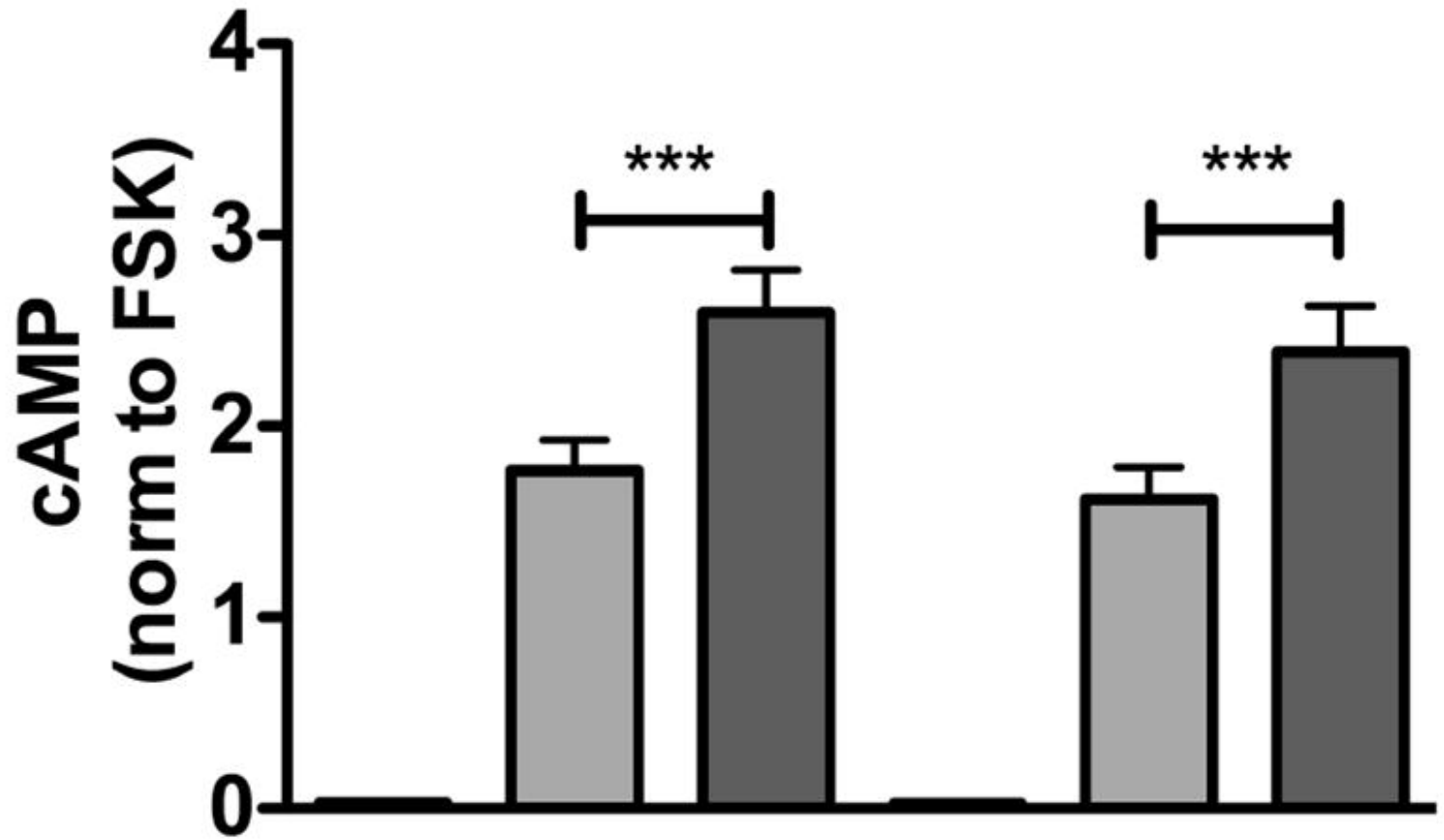
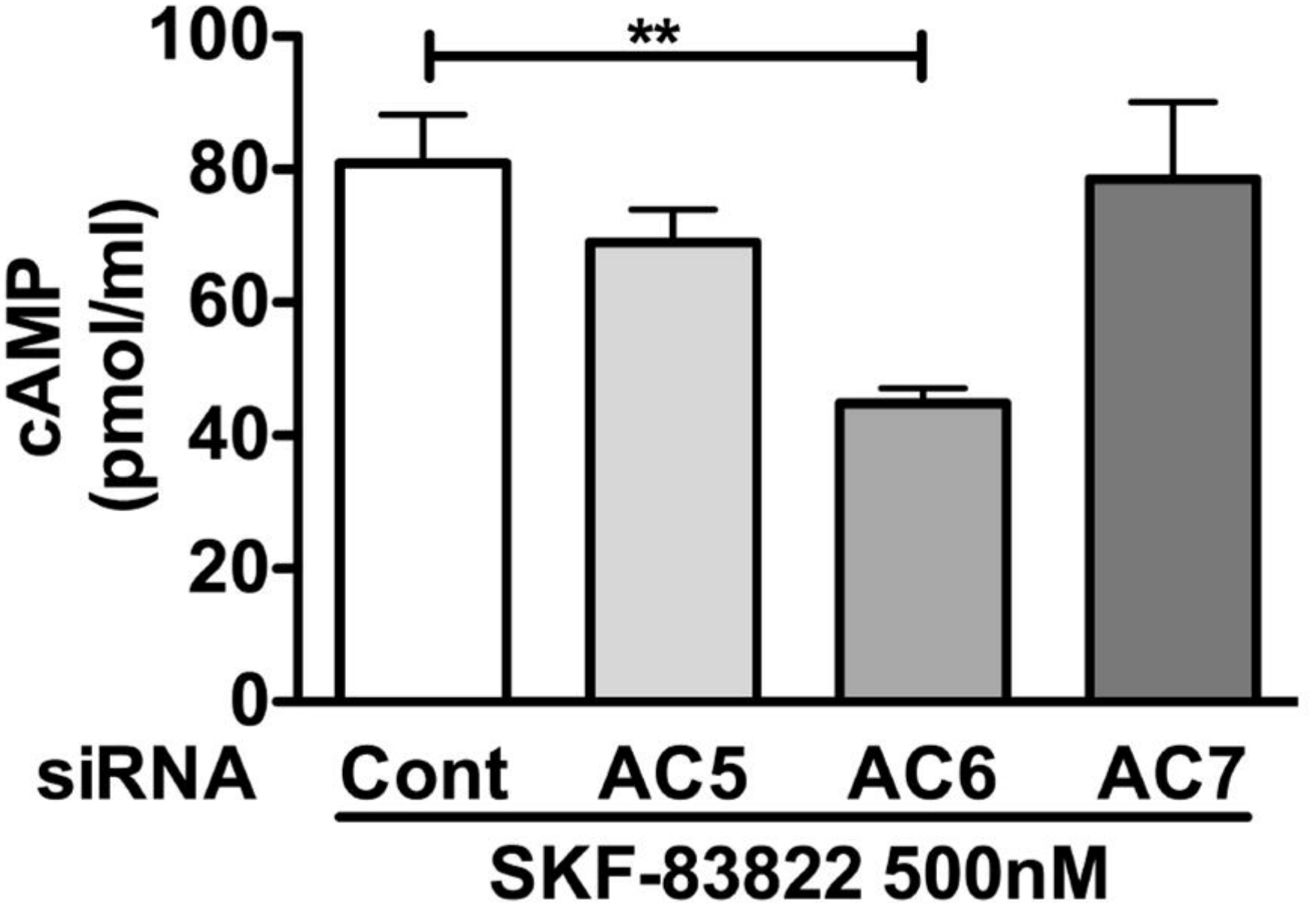

A
cAMP (norm to FSK)
2.5
2.0
1.5
1.0
0.5
0.0
***
**
SKF-83822 (nM) 0 1 10 500 0 1 10 500
(-)
ONO-DI-004 (10 μM)
(-)
B
cAMP (norm to FSK)
SKF-83822 (nM) 0 1 10 500 0 1 10 500
(-)
ONO-DI-004 (10 μM)
Gtα1
C
Effect of EP1 on D1 induced cAMP (%)
500
400
300
200
100
0
**
(-) Gtα1
SKF-83822 (10 nM)
ONO-DI-004 (10 μM)
Effect of EP1 on D1 induced cAMP (%)
200
150
100
50
0
*
(-) Gtα1
SKF-83822 (500 nM)
ONO-DI-004 (10 μM)
D
cAMP (norm to FSK)
3
2
1
0
*
**
**
*
SKF-83822 (nM) 10 100 500 10 100 500 10 100 500
Gβγ (-) + ++

Figure 6.



ONO-DI-004 (μM)	0	0	10	0	0	10
SKF-83822 (nM)	0	500	500	0	500	500
PTX (μg/ml)	0	0	0	0.2	0.2	0.2

Figure 7.


100
80
60
40
20
0
cAMP
(pmol/ml)
**
siRNA
Cont
AC5
AC6
AC7
SKF-83822 500nM

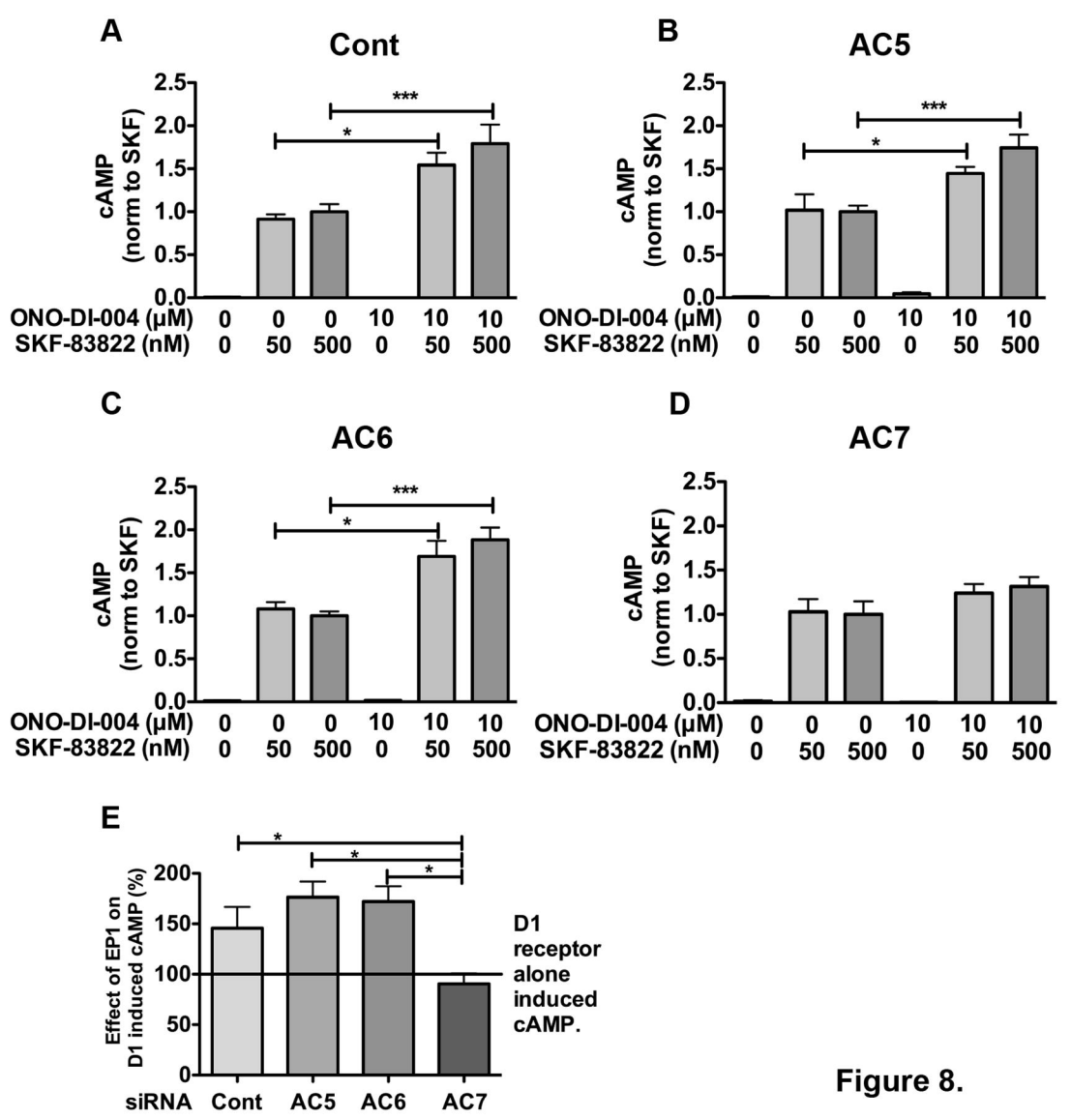

A
Cont
B
AC5
C
AC6
D
AC7
cAMP (norm to SKF)
2.5
2.0
1.5
1.0
0.5
0.0
*
***
ONO-DI-004 (μM) 0 0 0 10 10 10
SKF-83822 (nM) 0 50 500 0 50 500
E
Effect of EP1 on D1 induced cAMP (%)
200
150
100
50
0
siRNA Cont AC5 AC6 AC7
D1 receptor alone induced cAMP.

Figure 8.

Figure 9.