D₁-D₂ Dopamine Receptor Synergy Promotes Calcium Signaling via Multiple Mechanisms

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ABSTRACT

The D₁ dopamine receptor (D₁R) has been proposed to form a hetero-oligomer with the D₂ dopamine receptor (D₂R), which in turn results in a complex that couples to phospholipase C-mediated intracellular calcium release. We have sought to elucidate the pharmacology and mechanism of action of this putative signaling pathway. Dopamine dose-response curves assaying intracellular calcium mobilization in cells heterologously expressing the D₁ and D₂ subtypes, either alone or in combination, and using subtype selective ligands revealed that concurrent stimulation is required for coupling. Surprisingly, characterization of a putative D₁-D₂ heteromer-selective ligand, 6-chloro-2,3,4,5-tetrahydro-3-methyl-1-(3-methylphenyl)-1H-3-benzazepine-7,8-diol (SKF83959), found no stimulation of calcium release, but it did find a broad range of cross-reactivity with other G protein–coupled receptors. In contrast, SKF83959 appeared to be an antagonist of calcium mobilization. Overexpression of G₃ₛ with the D₁ and D₂ dopamine receptors enhanced the dopamine-stimulated calcium response. However, this was also observed in cells expressing G₃ₛ with only the D₁R. Inactivation of G₁ or G₃ with pertussis or cholera toxin, respectively, largely, but not entirely, reduced the calcium response in D₁R and D₂R cotransfected cells. Moreover, sequestration of G₁₃ subunits through overexpression of G protein receptor kinase 2 mutants either completely or largely eliminated dopamine-stimulated calcium mobilization. Our data suggest that the mechanism of D₁R/D₂R–mediated calcium signaling involves more than receptor-mediated G₃ₛ protein activation, may largely involve downstream signaling pathways, and may not be completely heteromer-specific. In addition, SKF83959 may not exhibit selective activation of D₁-D₂ heteromers, and its significant cross-reactivity to other receptors warrants careful interpretation of its use in vivo.

Introduction

Dopamine is a neurotransmitter that functions in the central nervous system to regulate neural processes that include motor control, cognition, and memory. Dysregulation of the dopamine (DA) system is associated with neurologic disorders such as Parkinson disease, schizophrenia, addiction, and attention deficit hyperactivity disorder. Five DA receptor (DAR) genes exist in mammals, each of which encodes a DAR subtype (D₁R–D₅R); these genes are grouped by structure and function into the D₁-like (D₁R and D₅R) and D₂-like (D₂R, D₃R, and D₄R) DAR families. The D₁-like receptors couple to the Gₛₒᵣl proteins to activate adenyl cyclase–mediated formation of cAMP, whereas the D₂-like receptors couple to the Gᵥₒᵣ proteins to inhibit adenyl cyclase (Sibley and Monsma, 1992; Missale et al., 1998). Several studies, however, have proposed DAR-mediated signaling pathways that do not involve activation of either Gᵥₒᵣ or Gₛₒᵣl proteins.

The first evidence for alternate signaling pathways came from multiple studies reporting “D₁-like” receptor stimulation of intracellular calcium mobilization, which was suggested to be a result of G₃ₛ-mediated activation of phospholipase C (PLC) (Mahan et al., 1990; Undie and Friedman, 1990; Wang et al., 1995; Pacheco and Jope, 1997). Subsequently, it was shown that in vitro cell cultures coexpressing the D₁R and D₂R could couple to intracellular calcium mobilization through the Gₛ₃ PLC-diacylglycerol

**ABBREVIATIONS:** CTX, cholera toxin; D₁R, dopamine receptor subtype; D₂R, dopamine receptor subtype; D₅R, D₂R long splice variant; D₇R, D₇R short splice variant; DA, dopamine; DAR, dopamine receptor; FDSS, Functional Drug Screening System; GRIK2, G protein receptor kinase 2; GPCR, G protein–coupled receptor; HEK293T, human embryonic kidney cells 293-tsa201; ICL3, third intracellular loop; l-Dopa, l-3,4-dihydroxyphenylalanine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NIMH, National Institute of Mental Health; PDS, Psychoactive Drug Screening Program; PLC, phospholipase C; PTX, pertussis toxin; SCH23390, (R)-(–)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; SKF83822, 6-chloro-2,3,4,5-tetrahydro-1-(3-methylphenyl)-3-(2-propenyl)-1H-3-benzazepine-7,8-diol; SKF83959, 6-chloro-2,3,4,5-tetrahydro-3-methyl-1-(3-methylphenyl)-1H-3-benzazepine-7,8-diol.
pathway (Lee et al., 2004; Rashid et al., 2007a). This calcium response required both coexpression and coactivation of both receptor subtypes. This led to the proposal of a "noncanonical" mechanism for DAR-mediated signaling wherein the D1R forms a heteromeric complex with the D2R and induces PLC-mediated intracellular calcium mobilization (Lee et al., 2004; Rashid et al., 2007b; Hasbi et al., 2011). The precise mechanism for this type of signaling and its prevalence in vivo, however, remain unclear.

In vivo, there is evidence both for (Surmeier et al., 1992, 1996; Lester et al., 1993; Ariano et al., 1997; Aizman et al., 2000; Lee et al., 2004) and against (Gerfen et al., 1990; Le Moine et al., 1991; Hersch et al., 1995; Le Moine and Bloch, 1995; Bertran-Gonzalez et al., 2008) the existence of neural cells coexpressing both D1R and D2R. Interestingly, some neurons that appear to coexpress D1R and D2R have neuronal projections that express only D1R or only D2R (Lee et al., 2004). This finding, along with the different methods of detection and visualization, may partially explain the incongruent reports of D1R and D2R colocalization. However, several recent studies using confocal FRET techniques argue for direct demonstration of the existence of D1-D2 heteromers in 10–20% of the cell bodies and presynaptic terminals of medium spiny neurons within the nucleus accumbens (Hasbi et al., 2009; Perreault et al., 2011, 2012a), and the two DARs have been shown to cointernalize after selective activation of either receptor (O’Dowd et al., 2005; So et al., 2005).

Interestingly, several agonists of the benzazepine family seem to exhibit differential effects on the D1R monomer compared with the proposed D1-D2 heteromer (Rashid et al., 2007b). One such compound, 6-chloro-2,3,4,5-tetrahydro-1-[(3-methylphenyl)-3-(2-propenyl)-1H-3-benzazepine-7,8-diol (SKF83882), has been proposed to selectively activate D1R-mediated cAMP production but have no effect on calcium mobilization (Rashid et al., 2007a,b). In contrast, another benzazepine, 6-chloro-2,3,4,5-tetrahydro-3-methyl-1-[(3-methylphenyl)-1H-3-benzazepine-7,8-diol (SKF83959), has been proposed to selectively activate the heteromer-mediated calcium release and have no effect on cAMP production (Rashid et al., 2007a,b; Hasbi et al., 2011). More recent studies have used this finding to interpret the results of systemic SKF83959 injections in mice, which resulted in increased Ca2+/calmodulin-independent protein kinase IIe phosphorylation and increased brain-derived neurotrophic factor expression in striatal neurons (Hasbi et al., 2009; Ng et al., 2010). It was also shown that expression of glutamate decarboxylase-67 and the vesicular transporter neuronal projections that express only D1R or only D2R (Lee et al., 2004; Rashid et al., 2007a). This calcium activation are multiple and complex and there is not, as yet, a selective pharmacology.

Materials and Methods

Human embryonic kidney 293-tesa201 (HEK293T) cells were a gift from Dr. Vania Ramakrishnan. A D1R expressing stable cell line was purchased from Codex Biosolutions, Inc. (Gaithersburg, MD). [3H]-N-methyl-DPAT (sodium salt, 7-C-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine, SCH23390 (80.5 Ci/mmol) and [3H]-N-methylspiperone (85.5 Ci/mmol) were obtained from PerkinElmer Life Sciences (Waltham, MA). Cell culture media and reagents were purchased from MediaTech/Cellogro (Manassas, VA). Cell culture flasks and materials and all assay plates were purchased from Sigma-Aldrich (St. Louis, MO) except where indicated.

Cell Culture and Transfection. HEK293T cells and D1R COX3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with a final concentration of 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 10 μg/ml gentamicin. Cells were incubated at 37°C, 5% CO2, and 90% humidity. They were passaged and plated mechanically using calcium-free Earle’s balanced salt solution and pelleted by centrifugation at 1000g for 10 minutes. For transfection studies, HEK293T cells were seeded in 150-mm plates at 10 × 106 cells per plate. After 24 hours, cells were transfected according to the manufacturer’s recommendations using Clontech’s CalPhos transfection kit (Clontech Laboratories, Inc., Mountain View, CA). The DAR plasmid constructs were FLAG-tagged rat D1R, D2R (D2 short splice variant), or D2LR (D2 long splice variant) in the pcDNA3 vector (Takebe et al., 1988; Monsma et al., 1990; Zhang et al., 1994) and D1R in pcDNA3.1(+) vector (Schetz and Sibley, 2001). Additional experiments were done using the G protein in the pcDNA3.1(+) vector (Missouri S&T cDNA Resource Center, Rolla, MO) and various functionally dominant negative G protein receptor kinase 2 (GRK2) mutants: GRK2 C-terminus 495–689 in pcDNA3(+), GRK2 K220R in pcDNA3(+), and empty pcDNA3.1(+) vector (Koch et al., 1994; Freedman et al., 1995). For all transfections, 5 μg of each DNA construct was used to transfect cells, with the exception of D1R, in which 10 μg was used.

Radioligand Binding Assays. Forty-eight hours after transfection, cells were dissociated from plates using calcium-free Earle’s balanced salt solution, and intact cells were collected by centrifugation at 800g for 10 minutes. Cells were resuspended and lysed using 5 mM Tris-HCl and 5 mM MgCl2 at pH 7.4 at 4°C. Cell lysate was pelleted by centrifugation at 20,000g for 30 minutes and resuspended in 5 mM Tris-HCl at pH 7.4; 100 μl of cell lysate (containing 8 μg of protein for D1R assays or 10 μg of protein for D2R assays) was incubated for 90 minutes at room temperature with various concentrations of [3H]-N-methyl-SCH23390 (D1R binding) or [3H]-N-methylspiperone (D2R binding) in a final reaction volume of 250 μl. Nonspecific binding was determined in the presence of 4 μM (+)-butaclamol. Bound ligand was separated from the unbound by filtration through a PerkinElmer Unifilter-96 GF/C 96-well microplate using the PerkinElmer Unifilter-96 Harvester, washing three times, 1 ml per well in ice-cold assay buffer. After drying, 50 μl of liquid scintillation cocktail (MicroScint FS, PerkinElmer) was added to each well, plates were sealed, and the plates were analyzed on a PerkinElmer TopCount NXT. For competition binding assays, a fixed concentration of 0.5 nM [3H]-N-methyl-SCH23390 was incubated with various concentrations of SKF83959, and the remainder of the assay was performed as described already herein. Ki values were calculated from observed IC50 values using the Cheng-Prusoff equation and a Kd value of 0.5 nM for SCH23390, as determined in independent
for radioligand binding inhibition by SKF83959 (10 μM). Forty-three G protein–coupled receptors (GPCRs) and neurotransmitter-related proteins were screened in the primary assay using radioligands with known binding properties. The percentage of inhibition was calculated by subtracting the percentage of specific binding in the presence of the test compound from the percentage of specific binding in the absence of the test compound (n = 4). Receptors whose corresponding radioligands had greater than 50% inhibition at 10 μM SKF83959 underwent secondary radioligand competition binding assays to generate full competition curves. Kᵢ determinations and receptor binding profiles were provided by the National Institute of Mental Health (NIMH) Psychoactive Drug Screening Program (PDSP), Contract HHSN-271-2008-00025-C. The NIMH PDSP is directed by Dr. Bryan L. Roth (University of North Carolina, Chapel Hill, NC) and by Project Officer Jamie Driscol (NIMH, Bethesda, MD). For experimental details, including radioligands used and associated Kᵢ values for each individual receptor, please refer to the PDSP website: http://pdsp.med.unc.edu/.

**Calcium Mobilization Assays.** HEK293T cells were transiently transfected as described; 24 hours after transfection, cells were plated in 384-well, optical, clear-bottom, black-walled plates (20 μl/well, 30,000 cells/well; Greiner Bio-One). Forty-eight hours after transfection, cells were incubated for 60 minutes at room temperature in the dark with Flu-8 NW calcium dye and an extracellular signal quencher to block any signal from extracellular calcium (Screen Quest Fluoro-8 NW Calcium Assay Kit; AAT Bioquest, Inc., Sunnyvale, CA), as recommended by the manufacturer. The plates were then treated with various concentrations of antagonist or agonists (diluted in the presence of 0.2 mM sodium metabisulfite) as indicated in the Results and figure legends. For agonist reads, plates were read kinetically in real-time (every 0.6 second) by recording a baseline read for 14 seconds before the addition of an agonist compound and then continually measured for 2 minutes after agonist addition. For agonist reads, plates were read kinetically in real-time (every 0.6 second) by recording a baseline reading for 20 seconds before the addition of that antagonist. Then, 3 minutes later, agonist compound was added, and the plates were read for an additional 3 minutes. All compound additions were done in unison using the 384-tip onboard robotics on a Functional Drug Screening System (FDSS) μCell (Hamamatsu, Bridgewater, NJ), and plates were continually read using the FDSS μCell from the bottom throughout the assay with an excitation wavelength of 480 nm and an emission wavelength of 540 nm. Data were recorded and quantified as maximum minus minimum (max–min) relative fluorescence units within the assay window using FDSS software. Data are expressed as a percentage of the control max–min relative fluorescence units for given studies as indicated in the figure legends. In these experiments, D₁R and D₂R receptor expression levels typically varied between 1 and 3 pmol/mg protein. We found that coexpressing both receptors sometimes affected their expression compared with expressing them alone (unpublished data). However, this did not affect the calcium mobilization response, which, although not studied in detail, appeared to require simply a minimum level of dual receptor expression.

**Statistical Analysis.** Data are expressed as a percentage of control values for individual experiments. Nonlinear regression of all data was conducted on GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA). Results are expressed as mean ± S.E.M.

See Supplemental Materials and Methods section for additional procedures.

**Results**

Previous studies have suggested that the D₁-D₂ receptor complex may signal as a heteromer and have implicated SKF83959 as a compound that may selectively activate this signaling complex (Lee et al., 2004; So et al., 2005; Rashid et al., 2007a,b). However, these findings have not been corroborated, and the mechanisms by which the D₁-D₂ receptor complex signals remain unclear. To investigate the apparent ability of D₁-D₂ receptor oligomerization to alter the G protein coupling of component receptors, we first transiently expressed the D₁R either alone or concurrently with either the short (D₂SR) or long (D₂LR) isoforms of the D₂R and measured intracellular calcium mobilization via kinetic fluorescence imaging. Preliminary immunoprecipitation experiments revealed that D₁-D₂ hetero-oligomers were indeed capable of forming under these expression conditions (Supplemental Fig. 1). When cells were transfected with the D₁R and D₂SR or the D₁R with D₂SR, a clear dose-dependent activation of calcium mobilization was observed in response to DA (Fig. 1). Importantly, we observed no difference in coupling efficacy or agonist potency between the short and long isoforms of the D₂R. However, when cells were transfected with any of the subtypes alone, the receptors failed to couple to calcium mobilization (Fig. 1). These data suggest that expression and activation of both the D₁R and D₂R are essential for coupling to calcium mobilization and signaling.

To investigate further that the activation of both receptor subtypes is required to stimulate calcium mobilization, we used receptor subtype–selective antagonists. Concentration response inhibition curves for the D₁R-selective (SCH23390) and the D₂R-selective (sulpiride) antagonists were generated for cells transfected with the D₁R and D₂R (Fig. 2, A and B). Cells were simultaneously stimulated with 1 μM DA and examined for calcium mobilization. We observed complete inhibition of the calcium signal with either SCH23390 or sulpiride treatment. The potencies of the antagonists (SCH23390 IC₅₀ ~8.0 nM, sulpiride IC₅₀ ~0.7 nM) are consistent with their known affinities for their selective subtypes as determined in our laboratory (unpublished data) as well as by other groups (Seeman and Van Tol, 1993; Millan et al., 2001). More
with D1R and D2R resulted in a calcium mobilization response, concurrent administration of a D1R-selective (SKF83822) protomer is required for calcium mobilization. As seen in Fig. 2, where the cotransfected cells were stimulated with SKF83822, further the coupling mechanism, subtype-selective agonists were used to determine whether indeed activation of both receptor subtypes is necessary for calcium mobilization. Taken together, these data indicate that stimulation of both receptor subtypes is necessary for calcium mobilization.

Figure 2. Inhibition of D1R + D2LR-mediated calcium mobilization by either D1R- or D2R-selective antagonists. HEK293T cells were transfected with D1R + D2LR as described and 24 hours later were plated in 384-well plates. Cells were incubated with the indicated concentrations of the D1R-selective antagonist SCH23390 (A) or the D2R-selective antagonist sulpiride (B) and then stimulated with an EC50 of DA (1 μM; SCH23390 IC50 = 8.0 nM, sulpiride, IC50 = 0.7 nM). Data are expressed as a percentage of the control (10 μM) DA response and are representative of two independent experiments performed with the same assay conditions on different days. Error bars indicate S.E.M. from multiple wells within the representative experiment.

Fig. 3. Stimulation of D1R + D2LR-mediated calcium mobilization by either D1R- or D2R-selective agonists. HEK293T cells were transfected with D1R + D2LR as described, plated 24 hours later in 384-well plates, and assayed for calcium accumulation the following day. Cells were stimulated with one of the following agonists as indicated: DA, the D1R-selective agonist SKF83822, the D2R-selective agonist quinpirole, or both SKF83822 and quinpirole (D1R + D2LR EC50 = 610.8 nM) combined. Control cells expressing the D1R, D2SR, or D2LR individually did not show a significant calcium response to concurrent agonist administration. Data are expressed as a percentage of control maximum DA-stimulated response and are representative of two independent experiments performed with the same assay conditions on different days. Error bars indicate S.E.M. from multiple wells within the representative experiment.

Importantly, complete inhibition of the calcium response is seen at antagonist concentrations that have no effect on the opposite receptor subtype. Thus, selectively blocking DA activation of either receptor subtype is sufficient to prevent calcium mobilization, further suggesting that both receptor protomers must be activated for this signaling to occur.

Whereas the studies using subtype-selective antagonists suggested that both D1R and D2R are required for calcium signaling, it might be possible that stabilizing one subtype into an inactive state within a heteromer might alter the conformation of the corresponding partner. Thus, to elucidate further the coupling mechanism, subtype-selective agonists were used to determine whether indeed activation of both protomers is required for calcium mobilization. As seen in Fig. 3, concurrent administration of a D1R-selective (SKF83822) and a D2R-selective (quinpirole) agonist to cells cotransfected with D1R and D2R resulted in a calcium mobilization response that nearly matched that of DA. In contrast, when D1R plus D2R-cotransfected cells were stimulated with quinpirole alone, no calcium mobilization was observed. Furthermore, when the cotransfected cells were stimulated with SKF83822, no calcium mobilization was seen at concentrations selective for D1R. A small response was observed at 10 μM, but this was at a concentration where SKF83822 loses receptor subtype selectivity and can begin to stimulate the D2R as well. Previous studies showed that SKF83822 has an affinity for D1R in the ~2 nM range and D2R in the ~200 nM range (O’Sullivan et al., 2004). Experiments done in our laboratory have demonstrated a D2R affinity that is greater than 10 μM (unpublished data), supporting the idea that the SKF83822-mediated calcium response seen at high concentrations is due to nonselective receptor activation. In addition, when cells were transfected with any of the subtypes individually, no signal was seen from any of the agonists (unpublished data). Taken together, these data indicate that stimulation of both receptor subtypes is necessary for calcium mobilization.

Previous studies suggested that SKF83959 may be a D1-D2 heteromer-selective compound, and a significant calcium response to this ligand has been reported in cells coexpressing the D1R and D2R (Lee et al., 2004; Rashid et al., 2007a,b; Beaulieu and Gainetdinov, 2011). This compound has also been reported to have seemingly paradoxical effects on the D1R, exhibiting both antagonist and agonist properties, depending on the system (Panchalingam and Undie, 2001; Cools et al., 2002; Zhang et al., 2005). In our current studies, we treated D1R and D2R cotransfected cells with SKF83959 and, surprisingly, were unable to elicit a calcium response (Fig. 4A). Furthermore, when SKF83959 was added in concert with the D2R selective agonist quinpirole, we were still unable to observe a significant calcium response. It should be noted that SKF83959 consistently failed to stimulate calcium mobilization even when this experiment was performed using different lots of compound from different vendors on separate days, as well as with different drug solvents (unpublished data). We also had one lot of compound chemically analyzed to verify its purity (unpublished data). To demonstrate that the SKF83959 (unpublished data).
likely have nanomolar $K_i$ values for these receptors. Table 1 summarizes the $K_i$ values for SKF83959 competition binding experiments against various G protein-coupled receptors. SKF83959 demonstrated very high (sub-100 nM) affinity for four of these GPCRs: the serotonergic receptor subtypes 5-HT2C, the adrenergic receptor subtype a2C, the D1, and D3 DAR subtypes, and the serotonin transporter. Notably, SKF83959 has also recently been shown to be a potent allosteric modulator of the $\sigma$-1 receptor (Guo et al., 2013). Taken together, these data indicate that SKF83959 has significantly high affinities for a wide number of receptors and thus caution should be taken when interpreting in vivo experimentation and the selectivity of this agent.

Whereas D1 and D2 receptors appear capable of signaling through calcium mobilization when both receptors are cotransfected, the D1R+D2LR system as described, plated 24 hours later in 384-well plates, and assayed for calcium accumulation the following day. (A) Cells were stimulated with one of the following conditions as indicated: DA, SKF83959, the D2R-selective agonist quinpirole, or both SKF83959 and quinpirole combined. (B) Cells were incubated with SKF83959 or the D2R-selective antagonist SCH23390, then stimulated with an $\sim EC_{50}$ of DA (1 $\mu M$). Data are expressed as a percentage of control maximum DA-stimulated response and are representative of two or three independent experiments performed with the same assay conditions on different days. Error bars indicate S.E.M. from multiple wells within the representative experiment. (C) HEK293 cells stably transfected with D1R (Codex Biosolutions, Inc., Gaithersburg, MD) were grown and membranes harvested as described in Materials and Methods. Membranes were incubated with various concentrations of SKF83959 and 0.5 nM $[^3]H$SCH23390 as indicated. Graph is representative of two independent experiments done on different days. Data are expressed as specific binding in units of fmol/mg. $K_i$ values were calculated using the Cheng-Prusoff equation and a radioligand $K_d$ value of 0.5 nM as determined via saturation binding isotherms (unpublished data). Average $K_i$ for SKF83959 on D1R was 2.6 nM ± 0.7.

### Table 1

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$K_i$ values for SKF83959 competition binding experiments against various G protein-coupled receptors were derived from radioligand binding competition curves generated against each of the above targets ($n = 2$) as described in Materials and Methods.
stimulated, the mechanism of transduction remains unclear. To understand more clearly the mechanisms involved, we tested the hypothesis that the receptors, perhaps within the context of a heteromer, may switch G protein-coupling selectivity and gain the ability to activate Gq. We first examined this possibility by overexpressing Gq, in cells expressing the D1R + D2R. Interestingly, the resulting DA-stimulated calcium signal was increased by 200% compared with cells transfected with the D1R + D2R alone (Fig. 5A). Expression of only the Gq protein in the absence of either receptor did not enable the ability of DA to stimulate calcium mobilization (Fig. 5A). In parallel studies, we examined how overexpression of Gq with the D1R or D2R alone could couple to intracellular calcium mobilization. Consistent with Fig. 1, cells transfected with D1R or D2R alone did not give a calcium response. However, when Gq was overexpressed, the D2R was able to elicit a DA-stimulated calcium signal in the absence of the D2R (Fig. 5B), although the calcium response was not as large as that seen with the D1R + D2R + Gq transfection (cf. Fig. 5, A and B). No such phenomenon was observed with the D2R. Taken together, these data suggest that the Gq protein may be involved in calcium mobilization mediated by a D1-D2 heteromer, but this interpretation is complicated by the fact that overexpression of Gq can also lead to monomeric D1R coupling.

Given our results with Gq overexpression, we re-evaluated SKF83959 stimulation of calcium mobilization under these conditions in the D1R and D2R coexpressed cells. We found that with Gq overexpression, SKF83959 is able to stimulate calcium mobilization in a manner similar to that of DA (Fig. 6A), whereas it is unable to stimulate such a response in cells lacking Gq, overexpression (Figs. 4 and 6A). Interestingly, SKF83959 was also able to stimulate calcium mobilization in cells expressing the D1R and overexpressing Gq, but not D1R alone (Fig. 6A). These results led us to test the antagonist sensitivity of the SKF83959 responses, as shown in Fig. 6B. We found that the D1R-selective antagonist SCH23390 could completely ablate SKF83959 stimulation of calcium mobilization in both D1R + Gq transfected and D1R + D2R + Gq transfected cells. However, in contrast to what we observed for DA stimulation of D1R + D2R cotransfected cells, the D2R-selective antagonist sulpiride was unable to block SKF83959 stimulation of calcium mobilization. These results suggest that overexpression of Gq enables SKF83959 to stimulate monomeric D1R present in the D1R and D2R cotransfected cells, rather than enabling it to gain function as a D1-D2 heteromeric-selective agonist.

Although the extant hypothesis, which our overexpression data support, is that Gq is central to the stimulation of calcium mobilization, the central question is whether direct coupling with a D1-D2 heteromer may be involved. An alternative hypothesis is that the D1R and D2R signal through downstream pathways that converge on the Gq protein or other components of the calcium mobilization process. To test whether D1-D2 synergistic signaling is independent of G1 or Gq protein function, we interfered with the activity of G1 and Gq by treatment with toxins. D1R and D2R cotransfected cells were incubated overnight in media containing pertussis toxin (PTX) to inhibit G1 protein function (Namkung et al., 2009) or cholera toxin (CTX) to interfere with Gq protein function (Mannoury la Cour et al., 2011). Cells were then assayed for calcium mobilization in response to DA stimulation. We found that treatment with CTX or PTX drastically, but not entirely, reduced the calcium response (Fig. 7). These data support the involvement of D1R-Gq- and D2R-G1-mediated mechanisms that majorly contribute to the calcium response in the D1R and D2R cotransfected cells.

Another possibility, however, may be that general G1-Gq “cross-talk” is occurring after receptor activation, which leads to PLC activation. Multiple cases of G1-Gq cross-talk in other receptor systems and cell types have been documented (Okajima et al., 1989; Carroll et al., 1995; Toms and Roberts, 1999; Rebres et al., 2011), and G1-Gq cross-talk in the D1-D2 receptor system could account for the PTX sensitivity of the calcium signal. In this model, any G1-linked GPCR, not just the D1R, would be able to support a Gq-mediated calcium response. To test this possibility, we used the D1R, a G1-linked DAR, which has not been found to form hetero-oligomers with the D1R (González et al., 2012). We cotransfected the D1R and D2R and compared the DA response with that in the D1R + D2R transfected cells (Fig. 8). In fact, the D1R did not support a calcium response in the presence of coexpressed D1R,
indicating that nonspecific G_{i}-G_{q} cross-talk, at least as previously described (Okajima et al., 1989; Carroll et al., 1995; Toms and Roberts, 1999; Rebres et al., 2011), does not explain the D_{1}-D_{2} heteromer-mediated calcium response.

The potential involvement of multiple G_{a}-proteins led us to also investigate other mechanisms by which D_{1}R and D_{2}R activation could stimulate calcium mobilization. Notably, G_{bg} subunits have been shown to increase cytoplasmic calcium concentrations by stimulating PLC\_b (Beaulieu and Gainetdinov, 2011). A recent publication reported that the ghrelin receptor-D2R dimer-linked calcium response was PTX sensitive, required PLC activity, and could be ablated by sequestering the G_{bg} subunits (Kern et al., 2012). To see whether G_{bg} plays a role in the D_{1}-D_{2} heteromer-mediated calcium release, we cotransfected the D1R and D2R with two different functionally dominant negative GRK2 mutants. The mutants we used were GRK2 K220R and the GRK2 C-terminal 495–689 peptide fragment (GRK2 c-term), both of which are unable to phosphorylate GPCRs but can bind to and sequester G_{bg} subunits (Koch et al., 1994; Freedman et al., 1995). We found that overexpression of GRK2 K220R was able to ablate completely DA-stimulated calcium mobilization in the D1R and D2R cotransfected cells (Fig. 9A). Similarly, overexpression of GRK2 c-term drastically reduced, but did not completely ablate, the DA-stimulated calcium response (Fig. 7B). These data suggest that the observed calcium mobilization occurring in response to D_{1}R and D_{2}R activation is largely dependent on free G_{bg} subunits.

**Discussion**

Receptor oligomers of many different GPCR types have been proposed to form homo- or hetero-oligomers with biochemical and functional characteristics that are unique to their oligomeric conformations (Ferre et al., 2009). These GPCR oligomers have been found not only to occur within a type of GPCR but also across different classes, families, types, and subtypes (Prinster et al., 2005). In addition to signaling, internalization and degradation of GPCRs in homo- and hetero-oligomers have been found to differ from their monomeric activities (Milligan, 2004; Terrillon and Bouvier, 2004).
It has been suggested that the coactivation of the D₁-D₂ complex causes a conformational change that results in the direct interaction between the C terminus of the D₁R and the third intracellular loop (ICL3) of the D₂R (ODowd et al., 2012). The ICL3 is the only region of difference between D₂L R and D₂S R, and there is evidence that it results in differences in the G protein coupling and signaling capabilities of each D₂R isoform (Kendall and Senogles, 2011). Recently, it was proposed that the ICL3 of D₂L R, but not the D₂S R, could form a complex with the D₁R (Pei et al., 2010), but the findings were based on the use of glutathione S-transferase and trans-activator of transcription–fused D₂R ICL3 fragments, which may not accurately mimic native receptor conformations and interactions. Later, it was shown that both D₂R splice isoforms were able to cointernalize with the D₁R (ODowd et al., 2012). Our results show that both D₂S R and D₂L R can couple with the D₁R to mobilize calcium (Fig. 1), and we have found that this is also true for both human (unpublished data) and rat DARs. We have also confirmed that both receptors must be expressed in the same cell and coactivated to induce a calcium response in HEK293T cells.

Our data also suggest that Gₙ protein signaling may play a role in the calcium response elicited by the D₁-D₂ complex. This was demonstrated by observing increased calcium mobilization in response to DA in cells transfected with the D₁R and D₂R plus Gₙ. However, we also observed that the D₁R alone may couple to Gₙ when the α subunit is expressed in significantly high amounts. This is likely due to the D₁R having a relatively low affinity for Gₙ; however, it may activate Gₙ-mediated calcium mobilization under conditions where Gₙ expression is very high. This is also supported by the enhanced calcium response we observed when the D₁R and D₂R are coexpressed in the presence of high levels of Gₙ protein, where the D₁R is the protomer within the heteromer that likely activates Gₙ; however, it may activate Gₙ-mediated calcium mobilization under conditions where Gₙ expression is very high. This is also supported by the enhanced calcium response we observed when the D₁R and D₂R are coexpressed in the presence of high levels of Gₙ protein, where the D₁R is the protomer within the heteromer that likely activates Gₙ; however, it may activate Gₙ-mediated calcium mobilization under conditions where Gₙ expression is very high. This is also supported by the enhanced calcium response we observed when the D₁R and D₂R are coexpressed in the presence of high levels of Gₙ protein, where the D₁R is the protomer within the heteromer that likely activates Gₙ; however, it may activate Gₙ-mediated calcium mobilization under conditions where Gₙ expression is very high.

2004; Prinster et al., 2005; Ferre et al., 2009; Missale et al., 2010). Like previously described receptor oligomers, it has been shown that the D₁R and D₂R can communoprecipitate with each other (Lee et al., 2004; Pei et al., 2010; Supplemental Fig. 1), and fluorescence imaging has shown that the two receptors cointernalize when one or the other receptor is stimulated (ODowd et al., 2005, 2012; So et al., 2005; Dziedzicka-Wasylew ska et al., 2006; Łukasiewicz et al., 2009). We have demonstrated that the calcium response is unique to cells that coexpress both D₁ and D₂ DARs and that the DARs must be costimulated, as an antagonist to either receptor blocks the transduction. However, the mechanism of action and whether heteromers or homomers form the functional units for calcium signaling remain unclear.

Fig. 9. GRK2 influence on DA-mediated D₁R + D₂R calcium mobilization. HEK293T cells were transiently transfected with D₁R + D₂R and either empty pcDNA vector the GRK2 catalytically inactive mutant GRK2 K220R (B; GRK2 c-term; D₁R + D₂R EC₅₀ = 90.4 nM, ECmax = 100% control; D₁R+D₂R + pcDNA EC₅₀ = 188.5 nM, ECmax = 106%; D₁R + D₂R + GRK2 c-term EC₅₀ = 288.1 nM, ECmax = 30% control, 70% inhibition), as indicated and described in Materials and Methods. Twenty-four hours later, cells were plated in 384-well plates and assayed the following day for calcium mobilization after stimulation by the indicated concentrations of DA. Data are expressed as a percentage of control maximum DA stimulation seen in cells transfected with D₁R + D₂R only and are representative of two or three independent experiments done with the same assay conditions on different days. Error bars indicate S.E.M. from multiple wells within the representative experiment.

It has also been suggested that SKF83959 may act as a D₁-D₂ heteromer-selective agonist, and it has been used as a putative heteromer-selective probe in vivo. However, these studies are not without controversy, as SKF83959 has a history of unusual pharmacology. Panchalingam and Undie (2001) found that SKF83959 inhibited D₁R-stimulated cAMP formation and also induced striatal intracellular calcium mobilization in rats and monkeys. It lacked the side effects typical to D₁R agonists that stimulate cAMP production but paradoxically seemed to cause typical D₁R agonist-like behaviors in rats (Perreault et al., 2010) and is an effective anti-Parkinsonian agent in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-lesioned monkeys unresponsive to L-Dopa (1-3,4-dihydroxyphenylalanine) (Andringa et al., 1999). In our hands SKF83959 did not stimulate a calcium response in cells...
transfected with both the D1R and D2R, despite the fact that it was active in binding to the D1R. In fact, it appeared to act as an antagonist of the DA-stimulated calcium response in D1R and D2R cotransfected cells. In contrast, when Gqa was overexpressed, SKF83959 stimulated a calcium response in cells cotransfected with the D1R and Gqa, as well as cells cotransfected with D1R, D2R, and Gqa. However, we observed that whereas the D1R-selective antagonist SCH23390 completely blocked the SKF83959-stimulated calcium response in both transfection conditions, the D2R-selective antagonist sulpiride was ineffective in the D1R and D2R cotransfection condition. This contrasts with sulpiride’s ability to block completely DA-stimulated calcium mobilization in the D1R and D2R cotransfected cells (cf. Figs. 2B and 6B). This finding suggests that SKF83959 is not activating the D1-D2 heteromer but rather is activating only D1R monomers that exist in the D1R and D2R cotransfected cells. This could be explained by the functionally selective or biased agonist properties of SKF83959 in that it can selectively activate D1R-Gqa signaling, provided there is sufficient Gqa present, but our current results do not support its ability to activate the D1-D2 heteromer.

It has also been proposed that D1-D2 heteromer activation via SKF83959 in vivo and in vitro results in increased Ca2+/calmodulin-dependent protein kinase IIα levels in the striatum and nucleus accumbens, further resulting in enhanced brain-derived neurotrophic factor expression and increased neuronal maturation and differentiation (Rashid et al., 2007a; Hasbi et al., 2009; Ng et al., 2010; Perreault et al., 2012b). Given that our experiments indicated that SKF83959 could not induce D1-D2 heteromer–selective calcium mobilization in a controlled cell environment, we conducted a single-point competition-binding screen against an array of 43 GPCRs and additional signaling proteins (Supplemental Table 1; Table 1). We observed that SKF83959 demonstrated considerably high affinity for multiple receptors and other signaling proteins, and we conducted secondary competition binding experiments on the ones for which it showed the highest affinity. Surprisingly, SKF83959 showed nanomolar affinities for many different GPCRs, including several serotonergic, adrenergic, dopaminergic, and muscarinic receptor subtypes (Table 1). This result, as well as our functional data, questions whether SKF83959 may be useful as a selective probe to study D1-D2 heteromer or even D1-like receptor signaling in vivo.

Our data also suggest that calcium signaling through the D1-D2 receptor complex is largely sensitive to Gi and Gs inhibition by PTX and CTX, respectively. This led us to investigate additional hypotheses for the mechanism of D1-D2 calcium signaling. Recently, Kern et al. (2012) showed that the ghrelin receptor could hetero-oligomerize with the D2R. This heteromer induced calcium release from internal cellular stores in a PLC-dependent and PTX-sensitive manner and seemed to require Gbg subunit activation. Previous studies have shown that GRK2 can bind to and sequester Gbg subunits (Koch et al., 1994), and catalytically inactive GRK2 mutants that retain Gbg binding have been used as tools to block Gbg signaling without the complication of added

Fig. 10. Various mechanisms of PLCβ activation that may occur when the D1R and D2R are coexpressed and coactivated.
receptor desensitization (Koch et al., 1994; Freedman et al., 1995). Our data demonstrated that the catalytically inactive GRK2 K220R mutant completely ablated the DA-stimulated calcium response in the D1R and D2R transfected cells, whereas GRK2 c-term (a truncated GRK2 protein that includes only the Gbg₃, binding domain) largely decreased the calcium response. Since activated Gbg₃ subunits can stimulate PLCβ activity (Camps et al., 1999), our results are consistent with the hypothesis that the DA-stimulated calcium response significantly involves Gbg₃ activation of PLCβ. Additionally, the N-terminal RGS domain of GRK2 has been shown to facilitate weak GTPase-activating protein-like activity on Gbg, inhibiting PLC activation. This may explain the difference in degree of calcium signal inhibition between the GRK2 K220R mutant and the truncated GRK2 c-term mutant (Carman et al., 1999). Therefore, the activation of PLCβ may be Gbg₃- as well as Gbg₃-dependent and due largely to synergistic crosstalk between the D1R and D2R.

Figure 10 represents several hypothetical signaling pathways for D1-D2 receptor-calcium signaling in HEK293T cells. Pathway A represents D1-D2 heterodimer activation of Gbg₃ leading to Gbg₃ activation of PLCβ, as has been hypothesized in the literature (Rashid et al., 2007b). Pathway B represents Gbg₃ activation of PLCβ, where free βγ subunits could arise through activation of either Gα, Gbg, or Gbg. Pathway C represents coactivation of D1R and D2R monomers and cross-talk between Gα and Gbg protein–mediated downstream signaling pathways, ultimately leading to PLCβ activation. Given that PTX and CTX can nearly eliminate the DA-stimulated calcium signaling, we believe that pathway A is largely inoperative in our system under basal conditions. Pathway C could readily account for the requirement for dual receptor activation, but the fact that Gbg₃ sequestration largely eliminates the DA calcium response suggests that pathway B is critically important. The PTX/CTX results further implicate Gα or Gbg, however, the requirement for dual receptor activation in pathway B is not completely clear. Certainly, additional work is required to answer these questions, but it is clear from these studies that D1-D2 receptors can dually activate calcium signaling through more than a single mechanism.

One additional consideration for D1-D2-calcium signaling, which does not necessarily exclude the possibility of heteromer formation, may involve the aggregation of the two DARs and their associated proteins in lipid rafts. Lipid rafts are a well-known but poorly understood platform for modulating certain protein-protein interactions in neurons as well as affecting GPCR ligand sensitivity, membrane trafficking, and signaling (Allen et al., 2007; Korade and Kenworthy, 2008; Björk and Svenningsson, 2011; Kong et al., 2011; Sebastião et al., 2011; Celver et al., 2012). Lipid rafts would readily enable cross-talk between the D1R and D2R and could assist in the multifaceted signaling profile of the D1-D2 receptor complex. In addition, differences in lipid raft composition, cell background, and assay detection may explain some of the differences observed between our data and the data generated by other groups. Despite the seeming complexity of the D1-D2 receptor signaling mechanisms, it may yet be useful to study how synergistic concurrent activation of the D1R and D2R may induce effects not seen when either receptor is expressed alone. This can be examined by coexpressing the dimeric (O'Dowd et al., 2012), and studying the effect of coactivation on the generation of a calcium signal. Additionally, a compound that can selectively bias both receptors toward a conformation that promotes PLC activation may be useful in providing a clearer understanding of the DAR system in vivo.

**Authorship Contributions**

*Participated in research design:* Chun, Free, Doyle, Sibley, Rankin, Huang.

*Conducted experiments:* Chun, Doyle, Rankin, Huang.

*Contributed new reagents or analytic tools:* Free.

*Performed data analysis:* Chun, Free, Doyle, Rankin, Huang.

*Wrote or contributed to the writing of the manuscript:* Chun, Free, Sibley, Rankin, Huang.

**References**


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