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D_1-D_2 Dopamine Receptor Synergy Promotes Calcium Signaling via Multiple Mechanisms[S]

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ABSTRACT

The D_1 dopamine receptor (D_1R) has been proposed to form a hetero-oligomer with the D_2 dopamine receptor (D_2R), 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 D_1 and D_2 subtypes, either alone or in combination, and using subtype selective ligands revealed that concurrent stimulation is required for coupling. Surprisingly, characterization of a putative D_1-D_2 heteromer-selective ligand, 6-chloro-2,3,4,5-tetrahydro-3-methyl-1-(3-methylphenyl)-1*H*-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 $G_{q\alpha}$ with the D_1 and D_2 dopamine receptors enhanced the dopamine-stimulated calcium response. However, this was also observed in cells expressing $G_{q\alpha}$ with only the D_1R. Inactivation of G_i or G_s with pertussis or cholera toxin, respectively, largely, but not entirely, reduced the calcium response in D_1R and D_2R cotransfected cells. Moreover, sequestration of $G_{\beta\gamma}$ 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 D_1R/D_2R–mediated calcium signaling involves more than receptor-mediated G_q protein activation, may largely involve downstream signaling pathways, and may not be completely heteromer-specific. In addition, SKF83959 may not exhibit selective activation of D_1-D_2 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 (D_1R–D_5R); these genes are grouped by structure and function into the D1-like (D_1R and D_5R) and D2-like (D_2R, D_3R, and D_4R) DAR families. The D1-like receptors couple to the $G_{s/olf}$ proteins to activate adenylyl cyclase–mediated formation of cAMP, whereas the D2-like receptors couple to the $G_{i/o}$ proteins to inhibit adenylyl 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 $G_{i/o}$ or $G_{s/olf}$ 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 G_q-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 D_1R and D_2R could couple to intracellular calcium mobilization through the G_q-PLC-diacylglycerol

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ABBREVIATIONS: CTX, cholera toxin; D_1R, D_1 dopamine receptor subtype; D_2R, D_2 dopamine receptor subtype; $D_{2L}R$, D_2R long splice variant; $D_{2S}R$, D_2R short splice variant; DA, dopamine; DAR, dopamine receptor; FDSS, Functional Drug Screening System; GRK2, G protein receptor kinase 2; GPCR, G protein–coupled receptor; HEK293T, human embryonic kidney cells 293-tsa201; ICL3, third intercellular loop; L-Dopa, L-3,4,-dihydroxyphenylaline; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydrapyridine; NIMH, National Institute of Mental Health; PDSP, 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-1*H*-3-benzazepine; SKF83822, 6-chloro-2,3,4,5-tetrahydro-1-(3-methylphenyl)-3-(2-propenyl)-1*H*-3-benzazepine-7,8-diol; SKF83959, 6-chloro-2,3,4,5-tetrahydro-3-methyl-1-(3-methylphenyl)-1*H*-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 D_1R forms a heteromeric complex with the D_2R 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 D_1R and D_2R. Interestingly, some neurons that appear to coexpress D_1R and D_2R have neuronal projections that express only D_1R or only D_2R (Lee et al., 2004). This finding, along with the different methods of detection and visualization, may partially explain the incongruent reports of D_1R and D_2R colocalization. However, several recent studies using confocal FRET techniques argue for direct demonstration of the existence of D_1-D_2 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 D_1R monomer compared with the proposed D_1-D_2 heteromer (Rashid et al., 2007b). One such compound, 6-chloro-2,3,4,5-tetrahydro-1-(3-methylphenyl)-3-(2-propenyl)-1*H*-3-benzazepine-7,8-diol (SKF83822), has been proposed to selectively activate D_1R-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)-1*H*-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 Ca^{2+}/calmodulin-dependent protein kinase IIα 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 glutamate transporters 1 and 2 in striatal neurons, when injected into rats, was altered by SKF83959 (Perreault et al., 2012b), which, again, was interpreted to be due to selective D_1-D_2 heteromer activation.

In the current study, we further investigated the biology and pharmacology of the proposed D_1-D_2 heteromer and the mechanism of calcium mobilization in heterologous expression systems. Although we found that coactivation of both D_1R and D_2R protomers is required for calcium mobilization to occur, there appear to be multiple mechanisms besides G_q activation through which this pathway is elicited. We also studied the functional characteristics of SKF83959 to determine its viability as a heteromer-selective in vivo ligand and found that it was significantly less selective than previously appreciated. In fact, we were not able to provide evidence for selective activation of the D_1-D_2 heteromer. These results indicate that D_1Rs and D_2Rs can synergize to induce calcium mobilization, although the mechanisms of activation are multiple and complex and there is not, as yet, a selective pharmacology.

Materials and Methods

Human embryonic kidney 293-tsa201 (HEK293T) cells were a gift from Dr. Vanitha Ramakrishnan. A D_1R expressing stable cell line was purchased from Codex Biosolutions, Inc. (Gaithersburg, MD). [^{3}H]*N*-methyl-(*R*)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH23390) (80.5 Ci/mmol) and [^{3}H]*N*-methylspiperone (85.5 Ci/mmol) were obtained from PerkinElmer Life Sciences (Waltham, MA). Cell culture media and reagents were purchased from MediaTech/Cellgro (Manassas, VA). Cell culture flasks and materials and all assay plates were purchased from Greiner Bio-One (Monroe, NC). SKF83959 and SKF83822 were purchased from Tocris Bioscience/RD Systems (Minneapolis, MN). All other compounds and buffer components were purchased from Sigma-Aldrich (St. Louis, MO) except where indicated.

Cell Culture and Transfection. HEK293T cells and D_1R CODEX 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% CO_2, and 90% humidity. They were passaged and plated mechanically using calcium-free Earle's balanced salt solution and pelleted by centrifugation at 1000*g* for 10 minutes. For transfection studies, HEK293T cells were seeded in 150-mm plates at 10×10^6 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 D_1R, $D_{2S}R$ (D_2 short splice variant), or $D_{2L}R$ (D_2 long splice variant) in the pCD-SRα vector (Takebe et al., 1988; Monsma et al., 1990; Zhang et al., 1994) and D_4R in pcDNA3.1(+) vector (Schetz and Sibley, 2001). Additional experiments were done using the G_q 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(+) (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 D_1R, 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 900*g* for 10 minutes. Cells were resuspended and lysed using 5 mM Tris-HCl and 5 mM $MgCl_2$ at pH 7.4 at 4°C. Cell lysate was pelleted by centrifugation at 20,000*g* 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 D_2R assays or 10 μg of protein for D_1R assays) was incubated for 90 minutes at room temperature with various concentrations of [^{3}H]*N*-methyl-SCH23390 (D_1R binding) or [^{3}H]*N*-methylspiperone (D_2R 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 PS; 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 [^{3}H]*N*-methyl-SCH23390 was incubated with various concentrations of SKF83959, and the remainder of the assay was performed as described already herein. K_i values were calculated from observed IC_{50} values using the Cheng-Prusoff equation and a K_d value of 0.5 nM for SCH23390, as determined in independent

saturation isotherms (unpublished data). Expression of the D_4R was determined in an identical assay format as that for the D_2R.

Competition Radioligand Binding Screen. A primary, single-point radioligand competition binding assay was performed to assay 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. K_i 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 K_d 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 Fluo-8 NW calcium dye and an extracellular signal quencher to block any signal from extracellular calcium (Screen Quest Fluo-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 antagonist 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 continuously 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, D_1R and D_2R 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 D_1-D_2 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 D_1-D_2 receptor complex signals remain unclear. To investigate the apparent ability of D_1-D_2 receptor oligomerization to alter the G protein coupling of component receptors, we first transiently expressed the D_1R either alone or concurrently with either the short ($D_{2S}R$) or long ($D_{2L}R$) isoforms of the D_2R and measured intracellular calcium mobilization via kinetic fluorescence imaging. Preliminary coimmunoprecipitation experiments revealed that D_1-D_2 hetero-oligomers were indeed capable of forming under these expression conditions (Supplemental Fig. 1). When cells were transfected with the D_1R and $D_{2L}R$ or the D_1R with $D_{2S}R$, 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 D_2R. 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 D_1R and D_2R 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 D_1R-selective (SCH23390) and the D_2R-selective (sulpiride) antagonists were generated for cells transfected with the D_1R and D_2R (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 IC_{50} ~8.0 nM, sulpiride IC_{50} ~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

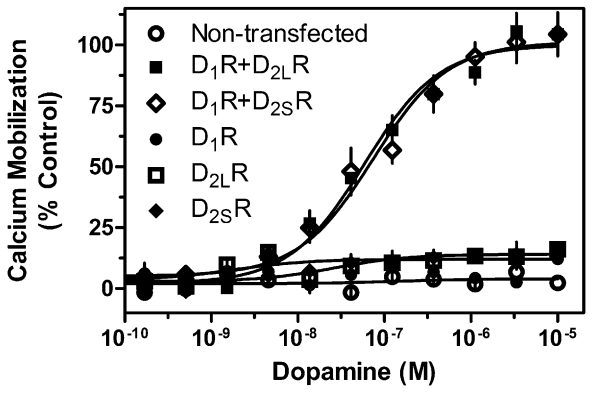

Fig. 1. Agonist-induced calcium mobilization in DA receptor–transfected cells. HEK293T cells were transiently transfected with D_1R, $D_{2L}R$, $D_{2S}R$, D_1R + $D_{2L}R$, or D_1R + $D_{2S}R$, as indicated and described in *Materials and Methods*. Twenty-four hours later, cells were plated in 384-well plates and assayed the following day for calcium mobilization after stimulation by DA (D_1R + $D_{2L}R$ EC_{50} = 73.8 nM, D_1R + $D_{2S}R$, EC_{50} = 58.2 nM). Data are representative of three independent experiments done with the same assay conditions on different days. Data are expressed as percentage of control, normalized to the maximum signal seen via DA stimulation of D_1R + $D_{2L}R$ transfected cells. Error bars indicate S.E.M. from multiple wells within the representative experiment.

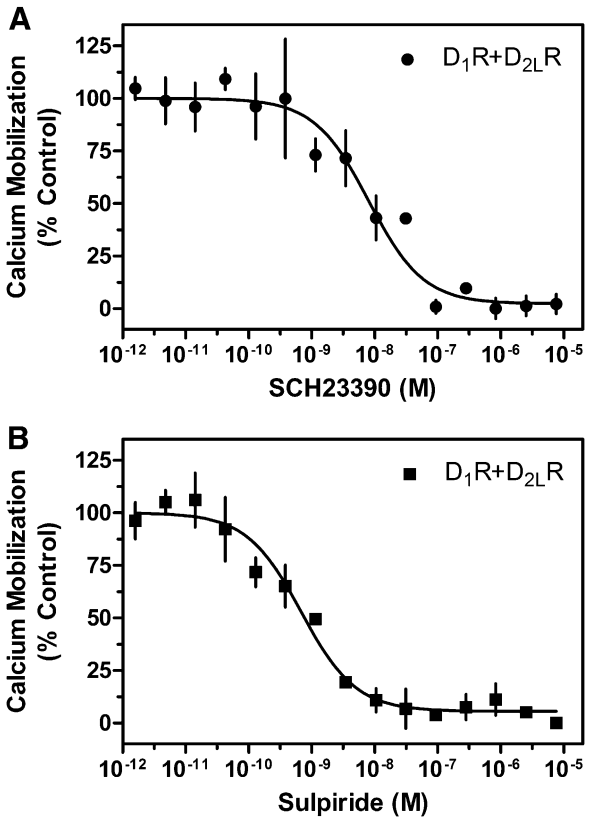

Fig. 2. Inhibition of $D_1R + D_{2L}R$–mediated calcium mobilization by either D_1R- or D_2R-selective antagonists. HEK293T cells were transfected with $D_1R + D_{2L}R$ as described and 24 hours later were plated in 384-well plates. Cells were incubated with the indicated concentrations of the D_1R-selective antagonist SCH23390 (A) or the D_2R-selective antagonist sulpiride (B) and then stimulated with an ~EC_{80} of DA (1 μM; SCH23390 IC_{50} = 8.0 nM, sulpiride, IC_{50} = 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.

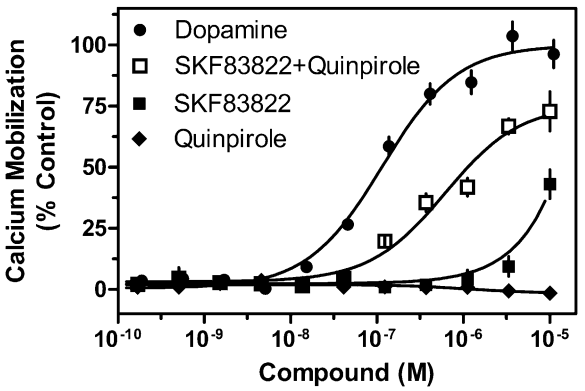

Fig. 3. Stimulation of $D_1R + D_{2L}R$–mediated calcium mobilization by either D_1R- or D_2R-selective agonists. HEK293T cells were transfected with $D_1R + D_{2L}R$ as described, plated 24 hours later in 384-well plates, and assayed for calcium accumulation the following day. Cells were stimulated with one of the following agonists as indicated: DA, the D_1R-selective agonist SKF83822, the D_2R-selective agonist quinpirole, or both SKF83822 and quinpirole ($D_1R + D_{2L}R$ EC_{50} = 610.8 nM) combined. Control cells expressing the D_1R, $D_{2S}R$, or $D_{2L}R$ 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 D_1R and D_2R 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 D_1R-selective (SKF83822) and a D_2R-selective (quinpirole) agonist to cells cotransfected with D_1R and D_2R resulted in a calcium mobilization response that nearly matched that of DA. In contrast, when D_1R plus D_2R–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 D_1R. 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 D_2R as well. Previous studies showed that SKF83822 has an affinity for D_1R in the ~2 nM range and D_2R in the ~200 nM range (O'Sullivan et al., 2004). Experiments done in our laboratory have demonstrated a D_2R 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 D_1-D_2 heteromer-selective compound, and a significant calcium response to this ligand has been reported in cells coexpressing the D_1R and D_2R (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 D_1R, 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 D_1R and D_2R cotransfected cells with SKF83959 and, surprisingly, were unable to elicit a calcium response (Fig. 4A). Furthermore, when SKF83959 was added in concert with the D_2R 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 compound was pharmacologically active in our hands, we performed two separate experiments. As shown in Fig. 4B, we stimulated calcium mobilization with DA and then

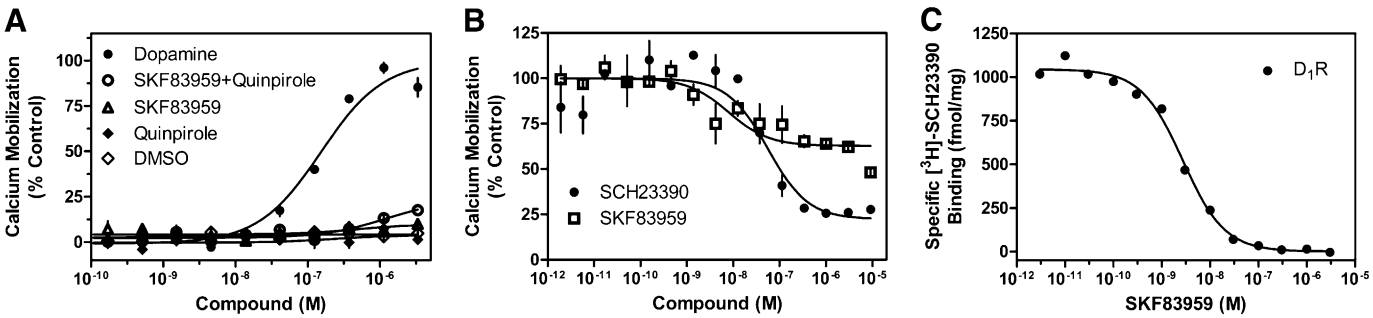

Fig. 4. Pharmacological characterization of SKF83959 on D_1R + $D_{2L}R$–mediated calcium mobilization. HEK293T cells were transfected with D_1R + $D_{2L}R$ as described, plated 24 hours later in 384-well plates, and assayed for calcium accumulation the following day. (A) Cells were stimulated with one of the following conditions as indicated: DA, SKF83959, the D_2R-selective agonist quinpirole, or both SKF83959 and quinpirole combined. (B) Cells were incubated with SKF83959 or the D_1R-selective antagonist SCH23390, then stimulated with an ~EC_{80} of DA (1 μM). 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 D_1R (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 value was calculated using the Cheng-Prushoff 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 D_1R was 2.6 nM ± 0.7.

dose dependently added either the D_1R-selective antagonist SCH23390 as a control (see Fig. 2A) or SKF83959 to see whether it might function as an antagonist in this system. In fact, it did, exhibiting even higher potency than SCH23390, although its efficacy of antagonism was less, exhibiting a maximum inhibition of ~50%. Finally, we performed a radioligand binding competition assay with SKF83959 and cells transfected with the D_1R (Fig. 4C). SKF83959 was able to compete potently and fully for radioligand binding to the D_1R. These experiments (Fig. 4, B and C) demonstrate that SKF83959 is active in binding to the monomeric D_1R, as well as active as a partial antagonist of the calcium response observed in D_1R and D_2R cotransfected cells. In contrast, it does not appear to function as an agonist with respect to stimulating calcium mobilization in the D_1R and D_2R cotransfected cells.

Given the apparent discrepancies of our findings with some previous studies (Lee et al., 2004; Rashid et al., 2007b; Hasbi et al., 2011) and the possibility that SKF83959 may not be as selective as previously thought, we sought to screen its selectivity against various GPCRs. This was accomplished through collaboration with the NIMH Psychoactive Drug-Screening Program (http://pdsp.med.unc.edu). For the primary screen, a single-point radioligand binding competition experiment was performed with 10 μM SKF83959 as a competitor against an appropriate receptor-specific radioligand of known properties. Forty-three GPCRs and signaling proteins were screened this way, and 20 of them resulted in >50% inhibition at 10 μM SKF83959 (Table 1). In contrast, 23 GPCR targets were found to have <50% inhibition at 10 μM SKF83959 and were therefore considered relatively "inactive/low affinity" for SKF83959 (Supplemental Table 1). The 20 "active" receptors/proteins underwent secondary radioligand competition binding experiments to generate full competition curves for SKF83959 and K_i values for these receptors were determined and are shown in Table 1. Of note is that the serotonin 5-HT2A, 5-HT2B, 5-HT2C, 5-HT5A, and 5-HT6 receptors; the adrenergic α2A, α2B, and α2C receptors; the D_1 D_2, and D_5 DARs; and the serotonin transporter all have nanomolar K_i values. SKF83959 demonstrated very high (sub-100 nM) affinity for four of these GPCRs: the serotonergic receptor subtypes 5-HT2C, the adrenergic receptor subtype α2C, the D_1, and D_5 DAR subtypes, and the serotonin transporter. Notably, SKF83959 has also recently been shown to be a potent allosteric modulator of the σ-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 D_1 and D_2 receptors appear capable of signaling through calcium mobilization when both receptors are

TABLE 1

SKF93959 competition binding experiments against various G protein–coupled receptors

K_i values were derived from radioligand binding competition curves generated against each of the above targets (n = 2) as described in *Materials and Methods*.

Target	SKF83959 K_i	S.E.M.
	nM	
5-HT1A	1648.0	352.3
5-HT2A	246.6	32.1
5-HT2B	405.0	145.1
5-HT2C	32.8	13.3
5-HT5A	277.8	141.8
5-HT6	546.0	56.0
α1A	1290.5	154.5
α1D	1115.5	232.4
α2A	323.7	120.6
α2B	163.1	17.8
α2C	31.1	7.6
D_1R	1.7	0.8
D_2R	567.0	150.0
D_3R	1018.3	109.8
D_4R	1975.7	756.4
D_5R	4.0	0.1
H2	1699.3	640.3
M4	5238.5	1985.5
M5	3484.0	114.0
SERT	365.6	79.2

α1A, α-adrenergic receptor subtype 1A; α1D, α-adrenergic receptor subtype 1D; α2A, α-adrenergic receptor subtype 2A; α2B, α-adrenergic receptor subtype 2B; α2C, α-adrenergic receptor subtype 2C; 5-HT1A, serotonergic receptor subtype 1A; 5-HT2A, serotonergic receptor subtype 2A; 5-HT2B, serotonergic receptor subtype 2B; 5-HT2C, serotonergic receptor subtype 2C; 5-HT5A, serotonergic receptor subtype 5A; 5-HT6, serotonergic receptor subtype 6; M4, muscarinic receptor subtype 4; M5, muscarinic receptor subtype 5; SERT, serotonin transporter.

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 G_q. We first examined this possibility by overexpressing $G_{q\alpha}$ in cells expressing the D_1R + D_2R. Interestingly, the resulting DA-stimulated calcium signal was increased by 200% compared with cells transfected with the D_1R + D_2R alone (Fig. 5A). Expression of only the $G_{q\alpha}$ 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 $G_{q\alpha}$ with the D_1R or D_2R alone could couple to intracellular calcium mobilization. Consistent with Fig. 1, cells transfected with D_1R or D_2R alone did not give a calcium response. However, when $G_{q\alpha}$ was overexpressed, the D_1R was able to elicit a DA-stimulated calcium signal in the absence of the D_2R (Fig. 5B), although the calcium response was not as large as that seen with the D_1R + D_2R + $G_{q\alpha}$ transfection (cf. Fig. 5, A and B). No such phenomenon was observed with the D_2R. Taken together, these data suggest that the G_q protein may be involved in calcium mobilization mediated by a D_1-D_2 heteromer, but this interpretation is complicated by the fact that overexpression of $G_{q\alpha}$ can also lead to monomeric D_1R coupling.

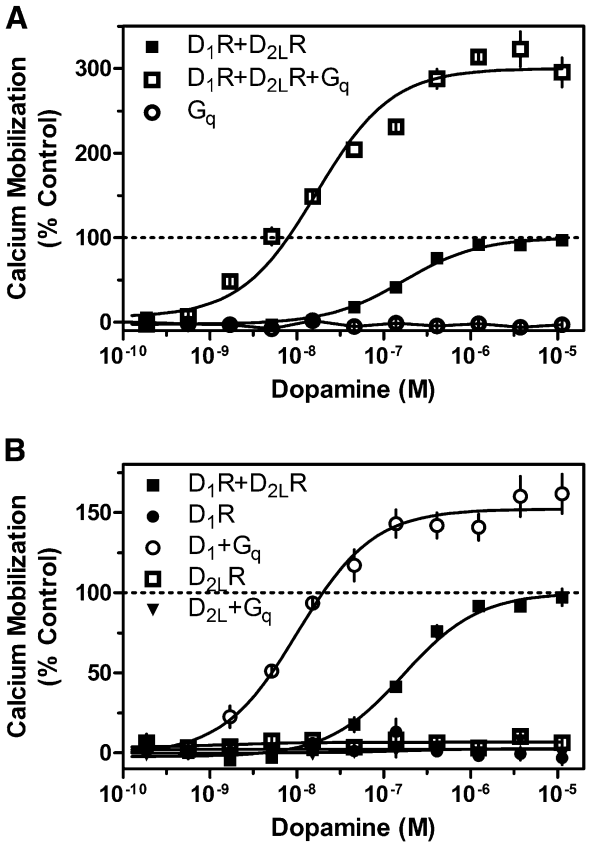

Fig. 5. Influence of $G_{q\alpha}$ protein overexpression on D_1R + $D_{2L}R$–mediated calcium mobilization. (A) HEK293T cells were transfected with D_1R + $D_{2L}R$ with and without $G_{q\alpha}$ or with $G_{q\alpha}$ alone (D_1R + D_2R EC_{50} = 168.3 nM, EC_{max} = 100%; D_1R + D_2R + G_q EC_{50} = 16.8 nM, EC_{max} = 300.1%). (B) HEK293T cells were transfected with D_1R + $D_{2L}R$, D_1R, or D_2R with and without $G_{q\alpha}$ (D_1R + G_q EC_{50} = 10.3 nM, EC_{max} = 152.2%). 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 for D_1R + $D_{2L}R$ alone 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.

Given our results with $G_{q\alpha}$ overexpression, we re-evaluated SKF83959 stimulation of calcium mobilization under these conditions in the D_1R and D_2R coexpressed cells. We found that with $G_{q\alpha}$ 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 $G_{q\alpha}$ overexpression (Figs. 4 and 6A). Interestingly, SKF83959 was also able to stimulate calcium mobilization in cells expressing the D_1R and overexpressing $G_{q\alpha}$, but not D_1R 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 D_1R-selective antagonist SCH23390 could completely ablate SKF83959 stimulation of calcium mobilization in both D_1R + $G_{q\alpha}$ transfected and D_1R + D_2R + $G_{q\alpha}$ transfected cells. However, in contrast to what we observed for DA stimulation of D_1R + D_2R cotransfected cells, the D_2R-selective antagonist sulpiride was unable to block SKF83959 stimulation of calcium mobilization. These results suggest that overexpression of $G_{q\alpha}$ enables SKF83959 to stimulate monomeric D_1R present in the D_1R and D_2R cotransfected cells, rather than enabling it to gain function as a D_1-D_2 heteromeric-selective agonist.

Although the extant hypothesis, which our overexpression data support, is that G_q is central to the stimulation of calcium mobilization, the central question is whether direct coupling with a D_1-D_2 heteromer may be involved. An alternative hypothesis is that the D_1R and D_2R signal through downstream pathways that converge on the G_q protein or other components of the calcium mobilization process. To test whether D_1-D_2 synergistic signaling is independent of G_i or G_s protein function, we interfered with the activity of G_i and G_s by treatment with toxins. D_1R and D_2R cotransfected cells were incubated overnight in media containing pertussis toxin (PTX) to inhibit G_i protein function (Namkung et al., 2009) or cholera toxin (CTX) to interfere with G_s 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 D_1R-G_s– and D_2R-G_i–mediated mechanisms that majorly contribute to the calcium response in the D_1R and D_2R cotransfected cells.

Another possibility, however, may be that general G_i-G_q "cross-talk" is occurring after receptor activation, which leads to PLC activation. Multiple cases of G_i-G_q 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 G_i-G_q cross-talk in the D_1-D_2 receptor system could account for the PTX sensitivity of the calcium signal. In this model, any G_i-linked GPCR, not just the D_2R, would be able to support a G_q-mediated calcium response. To test this possibility, we used the D_4R, a G_i-linked DAR, which has not been found to form hetero-oligomers with the D_1R (González et al., 2012). We cotransfected the D_1R and D_4R and compared the DA response with that in the D_1R + D_2R transfected cells (Fig. 8). In fact, the D_4R did not support a calcium response in the presence of coexpressed D_1R,

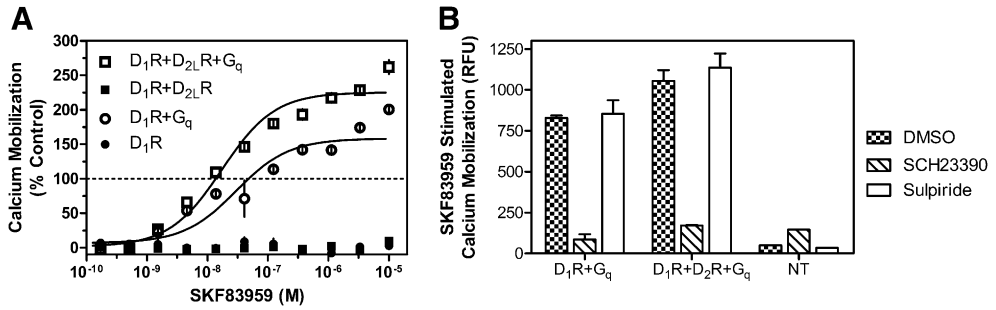

Fig. 6. SKF83959 stimulates D_1R-dependent calcium mobilization in the presence of $G_{q\alpha}$. HEK293T cells were transfected with D_1R + $D_{2L}R$, $G_{q\alpha}$ or with $G_{q\alpha}$ alone as described, plated 24 hours later in 384-well plates, and assayed for calcium accumulation the following day. (A) Cells were stimulated with SKF83959. The line at 100% denotes the maximal DA response of D_1R + $D_{2L}R$ cells. (B) Cells were incubated with the D_1R-selective antagonist SCH23390 (1 μM) or the D_2R-selective antagonist sulpiride (1 μM) and then stimulated with an ~EC_{80} of SKF83959 (100 nM). Error bars indicate S.E.M. from multiple wells within the representative experiment, which was replicated twice with similar results. DMSO, dimethylsulfoxide.

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_α-proteins led us to also investigate other mechanisms by which D_1R and D_2R activation could stimulate calcium mobilization. Notably, $G_{\beta\gamma}$ subunits have been shown to increase cytoplasmic calcium concentrations by stimulating PLCβ (Beaulieu and Gainetdinov, 2011). A recent publication reported that the ghrelin receptor-D_2R dimer-linked calcium response was PTX sensitive, required PLC activity, and could be ablated by sequestering the $G_{\beta\gamma}$ subunits (Kern et al., 2012). To see whether $G_{\beta\gamma}$ plays a role in the D_1-D_2 heteromer-mediated calcium release, we cotransfected the D_1R and D_2R 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 $G_{\beta\gamma}$ 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 D_1R and D_2R 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_1R and D_2R activation is largely dependent on free $G_{\beta\gamma}$ 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,

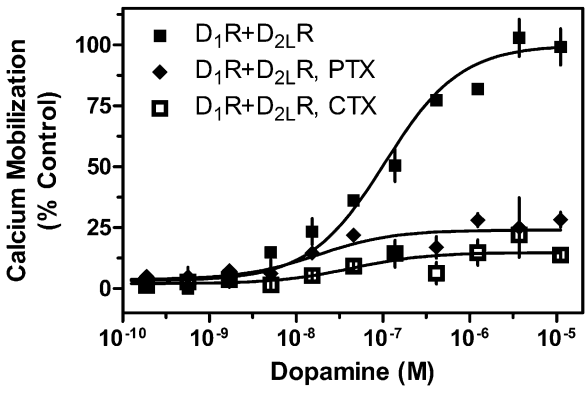

Fig. 7. G protein dependency of D_1R + $D_{2L}R$–mediated calcium mobilization. HEK293T cells were transfected with D_1R + $D_{2L}R$. Cells were incubated overnight in 1 μg/ml PTX or 1 μg/ml CTX; 48 hours post transfection, cells were assayed for calcium mobilization by stimulation with the indicated concentrations of DA (CTX EC_{max} = 14%, inhibition = 86% control, PTX EC_{max} = 24%, inhibition = 76% control). Data are expressed as a percentage of control maximum DA stimulation seen in untreated D_1R + $D_{2L}R$ cells 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.

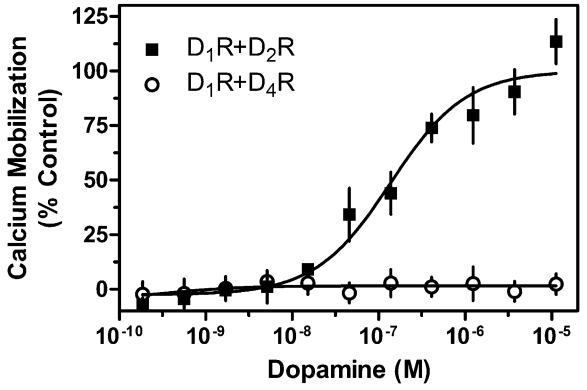

Fig. 8. Dopamine does not elicit a calcium response in cells co-expressing the D_1R and D_4R. HEK293T cells were transiently transfected with D_1R + $D_{2L}R$ or D_1R + D_4R, as indicated and described in *Materials and Methods*. Twenty-four hours later, cells were plated in 384-well plates and assayed for calcium mobilization through stimulation by the indicated concentrations of DA. Data are expressed as a percentage of control maximum DA stimulation seen in cells transfected with D_1R + $D_{2L}R$ only (EC_{50} = 162.0 nM) and are representative of two or three independent experiments done with the same assay conditions on different days. Expression of the D_4R was confirmed using radioligand binding assays as described in *Materials and Methods* and was similar to that of the D_2R. Error bars indicate S.E.M. from multiple wells within the representative experiment.

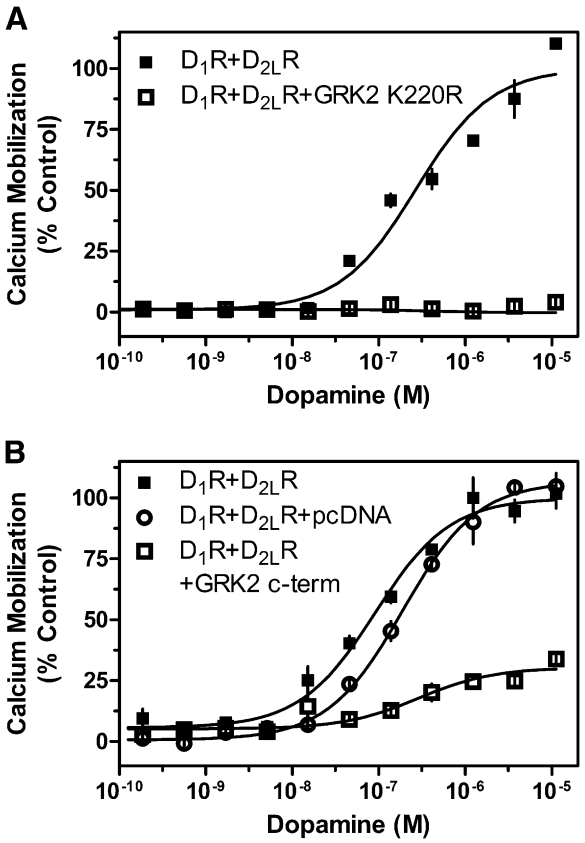

Fig. 9. GRK2 influence on DA-mediated D_1R + $D_{2L}R$ calcium mobilization. HEK293T cells were transiently transfected with D_1R + $D_{2L}R$ and either empty pcDNA vector the GRK2 catalytically inactive mutant GRK2 K220R (A) (D_1R + D_2R EC_{50} = 269.1 nM) or the GRK2 C-terminal 495–689 fragment (B; GRK2 c-term; D_1R + D_2R EC_{50} = 90.4 nM, EC_{max} = 100% control; D_1R+D_2R + pcDNA EC_{50} = 188.5 nM, EC_{max} = 106%; D_1R + D_2R + GRK2 c-term EC_{50} = 288.1 nM, EC_{max} = 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 D_1R + $D_{2L}R$ 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.

2004; Prinster et al., 2005; Ferre et al., 2009; Missale et al., 2010). Like previously described receptor oligomers, it has been shown that the D_1R and D_2R can coimmunoprecipitate 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 (O'Dowd et al., 2005, 2012; So et al., 2005; Dziedzicka-Wasylewska et al., 2006; Łukasiewicz et al., 2009). We have demonstrated that the calcium response is unique to cells that coexpress both D_1 and D_2 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.

It has been suggested that the coactivation of the D_1-D_2 complex causes a conformational change that results in the direct interaction between the C terminus of the D_1R and the third intracellular loop (ICL3) of the D_2R (O'Dowd et al., 2012). The ICL3 is the only region of difference between $D_{2L}R$ and $D_{2S}R$, and there is evidence that it results in differences in the G protein coupling and signaling capabilities of each D_2R isoform (Kendall and Senogles, 2011). Recently, it was proposed that the ICL3 of $D_{2L}R$, but not the $D_{2S}R$, could form a complex with the D_1R (Pei et al., 2010), but the findings were based on the use of glutathione S-transferase and transactivator of transcription–fused D_2R ICL3 fragments, which may not accurately mimic native receptor conformations and interactions. Later, it was shown that both D_2R splice isoforms were able to cointernalize with the D_1R (O'Dowd et al., 2012). Our results show that both $D_{2S}R$ and $D_{2L}R$ can couple with the D_1R 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 G_q protein signaling may play a role in the calcium response elicited by the D_1-D_2 complex. This was demonstrated by observing increased calcium mobilization in response to DA in cells transfected with the D_1R and D_2R plus $G_{q\alpha}$. However, we also observed that the D_1R alone may couple to $G_{q\alpha}$ when the α subunit is expressed in significantly high amounts. This is likely due to the D_1R having a relatively low affinity for $G_{q\alpha}$; however, it may activate G_q-mediated calcium mobilization under conditions where G_q expression is very high. This is also supported by the enhanced calcium response we observed when the D_1R and D_2R are coexpressed in the presence of high levels of G_q protein, where the D_1R is the protomer within the heteromer that likely activates $G_{q\alpha}$ (Rashid et al., 2007b). In this model, it is hypothesized that the D_2R allosterically modulates the D_1R (Rashid et al., 2007b; Hasbi et al., 2011). We believe, however, that the enhanced calcium mobilization seen in the D_1R + D_2R + $G_{q\alpha}$ transfected cells is not due solely to D_1R monomer activation of G_q, as the degree of calcium mobilization (300% of control, Fig. 5A) is twice that seen in the D_1R-$G_{q\alpha}$ transfected cells (Fig. 5B). Interestingly, another study has also reported D_1R-mediated calcium release from internal stores in mouse cells lacking thymidine kinase transfected with the human D_1R (Liu et al., 1992), indicating that this is not an event particular to our experimental paradigm. Thus, although G_q may play a role in the apparent ability of the D_1-D_2 heteromer to couple to calcium signaling, this role may be dependent on the level of G_q protein expression, either on a total cellular basis, which would thus be cell-type dependent, or this signaling may be localized to specific membrane microdomains (see discussion to follow).

It has also been suggested that SKF83959 may act as a D_1-D_2 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 D_1R-stimulated cAMP formation and also induced striatal intracellular calcium mobilization in rats and monkeys. It lacked the side effects typical to D_1R agonists that stimulate cAMP production but paradoxically seemed to cause typical D_1R 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-tetrahydrapyridine)-lesioned monkeys unresponsive to L-Dopa (L-3,4,-dihydroxyphenylaline) (Andringa et al., 1999). In our hands SKF83959 did not stimulate a calcium response in cells

transfected with both the D_1R and D_2R, despite the fact that it was active in binding to the D_1R. In fact, it appeared to act as an antagonist of the DA-stimulated calcium response in D_1R and D_2R cotransfected cells. In contrast, when $G_{q\alpha}$ was overexpressed, SKF83959 stimulated a calcium response in cells cotransfected with the D_1R and $G_{q\alpha}$, as well as cells cotransfected with D_1R, D_2R, and $G_{q\alpha}$. However, we observed that whereas the D_1R-selective antagonist SCH23390 completely blocked the SKF83959-stimulated calcium response in both transfection conditions, the D_2R-selective antagonist sulpiride was ineffective in the D_1R and D_2R cotransfection condition. This contrasts with sulpiride's ability to block completely DA-stimulated calcium mobilization in the D_1R and D_2R cotransfected cells (cf. Figs. 2B and 6B). This finding suggests that SKF83959 is not activating the D_1-D_2 heteromer but rather is activating only D_1R monomers that exist in the D_1R and D_2R cotransfected cells. This could be explained by the functionally selective or biased agonist properties of SKF83959 in that it can selectively activate D_1R-G_q signaling, provided there is sufficient $G_{q\alpha}$ present, but our current results do not support its ability to activate the D_1-D_2 heteromer.

It has also been proposed that D_1-D_2 heteromer activation via SKF83959 in vivo and in vitro results in increased Ca^{2+}/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 D_1-D_2 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 D_1-D_2 heteromer or even D1-like receptor signaling in vivo.

Our data also suggest that calcium signaling through the D_1-D_2 receptor complex is largely sensitive to G_i and G_s inhibition by PTX and CTX, respectively. This led us to investigate additional hypotheses for the mechanism of D_1-D_2 calcium signaling. Recently, Kern et al. (2012) showed that the ghrelin receptor could hetero-oligomerize with the D_2R. This heteromer induced calcium release from internal cellular stores in a PLC-dependent and PTX-sensitive manner and seemed to require $G_{\beta\gamma}$ subunit activation. Previous studies have shown that GRK2 can bind to and sequester $G_{\beta\gamma}$ subunits (Koch et al., 1994), and catalytically inactive GRK2 mutants that retain $G_{\beta\gamma}$ binding have been used as tools to block $G_{\beta\gamma}$ signaling without the complication of added

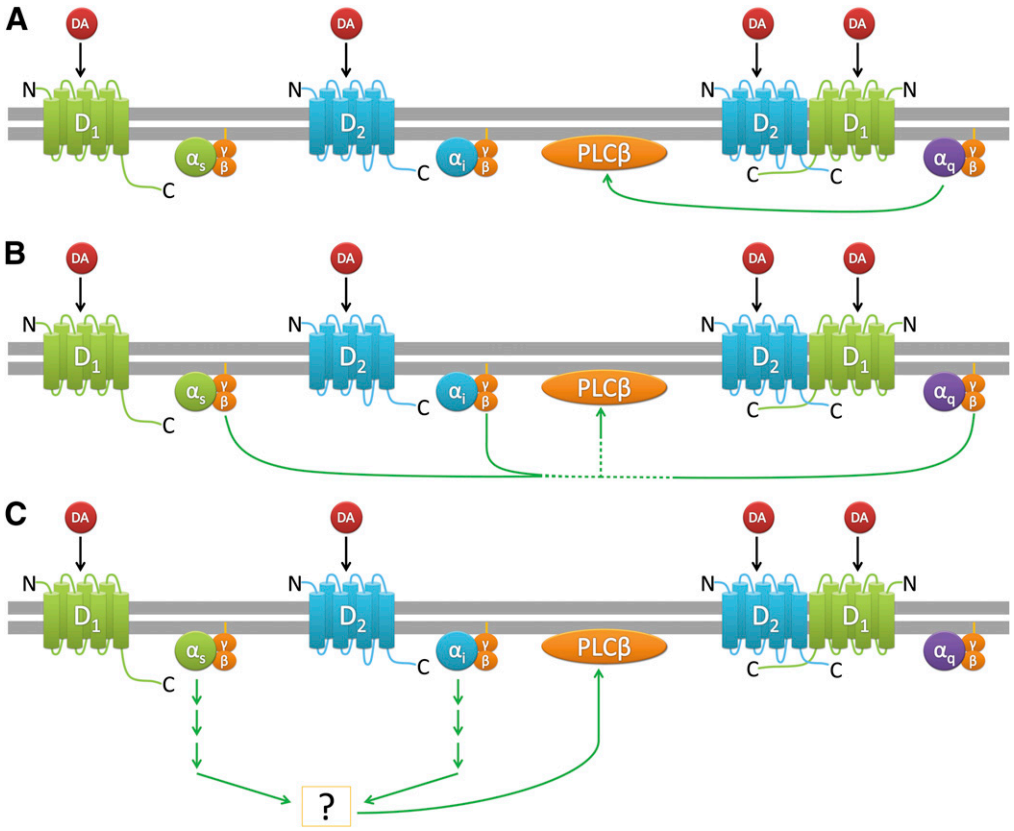

Fig. 10. Various mechanisms of PLCβ activation that may occur when the D_1R and D_2R are coexpressed and coactivated.

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 D_1R and D_2R transfected cells, whereas GRK2 c-term (a truncated GRK2 protein that includes only the $G_{\beta\gamma}$ binding domain) largely decreased the calcium response. Since activated $G_{\beta\gamma}$ subunits can stimulate PLCβ activity (Camps et al., 1992), our results are consistent with the hypothesis that the DA-stimulated calcium response significantly involves $G_{\beta\gamma}$ activation of PLCβ. Additionally, the N-terminal RGS domain of GRK2 has been shown to facilitate weak GTPase-activating protein-like activity on G_q, 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 $G_{q\alpha}$- as well as $G_{\beta\gamma}$-dependent and due largely to synergistic cross-talk between the D_1R and D_2R.

Figure 10 represents several hypothetical signaling pathways for D_1-D_2 receptor-calcium signaling in HEK293T cells. Pathway A represents D_1-D_2 heterodimer activation of G_q leading to $G_{q\alpha}$ activation of PLCβ, as has been hypothesized in the literature (Rashid et al., 2007b). Pathway B represents $G_{\beta\gamma}$ activation of PLCβ, where free β/γ subunits could arise through activation of either G_s, G_i, or G_q. Pathway C represents coactivation of D_1R and D_2R monomers and cross-talk between G_s and G_i 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 $G_{\beta\gamma}$ sequestration largely eliminates the DA calcium response suggests that pathway B is critically important. The PTX/CTX results further implicate G_s or G_i; 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 D_1-D_2 receptors can dually activate calcium signaling through more than a single mechanism.

One additional consideration for D_1-D_2-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 D_1R and D_2R and could assist in the multifaceted signaling profile of the D_1-D_2 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 D_1-D_2 receptor signaling mechanisms, it may yet be useful to study how synergistic concurrent activation of the D_1R and D_2R may induce effects not seen when either receptor is expressed alone. This can be examined by coexpressing mutants of the the D_1R and D_2R, which have been reported to be unable to form dimers (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.

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