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SKF-83959 is not a highly-biased functionally selective D_1 dopamine receptor ligand with activity at phospholipase C

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Abstract

SKF-83959 [6-chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine] is reported to be a functionally selective dopamine D_1 receptor ligand with high bias for D_1-mediated phospholipase C (PLC) versus D_1-coupled adenylate cyclase signaling. This signaling bias is proposed to explain behavioral activity in both rat and primate Parkinson's disease models, and a D_1-D_2 heterodimer has been proposed as the underlying mechanism. We have conducted an in-depth pharmacological characterization of this compound in dopamine D_1 and D_2 receptors in both rat brain and heterologous systems expressing human D_1 or D_2 receptors. Contrary to common assumptions, SKF-83959 is similar to the classical, well-characterized partial agonist SKF38393 in all systems. It is a partial agonist (not an antagonist) at adenylate cyclase *in vitro* and *ex vivo*, and is a partial agonist in D_1-mediated β-arrestin recruitment. Contrary to earlier reports, it does not have D_1-mediated effects on PLC signaling in heterologous systems. Because drug metabolites can also contribute, its 3-N-demethylated analog also was synthesized and tested. As expected from the known structure-activity relationships of the benzazepines, this compound also had high affinity for the D_1 receptor and somewhat higher intrinsic activity than the parent ligand, and also might contribute to *in vivo* effects of SKF-83959. Together, these data

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Conflict-of-interest
Richard Mailman has interests in issued and pending patents related to dopamine D_1 receptor mechanisms that constitute a conflict of interest for which there is University oversight. None of the other authors report a conflict-of-interest.

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demonstrate that SKF-83959 is not a highly-biased functionally selective D_1 ligand, and that its reported behavioral data can be explained solely by its partial D_1 agonism in canonical signaling pathway(s). Mechanisms that have been proposed based on the purported signaling novelty of SKF-83959 at PLC should be reconsidered.

Keywords

SKF-83959; Dopamine D_1 receptor; Dopamine D_5 receptor; Phospholipase C; Adenylate cyclase; Partial agonist; Functional selectivity; Ligand bias; Rat striatum

Introduction

One of the major conceptual changes in pharmacology the last decade has been the awareness that some ligands after interacting with a single molecular target can differentially affect the signaling pathways engaged by the target. This phenomenon is now generally termed functional selectivity (Urban *et al.*, 2007), and the degree of differential activation is termed the bias of a ligand. One of the first GPCR receptor systems in which functional selectivity was demonstrated was for dopamine receptors (Lawler *et al.*, 1999; Mailman *et al.*, 1998). The potential therapeutic utility of this mechanism has probably been shown most clearly by aripiprazole, a compound with a mechanism of action clearly differentiated from other approved antipsychotic drugs (Lawler *et al.*, 1999; Shapiro *et al.*, 2003; Mailman, 2007; Mailman and Murthy, 2010). Although aripiprazole is a partial D_2 agonist in some systems, in other systems (e.g., behavioral, electrophysiological, GTPγS binding) it behaves as a pure antagonist, and in others may have very high intrinsic activity (Kikuchi *et al.*, 1995; Lawler *et al.*, 1999; Shapiro *et al.*, 2003). It also has markedly different potencies at different assays in the same cell systems unlike typical partial agonists (Shapiro *et al.*, 2003; Urban *et al.*, 2007). These reports are consistent with the hypothesis that aripiprazole is functionally selective at D_2 receptors, but are inconsistent with it being simply a partial agonist (Burris *et al.*, 2002). Aripiprazole has a clinical profile somewhat different than earlier antipsychotics, and its D_2 functional selectivity may explain at least part of its atypicality.

Canonical signaling of D_1-like receptors (D_1, D_5) is thought to involve coupling to the G proteins $G\alpha_{OLF}$ or $G\alpha_S$ to stimulate adenylate cyclase, whereas D_2-like family receptors (D_2, D_3, D_4) couple to $G\alpha_I$ or $G\alpha_O$ to inhibit adenylate cyclase (Mailman *et al.*, 2001; Neve *et al.*, 2004). Thus, the cAMP resulting from D_1 receptor activation could initiate a host of downstream cascades such as from activation of cAMP/protein kinase A (PKA) signaling. There has been data, however, that challenges this notion, and postulates that behavioral effects of D_1 agonists in rodents can be due not to canonical pathways (Gnanalingham *et al.*, 1995a; Gnanalingham *et al.*, 1995b), but rather phospholipase C (PLC)-mediated calcium elevation (Undie and Friedman, 1990; Undie *et al.*, 1994; Andringa *et al.*, 1999; O'Sullivan *et al.*, 2004). This has led to the hypothesis that a functionally selective D_1 ligand highly biased against cAMP signaling would have an improved therapeutic index [e.g., for antiparkinson effects (Taylor *et al.*, 1991; Mailman *et al.*, 2001) or cognitive enhancement

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(Arnsten *et al.*, 1994)], without undesirable effects such as seizures (Starr, 1996) or rapid tolerance (Asin and Wirtshafter, 1993; Gulwadi *et al.*, 2001).

There is a prevailing view that SKF-83959 [6-chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine] represents such a highly biased D_1 ligand. SKF-83959 is purported to be a biochemical antagonist at D_1-coupled adenylate cyclase. Conversely, it is proposed to stimulate phospholipase C via $G\alpha_Q$ based on the fact that it stimulated phosphatidylinositol-4,5-biphosphate hydrolysis in membranes of rat frontal cortex in a D_1 receptor-dependent manner (Jin *et al.*, 2003; Panchalingam and Undie, 2001; Zhen *et al.*, 2005), and also induced PLC-mediated calcium elevation through the $G\alpha_Q$ protein in D_1-D_2 receptor heterodimer-expressing cells (Rashid *et al.*, 2007b; Hasbi *et al.*, 2009). SKF-83959 is behaviorally active in rat and primate Parkinson's disease (PD) models via its action at D_1 receptors (Arnt *et al.*, 1992; Jin *et al.*, 2003), and has behavioral activity in a variety of species that is known to be induced by D_1 full or partial agonists, but not by D_1 antagonists (Gnanalingham *et al.*, 1995a). This has led to the hypothesis that the behavioral actions of SKF-83959 may be mediated by its unique actions at PLC/calcium signaling at D_1-D_2 heterodimers (Rashid *et al.*, 2007a; Hasbi *et al.*, 2009; Downes and Waddington, 1993; Deveney and Waddington, 1995; Fujita *et al.*, 2010; Perreault *et al.*, 2010; Arnt *et al.*, 1992). This widespread belief that SKF-83959 is the first highly biased D_1 ligand has led many to consider it an important probe for studying the mechanisms related to D_1 signaling (Zhang *et al.*, 2009; Yu *et al.*, 2008; Zhang *et al.*, 2007; Zhang *et al.*, 2005; Perreault *et al.*, 2011).

We had previously used this compound, and contrary to its widely-cited pharmacology, consistently found that SKF-83959 was a partial agonist at D_1-adenylate cyclase (Ryman-Rasmussen *et al.*, 2005). Although one recent report offered data suggesting that in at least one system SKF-83959 may not be a highly-biased PLC preferring ligand (Chun *et al.*, 2013), the recent literature is replete with use of this compound for its novel properties. A rigorous reevaluation of its pharmacology in a variety of heterologous and native D_1 systems is therefore needed. In addition, the structure of SKF-83959 suggests that the N-demethylated analog ("desmethylSKF") is a probable metabolite that might contribute to its pharmacology *in vivo*. We therefore synthesized and characterized this potential metabolite. Our data clearly show that rather than being a highly-biased functionally selective D_1 ligand, SKF-83959 actually is a typical partial agonist of low intrinsic activity much like the prototype D_1 partial agonist SKF38393. Our data suggest that many hypotheses based on the purported signaling bias must be reconsidered.

Material and Methods

Materials

Both SKF-83959 and desmethylSKF [6-chloro-7,8-hydroxy-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine] (Figure 1) were synthesized according to procedures described in the supplemental data (Supplemental Scheme 1). In addition, SKF-83959 was also purchased commercially from Sigma/RBI. Samples from both sources behaved identically. [^{3}H]-SCH23390 and [^{3}H]-N-methylspiperone (NMS) were purchased from Perkin-Elmer Life Sciences Inc. (Boston, MA). SKF38393, quinpirole, and SCH23390 were

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obtained from Sigma/RBI (Natick, MA). Ham's F-12, penicillin, streptomycin, and geneticin (G418) were from Invitrogen Co. (Carlsbad, CA). Rat brains were either obtained from Sprague-Dawley rats euthanized according to procedures approved by the institutional IACUC committees, or purchased frozen (Pel-Freez Biologicals, Rogers, AR). Goat anti-cAMP antibody was obtained from Dr. Gary Brooker (George Washington University, Washington D.C.), and the donkey anti-goat secondary antibody was immobilized to Biomagnetic Particles (BMP) according to the manufacturer's procedure (Polyscience, Inc. Warrington, PA) for the filtration. All other reagents and materials were from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

Receptor source

All experiments with rats were conducted under protocols approved by our Institutional Animal Care and Use Committee. Rat striatal tissue was obtained from male Sprague Dawley rats (250–400 g) obtained from Charles River Laboratories, and housed under a 12 hour light/dark cycle and given food and water *ad libitum*. For experiments, the rats were briefly restrained in DecapiCones (Braintree Scientific), and the head removed using a rat guillotine with freshly-sharpened blade. Whole brains were removed rapidly, chilled briefly in ice-cold 0.9% (w/v) sodium chloride solution, and sliced into 1.2 mm coronal sections with the aid of a dissecting block similar to that described by Heffner et al. (Heffner *et al.*, 1980). The striatum was dissected and frozen immediately on dry ice, and stored at −70° C until the day of the assay.

Two different cell lines were used. HEK-293 cells were grown in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. HEK-293 cells were stably or transiently transfected with human D_1 receptors using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA) in OptiMEM medium according to the manufacturer's protocol. For transient transfection, cells were incubated for 48 h after transfection and used for radioreceptor and functional assays. For stable transfection, the cells were split into new plates 24 h after transfection, and the culture media were replaced every two or three days with fresh selection media containing 500 μg/mL G418. When stably transfected colonies were visible, the entire plate was resuspended and transferred to a new plate. These cells were maintained in DMEM (Hyclone Laboratories, Inc., South Logan, UT) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 500 μg/mL G418 for the binding and functional experiments. CHO cells stably expressing the human D_1 or D_{2L} receptor were maintained in Ham's F-12 medium (Hyclone Laboratories, Inc., South Logan, UT) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 500 μg/mL G418.

Membrane homogenates

Transfected cells and fresh or frozen rat striata were homogenized by several manual strokes in a Wheaton Teflon-glass homogenizer in 5 mL ice-cold lysis buffer (2 mM HEPES, 2 mM EDTA, 1 mM DTT, protease inhibitor cocktail, pH 7.4). The homogenate was centrifuged at 27,000 g for 20 min, the supernatant was discarded, and the pellet was homogenized (20 strokes) in ice-cold buffer and centrifuged again. The pellet was used as the membrane fraction for radioreceptor assays.

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Radioreceptor assays

Human D_1 or D_{2L} receptor density was assessed with saturation binding assay with [^{3}H]-SCH23390 or [^{3}H]-NMS, respectively. For saturation binding assay, membrane homogenates in triplicate were incubated with several concentrations of a radioligand in binding buffer (50 mM HEPES, 4 mM $MgCl_2$, 0.1% ascorbic acid, pH 7.4) for 15 min at 37°C. The affinity of a test ligand was assessed by competitive binding assays in which triplicate wells were incubated with increasing concentrations of a test ligand and a fixed concentration of a radioligand. Non-specific binding for the D_1 or D_2 receptor was determined by parallel incubations with 1 μM SCH23390 or 1 μM haloperidol, respectively. Binding reactions were terminated by rapid filtration onto 96-well filter plates (PerkinElmer, Boston, MA). Scintillation fluid Microscint 20 (PerkinElmer) was then added to each well, and plates were counted for tritium on a TopCount (PerkinElmer). The total protein concentration of membrane preparations used in the saturation binding assays was determined using BioRad protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA).

Adenylate cyclase assays

Essentially following a published procedure (Watts *et al.*, 1993), striatal tissue was homogenized with eight manual strokes in a Wheaton-Teflon glass homogenizer in 5 mM HEPES buffer (pH 7.5) containing 2 mM EGTA (50 mL/g tissue). Following the addition and mixing of another 50 mL/g of HEPES buffer, a 20 μL aliquot of this tissue homogenate was added to a prepared reaction mixture (final volume of 100 μL) containing 0.5 mM ATP, 0.5 mM isobutylmethylxanthine, 1 mM cAMP, 2 mM $MgCl_2$, 100 mM HEPES buffer, 2 μM GTP, and test drug(s), 10 mM phosphocreatine and 5 U creatine phosphokinase. Triplicate determinations were performed for each drug concentration. The reaction proceeded for 15 min at 30° C and was terminated by the addition of 100 μL of 3% sodium dodecyl sulfate (SDS). Proteins and much of the non-cyclic nucleotides were precipitated by addition of 300 μL each of 4.5% $ZnSO_4$ and 10% $Ba(OH)_2$. Samples were centrifuged (10,000 g for 8–9 min), and the supernatants assayed as described below.

For the studies in heterologous systems, we used two different assay methods to insure that assay issues were not affecting our results. Thus, both radioimmunoassay and the GloSensor™ cAMP system (Promega Corporation, Madison, WI) were used. Stably transfected CHO cells and transiently transfected HEK-293 cells were used for radioimmunoassay, and GloSensor™ cAMP system used transiently transfected HEK-293 cells. The procedures of radioimmunoassay have been previously published (Brown *et al.*, 2009; Harper and Brooker, 1975). Briefly, the transfected cells were seeded at a density of 10^5 cells/well in a 48-well plate and grown overnight. Next day, cell culture medium was aspirated off, and assay media (serum-free media containing 25 mM HEPES, 500 μM isobutylmethylxanthine, 100 μM propranolol, and 0.1% ascorbic acid) containing various concentrations of the test compounds were added. Plates were incubated for 15 min at 37°C. Then, cells were rinsed with assay media, and the reaction was halted by adding 250 μL of cold 0.1 M HCl, and the plates were stored at 4°C overnight.

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For the radioimmunoassay, sample aliquots (10 μL) were transferred to 96-well Skatron plates. Sodium acetate buffer (50 mM, pH 4.75) was added to the sample wells to bring the total volume up to 100 μL, and cAMP (0.3–25 nM) were used for the standard curve. Then, an acetylating mixture of triethlylamine/acetic anhydride (2:1 ratio) was added (10 μL) to the wells and vortexed. After 2 h of acetylation, ^{125}I-labeled cAMP (100 μL) and primary antibody for cAMP (100 μL) were added, and the plates were incubated overnight at 4°C. The following day, the secondary antibody labeled with magnetic beads (10 μL) was added, and the plates were incubated over 1 h at 4°C. Radioimmunoreaction was terminated by filtration with UniFilter-96 plates (Perkin Elmer) with diluted sodium acetate buffer (5 mM, pH 4.75). Radioactivity of each well was measured using TopCount NXT (PerkinElmer). A standard curve was generated using a sigmoidal model, and sample data were estimated by interpolation (Prism 4/5, GraphPad, Inc., San Diego, CA, USA) and converted to nmol cAMP values. For D_2 receptor-mediated cAMP inhibition, forskolin was added in assay media to elevate the basal level of cAMP, and haloperidol (10 μM) was used to block D_2 receptor activity. The inhibition of forskolin-induced cAMP was assessed as described above.

Assessment of activation of phospholipase C by D_1 receptors

The IP-One assay was used to determine inositol phosphate accumulation (Cisbio, Bedford, MA). In 96-well plates (pre-treated with poly-L-lysine), HEK-293 cells transiently transfected with the D_1 receptor were seeded at 80,000 cells/well and incubated overnight. Drug dilutions were performed in stimulation buffer and added to the plates, which were incubated for 1 h at 37°C, 5% CO_2. Cells were then incubated in lysis buffer for 30 min. Samples were transferred to the IP-One ELISA plate, and IP1 standards, α-IP1 mAb, and competitive IP1-HRP conjugate were added. The plate was incubated for 3 h at room temperature followed by six washings. The colorimetric reaction was initiated by adding TMB (tetramethylbenzidine), and the plate was incubated for 30 min. The reaction was terminated with stop solution. The 96-well plate was read at 450 nm/620 nm using a V_{max} plate reader (Molecular Devices, Sunnyvale, CA).

Assessment of β-arrestin activation at D_1 or D_2 receptors

β-arrestin activation in live CHO cells was monitored using PathHunter® β-arrestin assay (DiscoverX Corporation, Fremont, CA) as a D_1 or D_2 mediated non-cyclase signaling pathway. The platform of this assay is based on ligand binding to the D_1 or D_2 receptor that triggers the recruitment of intracellular β-arrestin to the activated receptors. The expression level in this kit was not available from DiscoverX, but they informed us that these engineered systems stably expressing D_1 or D_2 receptors have been standardized with well-known full and partial agonists and the potency and intrinsic activity are consistent with literature reports (personal communication).

Data and statistical analysis

The receptor binding and dose-response curves were analyzed by nonlinear regression using Prism 5.0 (GraphPad Software, San Diego, CA). $K_{0.5}$ for test ligands was calculated by Cheng-Prusoff equation with IC_{50} from the binding curves. Functional dose-response curves for all experiments (except D_1-mediated β-arrestin activation) were analyzed using a

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sigmoidal dose-response equation with a fixed slope to obtain apparent potency (EC_{50}) and maximal intrinsic activity (E_{max}). For D_1-mediated β-arrestin activation, a sigmoidal dose-response equation with a variable slope was used to better represent the original data. Student's t-tests or ANOVA with *post hoc* Tukey test were used for the statistical differences between two or multiple groups, respectively.

Results

Dopamine D_1 or D_2 receptor expression levels and SKF-83959 affinity

We used several human D_1 receptor expression systems (Table S1): stably transfected CHO and HEK-293 cells (0.7 pmol/mg protein and 5.2 pmol/mg protein, respectively) and transiently transfected HEK-293 cells (1.1 pmol/mg protein). The D_1 expression in rat striatal membranes was 1.95 pmol/mg protein. D_2 receptor expression in stably transfected CHO cells was 6.45 pmol/mg protein, which was higher than that in rat striatum (0.93 pmol/mg protein). SKF-83959 showed high affinity ($K_{0.5} = 2.5 \pm 0.2$ nM) for the D_1 receptor in rat striatum, whereas it had much lower affinity for the D_2 receptor ($K_{0.5} = 1.1 \pm 0.2$ μM) (Table 1). The affinity of SKF-83959 for human D_1 or D_2 receptor expressed heterologously was similar to that in rat striatum.

Partial intrinsic activity of SKF-83959 at D_1 receptor-mediated adenylate cyclase

Intrinsic activity for D_1-mediated cAMP synthesis was defined by the maximal response of dopamine. SKF-83959 had 35 ± 2% intrinsic activity at cAMP production in CHO cells expressing human D_1 receptors (Figure 2), whereas the prototypical D_1 partial agonist SKF38393 had ca. 50% intrinsic activity. In CHO cells, the prototypical D_1 receptor antagonist SCH23390 (10 μM) blocked cAMP elevation induced by SKF-83959 (Figure 3-Left) indicating that the cAMP production was a D_1 receptor-mediated response. SKF-83959 also had around 50% intrinsic activity at cAMP production in HEK-293 cells transiently expressing human D_1 receptors. When we assessed the cAMP elevation using cAMP-dependent luciferase activity in the same cells, SKF-83959 showed 42 ± 6% intrinsic activity, similar to results using radioimmunoassay (Figure 2). Again, the activity of SKF-83959 was significantly inhibited by SCH23390 (10 μM) (Figure 3-Middle and Right panels). These results demonstrate that SKF-83959 is a partial agonist much like SKF-38393 at D_1 receptor-mediated cAMP synthesis.

SKF-83959 fails to activate the D_2 receptor

Because SKF-83959 had some affinity for the D_2 receptor, we sought to characterize its D_2 functional effects by assessing its actions on D_2-mediated inhibition of cAMP synthesis. SKF-83959 has no intrinsic activity whatsoever against forskolin-stimulated adenylate cyclase activity (Figure 4), whereas the prototypical D_2 full agonist quinpirole and the partial agonist (–)3-PPP showed the expected full and partial activity, respectively.

Potency and intrinsic activity of SKF-83959 for β-arrestin activation at D_1 receptors

G protein–coupled receptor kinases (GRKs) have been known to phosphorylate dopamine receptors, and phosphorylated dopamine receptors can activate and recruit β-arrestin that plays a critical role in receptor desensitization and internalization (Gainetdinov *et al.*, 2004).

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β-arrestin recruitment to dopamine receptors can be a measure of non-cyclase dopamine receptor signaling. SKF-83959 was a partial agonist with 32 ± 2% intrinsic activity in D_1-mediated β-arrestin activation (Figure 5 Left), slightly less than the prototypical partial agonist SKF-38393. SCH23390 treatment (10 μM) significantly reduced the effect of SKF-83959 (Figure 5 Right) indicating that this β-arrestin activation was mediated by the D_1 receptor.

Potency and intrinsic activity of SKF-83959 for β-arrestin activation at D_2 receptors

SKF-83959 showed 19 ± 2% of the maximal response of quinpirole at D_2-mediated β-arrestin activation (Figure 6 Left). Consistent with its low D_2 affinity, the potency of SKF-83959 at the D_2 receptor (1.3 ± 0.2 μM) was much less than that at the D_1 receptor. A typical D_2 receptor antagonist haloperidol (10 μM) completely inhibited the activity of SKF-83959 (Figure 6 Right). These results indicate that SKF-83959 is also a partial agonist at β-arrestin signaling mediated by either D_1 or D_2 receptors.

Lack of SKF-83959 activity on D_1 receptor-mediated PLC stimulation

As noted in the Introduction, the major foundation for the proposed functional selectivity of SKF-83959 was its purported high bias and high intrinsic activity towards D_1-mediated PLC activation versus antagonism at D_1-mediated adenylate cyclase. Because of the potential issues that were discussed above (*vide infra*), this was examined in detail in a heterologous system using human D_1 receptor-transfected HEK-293 cells. As shown in Figure 7A, in hD_1-HEK-293 cells the muscarinic agonist carbachol (a positive control) caused marked stimulation of the production of IP1, and the potency we observed is consistent with its known micromolar-range affinity for muscarinic receptors (Cheng *et al.*, 2002; Jakubik *et al.*, 1997; Wood *et al.*, 1999). SKF-83959, however, produced no significant increase except for a slight trend at non-pharmacological concentrations (i.e., 300 μM). Moreover, there was no difference between the amount of IP1 produced by SKF-83959 alone and in combination with the D_1 antagonist SCH23390 (10 μM) (Figure 7B). Conversely, the stimulation of carbachol was blocked by atropine (10 μM), whereas atropine had no effect on the action of SKF-83959 (Figure 7C).

These studies were also repeated in stably transfected hD_1-HEK-293 cells using D_1 agonists from three chemical classes [benzazepines (SKF-83959 & SKF-83822), phenanthridine (dihydrexidine), or isochroman (A77636)]. None caused significant activation of PLC even at concentrations as high as 30 μM (Figure 8A). In addition, the effects of both carbachol and SKF-83959 were identical in both D_1 transfected and wild-type HEK-293 cells (Figure 8B and 8C).

Finally, the same experiments were also done in HEK-293 cells transiently co-transfected with the hD_1 and hD_{2L} receptors (Supplemental Figure 1). The dose-response curve for SKF-83959 was identical in hD1-expressing, in wild-type, or in cells co-expressing hD_1 and hD_{2L} receptors (Supplemental Figure 2). No significant stimulation of IP1 synthesis was found, and the slight increase sometimes seen at near mM concentrations was identical in all three cell lines.

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Activity of SKF-83959 and its N-demethylated analog in rat striatum

Because some of the novelty of SKF-83959 has been related to its actions *in vivo*, we also examined the pharmacology of the parent compound and a likely oxidative metabolite, the N-demethylated analog, both of which were synthesized as described in the Supplemental material. In rat striatal membranes, the prototypical partial agonist SKF-38393 (Watts *et al.*, 1993), SKF-83959, and its demethylated analog had high affinity for the rat D_1 receptor (Table 1). Interestingly, as shown in Figure 9, the N-demethylated analog had intrinsic activity (~60%) at D_1-mediated stimulation of adenylate cyclase significantly higher than SKF-83959 (~35%) (Student's t-test, $P<0.05$), with both compounds having sub-micromolar potency. The stimulation of cAMP synthesis was completely blocked by SCH23390 (Figure 9).

Discussion

There are no clinically-approved CNS penetrant, high intrinsic activity selective dopamine D_1 agonists, yet animal models have suggested that selective D_1 agonists may have utility for Parkinson's disease (Taylor *et al.*, 1991; Mailman *et al.*, 2001) and cognition (Arnsten *et al.*, 1994; Schneider *et al.*, 1994; Steele *et al.*, 1996; Steele *et al.*, 1997) among other disorders. In fact, for these two conditions therapeutic efficacy of D_1 agonists has been translated into the clinic with large effect sizes predicted by preclinical models (Rascol *et al.*, 1999; Rosell *et al.*, 2014). Despite this support for the clinical efficacy of D_1 agonists, there are reports of serious D_1-mediated side effects that may prevent approval of a D_1 agonist, including rapid tolerance (Asin and Wirtshafter, 1993), profound hypotension (Blanchet *et al.*, 1998) and seizures (Starr, 1996). During the past decade, it has become clear that one way of improving the pharmacological actions of a drug is to make it functionally selective at its targeted receptor(s) (Urban *et al.*, 2007; Neve, 2009; Kenakin, 2007; Mailman, 2007). If a D_1 agonist with high bias for different D_1-mediated signaling pathways were available, it would be a useful research tool that also might overcome some of the possible limitations associated with current D_1 agonists.

SKF-83959 is purported to be such a ligand, highly-biased towards D_1 stimulation of phosphoinositide hydrolysis (via a D_1-$G\alpha_Q$ mechanism), but with no intrinsic activity at $G\alpha_{OLF/S}$ mechanisms that stimulate adenylate cyclase (Undie and Friedman, 1992; Undie *et al.*, 1994; Gnanalingham *et al.*, 1995b; Arnt *et al.*, 1992). The literature is consistent with our finding that SKF-83959 has nanomolar affinity for the dopamine D_1 receptor in rat brain and the human D_1 receptor expressed heterologously (Table 1), and micromolar affinity for the cloned D_{2L} and rat brain D_2-like receptor. The question, then, is what functional profile does this ligand actually have?

D_1 effects on adenylate cyclase stimulation and β-arrestin activation

We studied D_1-mediated cAMP synthesis using the expressed human D_1 receptor in two different cell lines using two different assays, as well as in rat brain striatal homogenates. Both heterologously and *in situ*, SKF-83959 was a partial agonist with intrinsic activity slightly less than SKF38393, the prototypical partial agonist. These studies were performed both with compound synthesized by us (structure verified chemically), as well as material

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obtained commercially, and with two different assays for cAMP. Identical results were obtained with all combinations. We also studied these two compounds against a second accepted signaling system, β-arrestin-mediated signaling (Shenoy and Lefkowitz, 2005; Urs *et al.*, 2011). SKF-83959 also was a partial agonist at the D_1 receptor with intrinsic activity similar to the partial agonist SKF38393 and similar potency to its effects on adenylate cyclase, both in the nanomolar range. Together, this is consistent evidence that SKF-83959 appears to have properties expected of a partial D_1 agonist, not a highly-biased D_1 ligand. It is important to reiterate the fact of essentially identical intrinsic activity and potency seen with the D_1 receptor in rat striatum versus the human receptor expressed heterologously. Although receptor reserve clearly can influence measured intrinsic activity (Watts *et al.*, 1995), the agreement between the data obtained *ex vivo* versus *in vitro* strongly suggests that our conclusions are physiologically relevant.

How does one resolve the conundrum that many consider SKF-83959 an antagonist at D_1 cAMP signaling? A previous study from our lab also reported partial intrinsic activity for SKF-83959 in a hD_1 heterologous system (Ryman-Rasmussen *et al.*, 2005), and some of the published data are consistent with the current report when the data are examined (Gnanalingham *et al.*, 1995b; Chemel *et al.*, 2012; Rashid *et al.*, 2007b), yet other reports clearly show that SKF-83959 completely lacks intrinsic activity at D_1-stimulated adenylate cyclase (Jin *et al.*, 2003; Arnt *et al.*, 1992). Further independent studies are needed to arrive at a consensus.

D_1 effects on activation of phospholipase C

The second conundrum relates to the purported stimulation of PLC signaling by SKF-83959. The current data found no evidence that SKF-83959 causes any stimulation of PLC mediated by the D_1 receptor, even at concentrations five orders-of-magnitude higher than the K_D. This clearly contrasts with numerous reports that D_1 receptors directly stimulate the phosphoinositide signaling system in murine and primate brain (Liu *et al.*, 2009a; Liu *et al.*, 2009b; Yu *et al.*, 2008; Perreault *et al.*, 2014; Felder *et al.*, 1989a; Felder *et al.*, 1989b; Undie and Friedman, 1990; Dyck, 1990; Undie and Friedman, 1992; Vyas *et al.*, 1992; Undie and Friedman, 1994; Pacheco and Jope, 1997; Lee *et al.*, 2004; Banday and Lokhandwala, 2007; Liu *et al.*, 2009a; Zhang *et al.*, 2009; Mizuno *et al.*, 2012; Mahan *et al.*, 1990). We believe that there is a logical explanation for this lack of concordance. In examining the prior literature, the one constant was that effects of SKF-83959 on PLC signaling required concentrations of 10 μM and higher. Although SKF-83959 is a nanomolar affinity, selective D_1 ligand, it is known that in the micromolar range it (and its congeners) have numerous off-target activities (Setler *et al.*, 1978; Weinstock *et al.*, 1985; Neumeyer *et al.*, 2003; Chun *et al.*, 2013) some of which are to systems known to be coupled to PLC. In addition, despite the plethora of reports linking D_1 agonists to PLC activation, those studies only used D_1 agonists of the phenylbenzazepine family, and generally relied on SCH23390 (another phenylbenzazepine) as the D_1 antagonist. Such designs increase the likelihood of confounding off-target effects.

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Effects of D_1-D_2 heterodimers on phospholipase C activation

There has been an alternative hypothesis that D_1-D_2 heterodimers mediate PLC activation (Rashid *et al.*, 2007b; Hasbi *et al.*, 2009) although the existence of D_1-D_2 heteromers *in vivo* is still unclear. We noticed that most of those papers supporting this hypothesis also used the typically high SKF-83959 concentrations, and we feel that they must be approached skeptically due to the following reasons. 1) In brain the percentage of D_1 receptors that are colocalized with D_2 is very small (Aubert *et al.*, 2000; Gerfen *et al.*, 1990; Le Moine and Bloch, 1995; Thibault *et al.*, 2013; Lidow and Goldman-Rakic, 1994); 2) Because colocalization is a tiny fraction of the total D_1, one would predict that the magnitude of PLC or Ca^{2+} signals would be much less that from known $G\alpha_Q$ receptors directly linked to PLC and expressed at high levels, but it is not; 3) Co-expressing at high levels different receptors in heterologous systems can force protein interactions that would never occur *in situ*; 4) One paper from the George group used more reasonable (but still high, 100 nM) SKF-83959 concentrations in specifically isolated D_1/D_2 coexpressing neurons (Hasbi *et al.*, 2009), but while a Ca^{2+} response was reported, PLC was not tested directly and the response could have been secondary to other signaling; 5) A recent paper from Sibley's group failed to replicate some of the effects predicted from the George hypothesis (Chun *et al.*, 2013).

Above and beyond the fact that we were unable to demonstrate activation of PLC in cells co-expressing D_1 and D_2 receptors (Supplemental Figure 2), it is important to note that the heterodimer hypothesis is actually secondary to the first hypothesis of our manuscript: that SKF-83959 and a possible metabolite both are partial D_1 agonists and activate adenylate cyclase. Accepting the PLC hypothesis would seem to require first showing that cAMP-PKA-dependent signaling was not involved, but this has never been done. Our secondary target related to D_1 stimulation of PLC, and here one well-controlled experiment was offered, supplemented by a careful look at the literature that strongly suggests that PLC activation is highly likely a result of non-D_1 effects caused by the use of suprapharmacological concentrations.

Summary

To our knowledge, this is the first rigorous characterization of the pharmacology of SKF-83959 in several systems. Although we are confident in our current data, we recognize the possibility that unknown experimental differences might explain why we feel that rather than being a highly-biased functionally selective D_1 ligand, SKF-83959 is a typical partial agonist. Our data suggest that at relevant pharmacological concentrations, SKF-83959 does not activate PLC, and we hypothesize that the prior reports were detecting off-target effects. The use of 10–300 μM concentrations of a nanomolar affinity ligand would thereby affect many known, as well as unknown, targets. We recognize that there are many hypotheses that could explain why D_1 receptor activation might directly stimulate PLC (homomeric D_1, D_1-D_2 dimer, D_1 heteromer with some other receptor, etc.), but all of these depend on the D_1. Of particular relevance, therefore, is a study that actually compared the PLC stimulation of the 1-phenyl-3-benzazepines in wild-type and D_1 knockout mice (Friedman *et al.*, 1997). Those authors report no difference in PLC stimulation between wild-type and D_1 knockout mice, yet rather than reject the D_1-PLC hypothesis, they postulated that it was caused by a novel, unknown D_1-like receptor (Friedman *et al.*, 1997). This novel explanation seems

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extremely unlikely as there are essentially no D_5 receptors in the mouse striatum and no other receptor (characterized or orphan) in the mouse genome GPCR that has D_1-like properties.

These data impact the many studies that have examined aspects of D_1 function based on novel properties of SKF-83959 (Yu *et al.*, 2008; Guo *et al.*, 2013; Lee *et al.*, 2004; Hasbi *et al.*, 2009; Hasbi *et al.*, 2011; Rashid *et al.*, 2007a; Rashid *et al.*, 2007b), including suggestions that downplay the role of cAMP signaling in behavioral actions of D_1 ligands (Arnt *et al.*, 1992; Downes and Waddington, 1993; Gnanalingham *et al.*, 1995a). If our assessment of the pharmacology of SKF-83959 is correct, it may affect the validity of the conclusions that were drawn from such studies. In addition, we have shown that at least one predicted metabolite of SKF-83959 has actually somewhat higher intrinsic activity than the parent molecule, offering another possible confound when used in behavioral or physiological studies. We urge investigators to consider the current data and to review the supporting literature for themselves, before utilizing this compound for properties that it may, or may not, have.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

AC	adenylate cyclase
DA	dopamine
DHX	dihydrexidine; (trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo-[*a*]phenanthridine)
GPCR	G protein-coupled receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBMX	3-Isobutyl-1-methylxanthine
IP3	Inositol triphosphate
IP1	Inositol monophosphate
$K_{0.5}$	Apparent affinity constant
NMS	N-methylspiperone

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PLC	Phospholipase C
SKF-83959	6-chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine
desmethylSKF	6-chloro-7,8-hydroxy-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine

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Highlights

- SKF-83959 is a D_1 receptor partial agonist at cAMP and β-arrestin signaling.
- SKF-83959 does not stimulate PLC activity via D_1 receptors.
- Behavioral effects of SKF-83959 can be explained by partial D_1 agonism.
- SKF-83959 is not a highly-biased functionally selective D_1 receptor ligand.
- The importance of direct D_1 receptor activation of PLC signaling is unclear.

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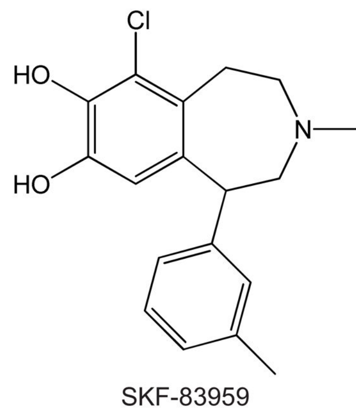

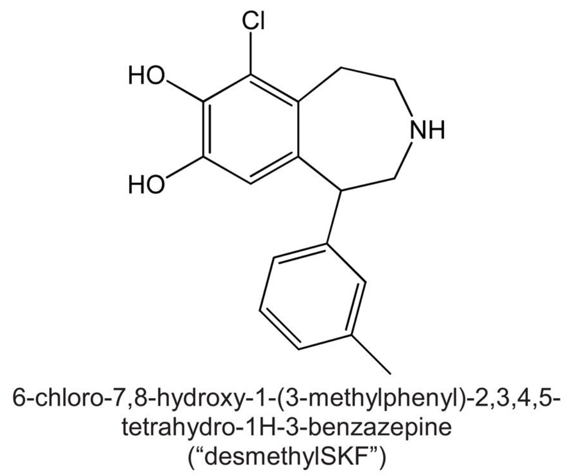

Figure 1.
Structures of SKF-83959 and a postulated demethylated metabolite.

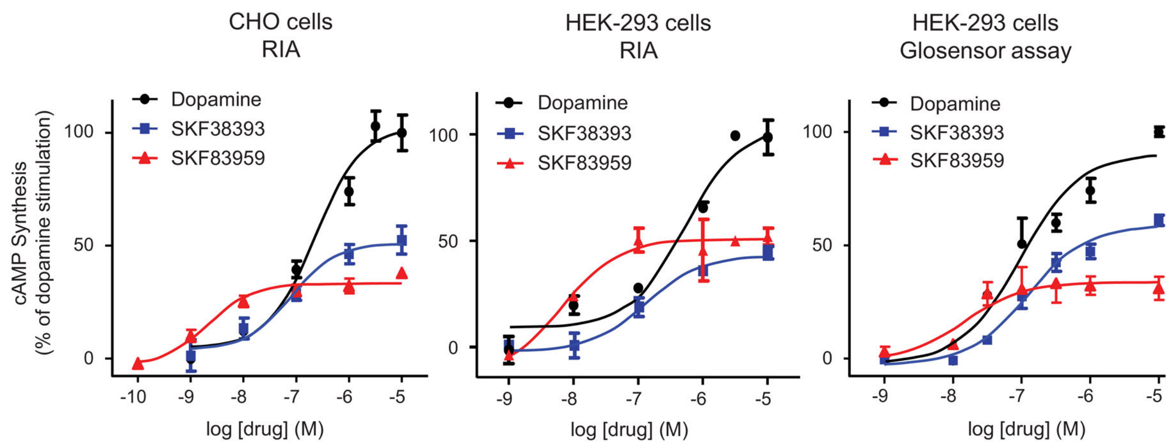

Figure 2.

D_1-mediated adenylate cyclase activation by SKF-83959 in heterologous expression systems. D_1 receptor activation in: (Left) CHO cells and (Middle) HEK-293 cells expressing D_1 receptors was assessed by radioimmunoassay. (Right) D_1 receptor activation in transiently transfected HEK-293 cells was also assessed by the Glosensor assay. Representative curves of at least three independent experiments are shown.

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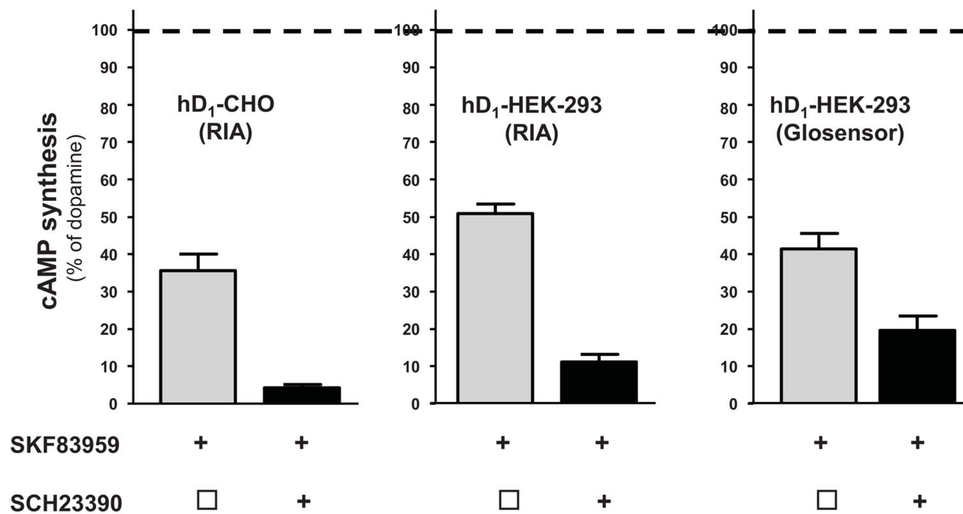

Figure 3.

Inhibition of SKF-83959-stimulated cAMP synthesis by SCH23390 (10 μM). Left panel: CHO cells. Middle and right panels: HEK-293 cells. Both cell types were transfected with the human D_1 receptor as noted in the methods.

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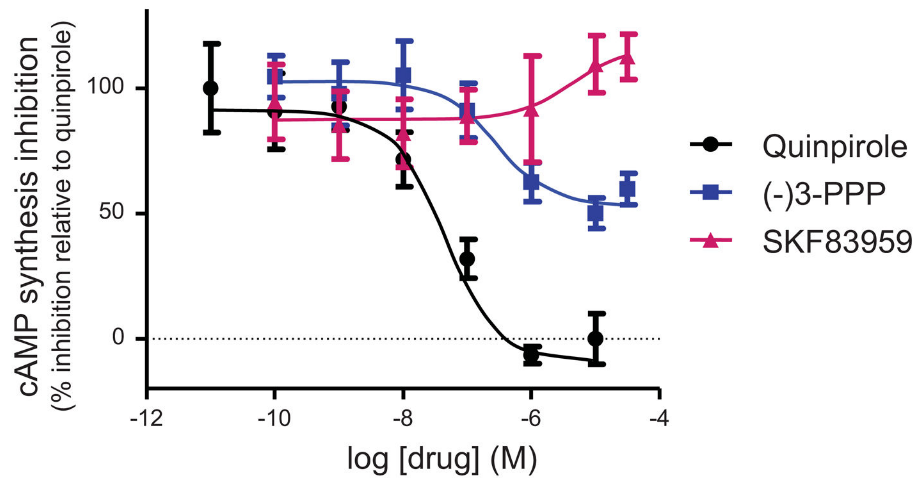

Figure 4.

D_2-mediated adenylate cyclase inhibition. D_2 receptor activation was assessed by radioimmunoassay in CHO cells stably transfected with human D_{2L} receptors. Representative curves of at least three independent experiments are shown.

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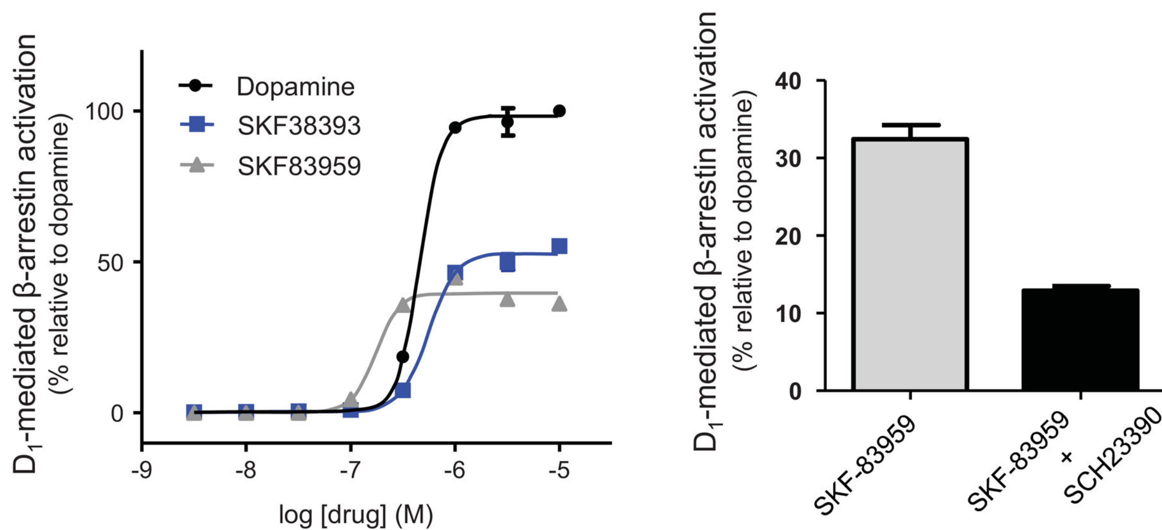

Figure 5.

Induction of β-arrestin activation at D_1 receptors by SKF-83959. SKF-83959 was a partial agonist at β-arrestin activation at D_1 (Left), and this was reversed by SCH23390 (Right). Representative curves of at least three independent experiments are shown. For right panel, 10 μM concentrations were used for SKF-83959 and SCH23390.

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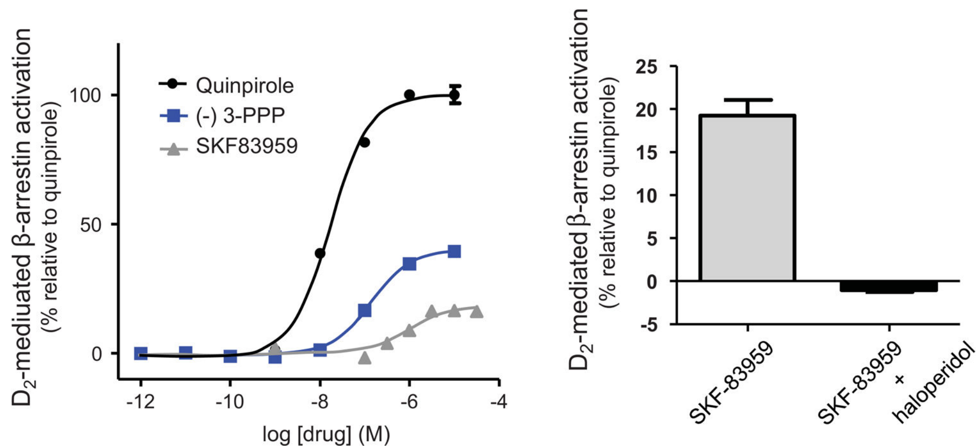

Figure 6.

Partial agonism of SKF-83959 on induction of β-arrestin by D_2 receptors. (Left) SKF-83959 causes ca. 20% activation, somewhat less than the known partial agonist 3-PPP. (Right)The D_2 antagonist haloperidol (10 μM) completely inhibits the SKF-83959-mediated activation of β-arrestin.

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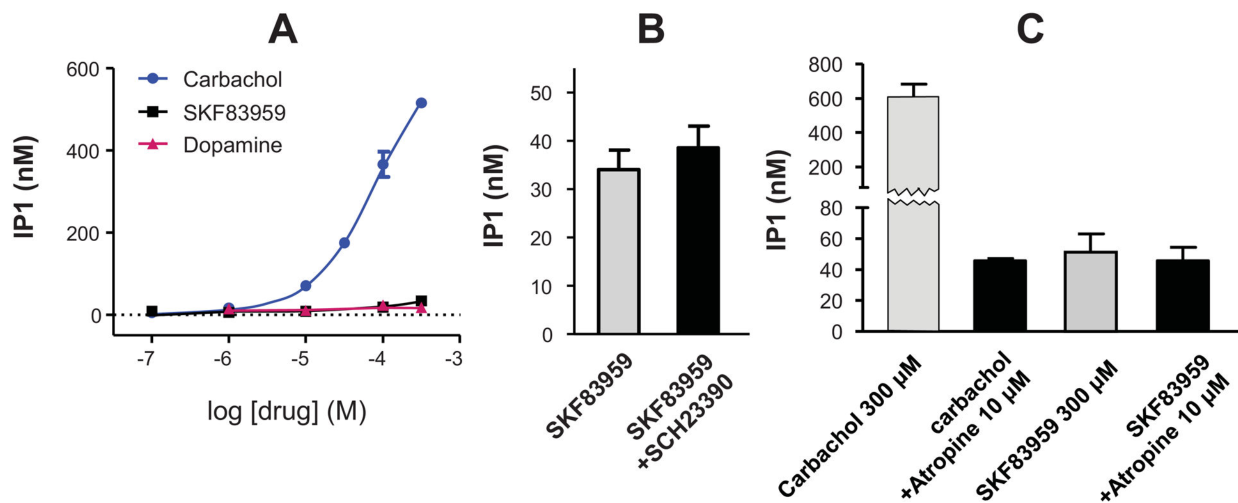

Figure 7.

SKF-83959 does not stimulate phospholipase C activity via D_1 receptors in hD_1-HEK-293 cells. (Panel A) SKF-83959 could not induce IP1 production considerably in transiently transfected hD_1-HEK-293 cells. Representative curves of at least three independent experiments are shown. (Panel B) The slight increase seen at 300 μM SKF-83959 was not inhibited by the D_1 antagonist SCH23390 (10 μM). (Panel C) The stimulation by the muscarinic agonist carbachol was inhibited by the muscarinic antagonist atropine (10 μM), and there were no effects on the action of SKF-83959 (300 μM).

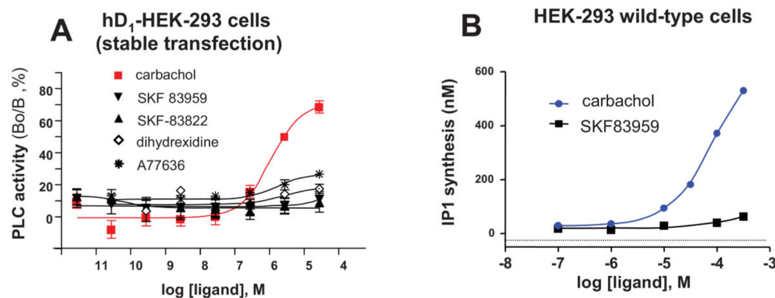

Figure 8.

Selective D1 agonists including SKF-83959 and dopamine fail to stimulate PLC activity at physiologically-relevant concentrations. (Panel A) In stably transfected hD_1-HEK-293 cells, D_1 agonists from three chemical classes [benzazepines (SKF-83959 & SKF-83822), phenanthridine (dihydrexidine), or isochroman (A77636)] failed to activate PLC. (Panel B) In wild-type HEK-293 cells, carbachol caused significant PLC activation whereas SKF-83959 did not, and both compounds caused identical results to those shown in Figure 7A). Representative curves of at least two independent experiments are shown.

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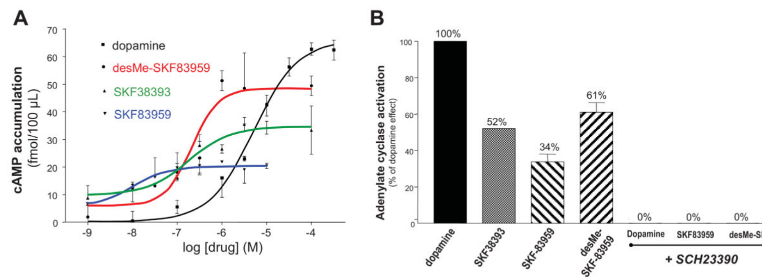

Figure 9.

Partial agonist properties of SKF-83959 and desMe-SKF-83959 on D_1-stimulated adenylate cyclase of rat striatum. The benzazepine ligands were used at 1 μM concentrations, dopamine at 10 μM, and SCH23390 at 10 μM.

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Table 1

Summary of SKF-83959 binding affinity.

		SKF-83959	**desmethylSKF**
Receptor	**Source, System**	*[$K_{0.5}$ (nM)]*	
$D_{1\text{-like}}$	rat striatum	2.5 ± 0.2	4.0 ± 0.7
D_1	human, CHO	1.1 ± 0.1	--
D_1	human, HEK-293	0.8 ± 0.1	--
$D_{2\text{-like}}$	rat striatum	1,080 ± 225	> 1 μM
D_{2L}	human, CHO	1,080 ± 115	--

Data are from at least three independent experiments and expressed as mean ± SEM.