Minireview

Dopamine D₁ Receptor Signaling: Does G α_Q –Phospholipase C Actually Play a Role?

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ABSTRACT

Despite numerous studies showing therapeutic potential, no central dopamine D₁ receptor ligand has ever been approved, because of potential limitations, such as hypotension, seizures, and tolerance. Functional selectivity has been widely recognized as providing a potential mechanism to develop novel therapeutics from existing targets, and a highly biased, functionally selective D₁ ligand might overcome some of the past limitations. SKF-83959 [6-chloro-3-methyl-1-(*m*-tolyl)-2,3,4,5-tetrahydro-1*H*-benzo[*d*]azepine-7,8-diol] is reported to be a highly biased D₁ ligand, having full agonism at D₁-mediated activation of phospholipase C (PLC) signaling (via G α_Q) and antagonism at D₁-mediated adenylate cyclase signaling (via G $\alpha_{OLF/S}$). For this reason, numerous studies have used this compound to elucidate the physiologic role of D₁-PLC signaling,

including a novel molecular mechanism (G α_Q -PLC activation via D₁-D₂ heterodimers). There is, however, contradictory literature that suggests that SKF-83959 is actually a partial agonist at both D₁-mediated adenylate cyclase and β -arrestin recruitment. Moreover, the D₁-mediated PLC stimulation has also been questioned. This Minireview examines 30 years of relevant literature and proposes that the data strongly favor alternate hypotheses: first, that SKF-83959 is a typical D₁ partial agonist; and second, that the reported activation of PLC by SKF-83959 and related benzazepines likely is due to off-target effects, not actions at D₁ receptors. If these hypotheses are supported by future studies, it would suggest that caution should be used regarding the role of PLC and downstream pathways in D₁ signaling.

The Potential Utility of a Functionally Selective D₁ Ligand

Animal models have suggested the utility of selective D₁ agonists for a variety of therapeutic targets, including Parkinson's disease (Taylor et al., 1991; Mailman and Nichols, 1998; Mailman et al., 2001), cognition (Arnsten et al., 1994; Schneider et al., 1994; Steele et al., 1996, 1997; Goldman-Rakic et al., 2004; Toda and Abi-Dargham, 2007; Rosell et al., 2014), and other disorders. In fact, for Parkinson's disease, the efficacy of D₁ agonists in animal models has translated into clinical trials with large effect sizes similar to those seen in the pre-clinical models (Rascol et al., 1999, 2001). A recent preliminary clinical study (Rosell et al., 2014) also reported significant effects on working memory consistent with predictions from

preclinical models (Arnsten et al., 1994; Schneider et al., 1994; Steele et al., 1996, 1997). Despite this support for the clinical efficacy of D₁ agonists, there are reports of serious D₁-mediated side effects that may prevent approval of a D₁ agonist, including rapid tolerance (Asin and Wirtshafter, 1993; Gulwadi et al., 2001), profound hypotension (Blanchet et al., 1998), and seizures (Starr, 1996).

During the past decade, it has become clear that one way of improving a pharmacological profile is to discover functionally selective ligands for the target receptor (Kenakin, 2007; Mailman, 2007; Urban et al., 2007; Neve, 2009). Functional selectivity describes the property of some ligands to interact with a single receptor and differentially affect the signaling pathways engaged by that target. The degree to which the ligand differentially affects signaling pathways is termed the bias of the ligand. The first use of the term "functional selectivity" in the context of a single receptor was with dopamine receptor ligands (Mailman et al., 1998; Lawler et al., 1999), and the earliest therapeutic utility of this mechanism was suggested by aripiprazole, a D₂-preferring compound with a mechanism of action clearly differentiated from earlier approved antipsychotic drugs (Kikuchi et al., 1995; Lawler et al., 1999; Shapiro et al.,

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ABBREVIATIONS: PLC, phospholipase C; SCH23390, (*R*)-8-chloro-3-methyl-5-phenyl-2,3,4,5-tetrahydro-1*H*-benzo[*d*]azepin-7-ol; SKF-38393, (*R*)-1-phenyl-2,3,4,5-tetrahydro-1*H*-benzo[*d*]azepine-7,8-diol; SKF-83959, 6-chloro-3-methyl-1-(*m*-tolyl)-2,3,4,5-tetrahydro-1*H*-benzo[*d*]azepine-7,8-diol.

2003; Mailman, 2007; Mailman and Murthy, 2010). Thus, if a D₁ agonist with high bias for D₁-mediated signaling pathways were available, it would be an excellent investigational tool, and it also might overcome some of the possible limitations sometimes thought to be obligatory with D₁ agonists.

SKF-83959, the First Functionally Selective D₁ Ligand?

Dopamine receptors were originally classified on the basis of their coupling to adenylate cyclase (Kebabian et al., 1972; Garau et al., 1978; Kebabian and Calne, 1979), and this canonical signaling of the D₁-like receptors (D₁, D₅) is thought to involve coupling to the G proteins G α_{OLF} or G α_S (Neve et al., 2004; Mailman and Huang, 2007). Thus, the cAMP resulting from D₁ receptor activation could initiate a host of downstream cascades, such as those engaged by the activation of cAMP/protein kinase A signaling. The importance of this pathway was challenged, however, with reports that the behavioral effects of some D₁ agonists were unrelated to cAMP/protein kinase A signaling (Gnanalingham et al., 1995a,b), but rather involved non-cAMP-mediated signaling, including phospholipase C (PLC)-mediated calcium elevation (Undie and Friedman, 1990; Undie et al., 1994; Andringa et al., 1999b; O'Sullivan et al., 2004). This has led to the hypothesis that a functionally selective D₁ ligand highly biased against cAMP signaling might have an improved therapeutic index.

Much of this research has focused on SKF-83959 [6-chloro-3-methyl-1-(*m*-tolyl)-2,3,4,5-tetrahydro-1*H*-benzo[*d*]azepine-7,8-diol] because it was purported to be a highly biased D₁ ligand that was a biochemical antagonist at D₁-coupled adenylate cyclase, but a full agonist at D₁-stimulated PLC via G α_Q (Panchalingam and Undie, 2001; Jin et al., 2003; Zhen et al., 2005; Rashid et al., 2007b; Hasbi et al., 2009) (see Fig. 1 for structures of SKF-83959 and other relevant ligands). SKF-83959 is behaviorally active in rat and primate Parkinson's disease models via its action at D₁ receptors (Arnt et al., 1992; Jin et al., 2003), and it has behavioral activity in several animal species that is known to be induced by D₁ full or partial agonists but not by D₁ antagonists (Gnanalingham et al., 1995a).

The concatenation of these data led to the hypothesis that the behavioral actions of SKF-83959 were independent of D₁-mediated cAMP signaling, but depended on D₁ actions at PLC/calcium signaling (Downes and Waddington, 1993; Deveney and Waddington, 1995; Rashid et al., 2007a; Hasbi et al., 2009; Fujita et al., 2010; Perreault et al., 2010), possibly mediated by D₁-D₂ heterodimers (Rashid et al., 2007a; Hasbi et al., 2009; Perreault et al., 2010; Chun et al., 2013). This would mean that SKF-83959 is the first highly biased, functionally selective D₁ ligand (Arnt et al., 1992; Undie and Friedman, 1992; Undie et al., 1994; Gnanalingham et al., 1995b) and would make it an important probe for studying mechanisms related to D₁ signaling (Zhang et al., 2005, 2007, 2009; Yu et al., 2008; Liu et al., 2009a,b; Perreault et al., 2011, 2014) as is often explicitly stated: "SKF 83959 robustly stimulat[es] the D₁-D₂ heteromer-mediated calcium signal and [does] not activat[e] adenylyl cyclase by the D₁-D₁ homomer" (Perreault et al., 2014); SKF-83959 is "a selective [phosphatidylinositol]-linked D₁-like receptor agonist" (Zhang et al., 2009); and there are "potential stimulant-antagonist actions, as observed with SKF-83959" (Neumeyer et al., 2003).

Some years ago, Pacheco and Jope (1997) noted some contradictions in the literature, but speculated that "variations in experimental methods likely contribute to the differing results," although these issues were not identified. They themselves reported that "dopamine D₁ receptors directly stimulate the [phosphoinositide] signaling system" in the human brain (Pacheco and Jope, 1997). In noting such inconsistencies, we had offered a skeptical view of the hypothesis that SKF-83959 was a highly biased, functionally selective ligand (Huang et al., 2001). Yet the increasing numbers of papers whose data depend in large part on the highly biased signaling of SKF-83959 have made it important to re-examine the pharmacology of this compound. In evaluating the evidence for SKF-83959 being a highly biased ligand, one must also consider the role of D₁-PLC signaling. We have chosen to do this by formulating a series of hypotheses that together form the basis for accepted dogma about the direct role for D₁ receptors in stimulating G α_Q -PLC and, more recently, the role of D₁-D₂ heterodimers in affecting PLC/Ca²⁺-mediated signaling.

Hypothesis: SKF-83959 Is a Selective, High-Affinity D₁ Ligand

There is universal agreement that SKF-83959 has nanomolar affinity for both rat and human D₁ receptors, heterologously expressed or in situ (Arnt et al., 1992; Chun et al., 2013; Lee et al., 2014), and also that the ligand has micromolar affinity for D₂ receptors (Arnt et al., 1992; Chun et al., 2013; Lee et al., 2014), where it is an antagonist at cAMP signaling and a low-intrinsic-activity partial agonist at β -arrestin recruitment (Lee et al., 2014). Similar D₁-D₂ selectivity with a wide range of D₁ intrinsic activities has been shown for other ligands with similar benzazepine structures [i.e., ligands with substituents on a backbone of 1-phenyl-2,3,4,5-tetrahydro-1*H*-benzo[*d*]azepine] (Setler et al., 1978; Iorio et al., 1983; Weinstein et al., 1985; Neumeyer et al., 2003). Because the D₂ affinities of most benzazepines are in the micromolar range, this provides an obvious off-target mechanism when high concentrations or doses are used experimentally. Ligands of this family also have measurable affinity for other neuroreceptors. Andringa et al. (1999a) reported that SKF-83959 had micromolar affinity for all α -adrenoreceptors and the norepinephrine transporter, and Neumeyer et al. (2003) noted that it also had affinity for both 5-HT_{2A} (K_i = 88 nM) as well as the α_2 -adrenergic (K_i , ~3000 nM) receptors. More recently, Chun et al. (2013) reported the results of a broader screen, reporting micromolar affinity for dozens of G protein-coupled receptors, including K_i < 10 μ M for many receptors that couple directly to G α_Q , such as the 5-HT_{2A} and 5-HT_{2C} serotonin, α_1 -adrenergic, H₁ histamine, and M₅ muscarinic receptors, among others. Thus, the literature shows that although SKF-83959 has its highest affinity for D₁/D₅ receptors, at micromolar concentrations it will interact both with D₂-like dopamine receptors and a variety of other targets. Additionally, while the focus of this Minireview was on the numerous studies in which SKF-83959 was used at micromolar concentrations, a subset of off-target receptors (Neumeyer et al., 2003; Chun et al., 2013) will be engaged at much lower concentrations; thus, the need for proper experimental controls remains unobviated. The implications of such off-target actions are discussed below.

Fig. 1. Research ligands and drugs that have properties relevant to these issues.

Hypothesis: SKF-83959 Has No Intrinsic Activity at D₁-Mediated Stimulation of Adenylate Cyclase

Although purported to have no intrinsic activity at D₁-mediated adenylate cyclase, we previously used SKF-83959 and found it to be a partial agonist (Ryman-Rasmussen et al., 2005). Because of the continuing use of this ligand for its “novel” properties, we recently did a rigorous evaluation of SKF-83959 pharmacology in a variety of heterologous and native D₁ systems, studying both the parent ligand and a potential *N*-demethylated metabolite, 6-chloro-1-(*m*-tolyl)-2,3,4,5-tetrahydro-1*H*-benzo[*d*]azepine-7,8-diol, that might contribute to pharmacological effects in vivo (Lee et al., 2014). Both in vitro and ex vivo, SKF-83959 was a partial agonist at D₁-mediated adenylate cyclase in two different cell lines transfected with the human D₁ receptor, as well as in rat striatal homogenates. Its potency and affinity were very similar in all of these systems. The functional effects were inhibited by SCH23390 [(*R*)-8-chloro-3-methyl-5-phenyl-2,3,4,5-tetrahydro-1*H*-benzo[*d*]azepine-7-ol], as well as by structurally dissimilar D₁ antagonists, and SKF-83959 had no effect in untransfected cells. As noted above, while D₁-selective, SKF-83959 also had micromolar affinity for D₂ receptors, where it was an antagonist at cAMP signaling (Lee et al., 2014).

In some cases, the published data actually agree with the findings of Lee et al. (2014). For example, Gnanalingham et al. (1995b) reported that SKF-83959 did not cause significant stimulation of adenylate cyclase, yet their data clearly show measurable (~50%) intrinsic activity, with some data points marked as significant. In addition to our previous study showing >40% intrinsic activity in a human D₁ heterologous expression

system (Ryman-Rasmussen et al., 2005), Chemel et al. (2012) reported high intrinsic activity in an overexpressed human D₁ receptor system. Although Rashid et al. (2007b) showed a “flat” dose-response curve for SKF-83959, only relative activity units were provided, making it impossible to know the level of basal synthesis. The lowest SKF-83959 concentration tested was 10 nM (Rashid et al., 2007b), making it possible that maximal stimulation had already been caused. Without either basal synthesis levels or lower concentrations, the only valid conclusion from those data is that SKF-83959 was not a full agonist. There are, however, a few studies reporting no intrinsic activity for SKF-83959 for which obvious experimental or interpretational confounds are not evident (Arnt et al., 1992; Jin et al., 2003). It will be useful for other laboratories using this compound to independently determine whether SKF-83959 has measurable intrinsic activity in canonical assays.

Signaling via β -arrestin also represents an important non-G protein signaling pathway (Shenoy and Lefkowitz, 2005), one that can be evoked by D₁ receptors (Urs et al., 2011). SKF-83959 also is a partial agonist at the D₁-mediated β -arrestin activation, with intrinsic activity similar to the partial agonist SKF-38393 [(*R*)-1-phenyl-2,3,4,5-tetrahydro-1*H*-benzo[*d*]azepine-7,8-diol] and with similar potency as seen with adenylate cyclase activation (Lee et al., 2014). Importantly, its potency in both D₁-mediated adenylate cyclase and β -arrestin assays was in the nanomolar range, similar to its affinity (Lee et al., 2014). Interestingly, at the D₂ receptor, SKF-83959 had no intrinsic activity at adenylate cyclase but modest agonist activity at D₂-mediated β -arrestin activation (Lee et al., 2014). At both of these D₁-mediated functions, the potency of SKF-83959

was, again, similar to its affinity, a point of relevance to the following section. Thus, the weight of the evidence suggests that SKF-83959 is a typical partial D₁ agonist, not a highly biased, noncyclase-preferring D₁ ligand.

Hypothesis: SKF-83959 Activates PLC via a D₁-Gα_Q Mechanism

Although SKF-83959 may have partial agonist properties at Gα_{OLF/S}-mediated stimulation of cAMP, it might still be useful as a moderately biased, functionally selective ligand if it were a full agonist in activating PLC. Some years ago, we briefly reviewed the evidence for D₁ linkage to PLC (Huang et al., 2001) and suggested flaws with that hypothesis, yet there has been a continued use of this compound based on this property. We recently investigated whether D₁ signaling was mediated by PLC activation. There was no significant stimulation caused by SKF-83959 in either D₁-transfected or wild-type HEK293 cells. Conversely, in both the wild-type and D₁-transfected cells, the positive Gα_Q control carbachol markedly stimulated inositol monophosphate synthesis with a potency consistent with its affinity, and the response was completely blocked by atropine.

In attempting to resolve the discrepancy between these recent data (Lee et al., 2014) and a sizable literature, we carefully reviewed the experimental protocols. Strikingly, in all prior publications reporting direct D₁ stimulation of PLC activity, the concentrations of SKF-83959 or other benzazepines required to elicit these effects were in the high micromolar/low millimolar range (Felder et al., 1989a,b; Dyck, 1990; Mahan et al., 1990; Undie and Friedman, 1990, 1992, 1994; Vyas et al., 1992; Pacheco and Johe, 1997; Lee et al., 2004; Banday and Lokhandwala, 2007; Liu et al., 2009a; Zhang et al., 2009; Mizuno et al., 2012). No rationale was offered for use of SKF-83959 and chemically related benzazepines at concentrations far greater than their nanomolar K_D values. For SKF-83959 in particular, this raises a conundrum. The functional potency reported for D₁-mediated PLC activation is about 4 orders of magnitude greater than either its D₁ affinity or its D₁ potency for stimulating cAMP synthesis or activating β-arrestin.

Earlier we summarized the off-target activity of SKF-83959 and other benzazepines, noting the many receptors and transporters whose K_D values are in the range of 1–10 μM. Thus, when concentrations of 100–300 μM are used, these (as well as other) targets are probably engaged unintentionally. It would seem prudent that there be tests of the obvious alternate hypothesis that activation of PLC, when found, is mediated by one or several of these off-target receptors. In fact, some data already support this latter hypothesis. Yu et al. (1996) reported that 5 μM fenoldopam (an SKF-83959 analog that has high D₁ intrinsic activity) increased PLC activity, but only by 30–50%, and that this increase was mediated by cAMP, not direct PLC activation. Similarly, Jin et al. (2003) reported that the D₁ receptor linked to PLC was found in striatum, hippocampus, frontal cortex, and cerebellum. Because neither D₁-binding sites nor D₁ mRNA are found in the cerebellum, this suggests an off-target, rather than D₁, mechanism. Finally, in almost every study using pharmacological antagonism of D₁-PLC effects, the sole antagonist employed was the structurally similar benzazepine SCH23390 (Felder et al., 1989a,b; Dyck, 1990; Mahan et al., 1990; Undie and Friedman, 1990, 1992, 1994; Vyas et al., 1992;

Pacheco and Johe, 1997; Lee et al., 2004; Banday and Lokhandwala, 2007; Liu et al., 2009a; Zhang et al., 2009; Mizuno et al., 2012). Yet when structurally diverse antagonists were used, a very different picture emerges. For example, Jin et al. (2003) reported that the antagonist *cis*-flupenthixol was only half as effective as SCH23390 in blocking purported D₁-PLC activation; moreover, the D₁/D₂ antagonist (+)-butaclamol failed to block these effects at all.

In our view, the most compelling evidence comes from the study of Friedman et al. (1997), who found that the PLC activation *ex vivo* was unaffected by knockout of the D₁ receptor. Rather than interpreting this as evidence for a non-D₁ action of the benzazepines, Friedman et al. (1997) proposed that there was a novel D₁-like receptor that was responsible, despite inconsistencies with both the known expression of the D₅ receptor and the likely properties of then-orphan receptors in the mouse genome. Recalling that every published study over the last three decades failed to find D₁-mediated PLC activation at theoretical fractional occupancies even of 95%, there seems compelling support for the alternate hypothesis that off-target effects were the actual mechanism. The original idea, however, has morphed into a newer second hypothesis that D₁-D₂ heterodimers actually activate the Gα_Q-PLC-Ca²⁺ cascade. Although we feel the foundation for this novel hypothesis was flawed, it must be evaluated on its merits.

Hypothesis: D₁-D₂ Heterodimer-Mediated Activation of Gα_Q-PLC-Ca²⁺ by D₁-Selective Ligands Is an Important Signaling Mechanism

George and coworkers have recognized the discrepancy between functional potencies of SKF-83959 and its affinity, and hypothesized that whereas D₁ receptors alone (monomer and/or homodimer) could not stimulate PLC signaling, this would occur via coactivation of a D₁-D₂ heterodimer in a Gα_Q protein-dependent manner (Lee et al., 2004; Rashid et al., 2007a,b; Hasbi et al., 2009, 2011). Their view is that the limiting factor is the micromolar affinity of SKF-83959 for the D₂ receptor (Rashid et al., 2007b; Lee et al., 2014). There are several lines of evidence, however, that are inconsistent with this D₁-D₂ hypothesis.

First, if the effects are dependent on D₂ receptors, it is unclear how one can conclude that the occupancy of the D₁ receptor is important as the D₁ receptor is essentially saturated at the concentrations at which there is even partial fractional occupancy of the D₂. In addition, the reports of PLC activation in either kidney or brain (Felder et al., 1989a,b; Dyck, 1990; Mahan et al., 1990; Undie and Friedman, 1990, 1992, 1994; Vyas et al., 1992; Pacheco and Johe, 1997; Lee et al., 2004; Banday and Lokhandwala, 2007; Liu et al., 2009a; Zhang et al., 2009; Mizuno et al., 2012) show no effect at concentrations that would essentially saturate both D₁ and D₂ receptors (e.g., 10 μM). The rare reports showing functional Ca²⁺ effects at more reasonable concentrations also have anomalies. For example, one study reported essentially a quantal-like response for SKF-83959 in which no significant effects were seen at 30 nM concentrations, but a maximal effect at 100 nM (see Fig. 1B in Hasbi et al., 2009). This type of dose-response behavior is inconsistent with a Gα-type of mechanism that almost always yields a graded response.

More recently, it has been suggested that there is a unique role of D₁-D₂ heterodimers in subsets of coexpressing striatal

neurons (Perreault et al., 2012, 2014). Although this is a change from the original hypothesis of direct D₁-Gα_q-PLC activation by SKF-83959, it is important to consider its merits. One line of evidence that argues against the D₁-D₂ heterodimer hypothesis is that Chun et al. (2013) failed to find the predicted calcium signaling. Secondly, in HEK293 cells, SKF-83959 is an antagonist at D₂-mediated adenylate cyclase and a low-intrinsic-activity partial agonist at D₂-mediated β-arrestin recruitment (Lee et al., 2014), inconsistent with a dopamine-like physiologic effect. Thirdly, there was no PLC activation by SKF-83959 in HEK293 cells coexpressing D₁ and D₂ receptors (Lee et al., 2014). Finally, although there may be some coexpressing D₁ and D₂ cells (especially early in development), the vast majority of adult striatal neurons [where D₁-PLC activation was first reported (Undie and Friedman, 1990, 1992; Friedman et al., 1993)] are highly segregated. Medium spiny neurons of the direct pathway are largely D₁-expressing, those of the indirect pathway largely D₂-expressing, and such segregation also occurs elsewhere in the brain (Gerfen et al., 1990; Harrison et al., 1990; Scibilia et al., 1992; Rappaport et al., 1993; Le Moine and Bloch, 1995; Aubert et al., 2000; Deng et al., 2006; Bateup et al., 2008; Matamalas et al., 2009; Bertran-Gonzalez et al., 2010). In summary, while the D₁-D₂ heterodimer hypothesis is enticing, there is a significant body of evidence that suggests it may not be valid.

Hypothesis: D₁-PLC Signaling of SKF-83959 Causes Novel Behavioral Effects

One purportedly novel characteristic of SKF-83959 is its D₁-like behavioral activity in the absence of the ability to activate adenylate cyclase. This has been reported in both murine (Arnt et al., 1992; Downes and Waddington, 1993) and primate species (Gnanalingham et al., 1995a) and has been used as *prima facie* functional evidence for the importance of the D₁-Gα_q-PLC and/or D₁-D₂ heterodimer-Gα_q-PLC-Ca²⁺ signaling pathway. There are, however, two lines of evidence that suggest this hypothesis may not be true. These relevant *in vivo* studies have relied solely on the benzazepine family of D₁ agonists that, like SKF-83959, are behaviorally effective at quite low doses. For example, Arnt et al. (1992) reported that SKF-83959 caused maximal turning in the unilateral 6-hydroxydopamine model at a dose of 0.3 μmol/kg, whereas Deveney and Waddington (1995) found that yet lower doses were active in evoking D₁ behaviors in unlesioned rats. Yet even assuming no metabolism (and these catechols are rapidly metabolized), this yields a predicted concentration at the D₁ receptor far lower than required *in vitro* to activate either the hypothesized D₁-PLC or D₁-D₂ heterodimer systems. More recently, Medvedev et al. (2013) concluded that PLCβ via D₁ mechanisms regulated forward locomotion in mice, yet the sole experimental selective D₁ agonist, SKF-81297, was used at doses of 10 mg/kg, far higher than the 0.001–0.3 mg/kg known to produce typical D₁-like behavioral activity (Vermeulen et al., 1994; Cai and Arnsten, 1997; Diaz Heijtz and Castellanos, 2006). A parsimonious deduction would seem to be that D₁-like behavioral effects are irrelevant to these purported PLC signaling mechanisms requiring near-millimolar concentrations.

Another important potential confound also relates to pharmacokinetic factors. For example, a classic phase I metabolic reaction is *N*-demethylation, and SKF-83959 has the chemical

properties that suggest it would be a substrate for this type of cytochrome P450-mediated reaction (see Fig. 1). Recently, we demonstrated that the *N*-demethylated product of SKF-83959 actually has somewhat greater D₁ intrinsic activity than the parent compound (Lee et al., 2014). Thus, even if one still posits that the behavioral effects of SKF-83959 cannot involve adenylate cyclase signaling, the possibility that all of its behavioral actions might be explained by one or more active metabolites must be addressed.

Reconciliation of the Role of D₁ Receptors and Gα_q-PLC Activation

Popper (1959) has written, “Whenever the ‘classical’ system of the day is threatened by the results of new experiments which might be interpreted as falsifications..., the system will appear unshaken to the conventionalist. He will explain away the inconsistencies which may have arisen; perhaps by blaming our inadequate mastery of the system. Or he will eliminate them by suggesting ad hoc the adoption of certain auxiliary hypotheses, or perhaps of certain corrections to our measuring instruments.” It is with due irony that this seems to encapsulate the issues addressed in this Minireview. We have not sought to be provocative, but rather to follow the notion that it is appropriate, and even desirable, to be skeptical (not cynical) about hypotheses (or even theories or laws). Devising experiments to disprove a hypothesis honors the original conceptualization if those experiments fail to do so. Thus, the fundamental question is whether SKF-83959, or indeed any other D₁ agonist, causes PLC activation via actions at the D₁ receptor. We have encapsulated the issues at hand into three interrelated hypotheses illustrated in Fig. 2.

The first hypothesis (Fig. 2, left) deals with whether SKF-83959 is highly biased because of a lack of activity at canonical signaling pathways, a property used to explain why D₁-PLC signaling was important for behavioral/physiologic effects of SKF-83959 and related compounds. As we have reviewed, the weight of the literature (although not unanimous) suggests that the compound is actually a partial agonist at D₁ receptors in both heterologous systems and in the striatum, with activity in at least three D₁-mediated pathways: adenylate cyclase, β-arrestin, and receptor internalization (Gnanalingham et al., 1995b; Ryman-Rasmussen et al., 2005; Chemel et al., 2012; Lee et al., 2014). Thus, above and beyond the hypothesis that the D₁ receptor engages Gα_q-PLC, SKF-83959 would, at most, have modest bias, and canonical mechanisms would have to be ruled out before assigning physiologic/behavioral effects to PLC signaling.

The second hypothesis (Fig. 2, center) posits that PLC-mediated signaling is an important mechanism for SKF-83959 and other D₁ agonists. The studies in both kidney and brain that have supported a role of D₁ activation of PLC have been consistent in the use of ligands only from the benzazepine family and have employed these agonists at concentrations orders of magnitude higher either than their *K_D* or than their functional potency in canonical assays. Moreover, they generally used only the structurally similar SCH23390 as an antagonist (see references cited earlier). Unfortunately, the use of suprapharmacological concentrations of SKF-83959 and failure to consider non-D₁ mechanisms is not unique to studies of PLC signaling (Yu et al., 2008; Guo et al., 2013). It is unclear

Fig. 2. Schematic representation of three hypotheses related to D_1 signaling by SKF-83959. ACCase, adenylate cyclase; CREB, cAMP response element-binding protein; DAG, diacylglycerol; IA, intrinsic activity; IP₃, inositol triphosphate; PKA, protein kinase A.

why off-target actions, an obvious factor in pharmacological designs, have not generally been considered, especially as many of the likely targets are $G\alpha_Q$ -linked receptors.

To complicate matters, numerous studies failing to show D_1 -mediated stimulation of phosphoinositide hydrolysis (Kelly et al., 1988; Dearry et al., 1990; Rubinstein and Hitzemann, 1990; Pedersen et al., 1994; Demchyshyn et al., 1995; Kimura et al., 1995; Lee et al., 2014) have often been ignored. Indeed, Wallace and Claro (1990) noted that dopamine (at concentrations that stimulate cAMP synthesis) actually inhibited muscarinic stimulation of phosphoinositide hydrolysis. The most telling piece of data is the fact that PLC activation by SKF-83959 and other benzazepines was absolutely preserved in tissue from mice that had the D_1 receptor knocked out genetically (Friedman et al., 1997). Together, the available data suggest that the second hypothesis in Fig. 2 is false—SKF-83959 and other benzazepines do not stimulate PLC activity via the D_1 receptor.

The third hypothesis (Fig. 2, right) posits a critical role for D_1 - D_2 heterodimers, and is the one of the greatest of current interest because of the novel underlying molecular mechanism. In Popper's words, it may be considered as a "correction" of the initial hypothesis (Fig. 2, center). Although it was based originally on the D_1 -PLC mechanism and the use of SKF-83959, it has now taken on a life of its own because of the hypothesized unique role of D_1 - D_2 heterodimers in subsets of coexpressing striatal neurons (Perreault et al., 2012, 2014). We have reviewed the available data that we feel can be construed as weakening this hypothesis. Above and beyond the experimental issues [including our failure to be able to see evidence of PLC signaling in D_1 - D_2 -cotransfected HEK293 cells (Lee et al., 2014)], there is the question of the degree to which D_1 - D_2 colocalization occurs in the mature nervous system. Thus, our view is that hypothesis 3 is also false. Some may view this

conclusion as premature, yet a recent paper concluded: "the mechanism of D_1 [receptor]/ D_2 [receptor]-mediated calcium signaling involves more than receptor-mediated Gq protein activation, may largely involve downstream signaling pathways, and may not be completely heteromer-specific. In addition, SKF-83959 may not exhibit selective activation of D_1 - D_2 heteromers, and its significant cross-reactivity to other receptors warrants careful interpretation" (Chun et al., 2013). Unfortunately, this may be interpreted by some as simply being an experimental difference between investigators. A more skeptical view is that an extensive literature has already provided adequate evidence to falsify all of these prevailing hypotheses, much as we suggested years ago (Huang et al., 2001).

Conclusions

Although we have tried to offer a critical review of the available literature, there are several issues that we could not address. An obvious one with a pharmacological basis has to do with receptor reserve, a well known mechanism that can affect interpretation of functional studies and might be thought to be a reason for the disparate literature. Yet it is important to remember that much of the PLC hypothesis has been based on studies done *ex vivo* using brain or kidney tissue, where presumably all investigators were dealing with both physiologically relevant and similar receptor reserve. Thus, the huge difference in D_1 potencies between canonical functional assays and PLC activation is unlikely to involve receptor reserve. Moreover, the near-millimolar concentrations of D_1 agonists required to cause PLC activation *ex vivo* will never be achieved *in vivo* when administering D_1 dopamine agonists to animals at

behaviorally active concentrations (Taylor et al., 1991; Arnsten et al., 1994; Schneider et al., 1994; Mottola et al., 2002).

In addition to our recent work (Lee et al., 2014), there are other experiments that might be useful if one felt that the available data were not adequately conclusive. The two most obvious are those with a pharmacological basis. First, future studies should take advantage of the structurally diverse D₁ agonists and antagonists that are available (e.g., Fig. 1). Second, when there is a question of off-target engagement, studies done in vivo or ex vivo should measure drug concentrations in tissue. In addition, a host of molecular approaches might also be useful. The most challenging relates to the idea that D₁-D₂ heterodimers are the mechanistic key. Although we feel that this hypothesis is not well supported, others may feel that further studies are required to reach firm conclusions. Techniques such as proximity ligation assays (Trifileff et al., 2011) can be used to look for molecular proximity between D₁ and D₂ in brain slices, and bioluminescence resonance energy transfer can be used to determine if Gα_Q is activated by the D₁ alone or when complexed with D₂ (Urizar et al., 2011). Indeed, there are recent data using such approaches that failed to find evidence for either D₁ agonist engagement of Gα_Q or molecular proximity of D₁ and D₂ in mouse brain (J. Javitch et al., personal communication). Such data, while “negative,” are compelling evidence for the perspectives we have offered.

There may be substantial impact to resolution of this controversy. The potential clinical utility of D₁ agonists has slowly been receiving human validation (Rascol et al., 1999; Rosell et al., 2014), although the development of an approvable drug has been inhibited by issues including pharmacokinetics, seizures, and hypotension. A functionally selective D₁ agonist could theoretically have advantages (Mailman, 2007), yet whereas some existing D₁ ligands do have some degree of functional selectivity (Lewis et al., 1998; Ryman-Rasmussen et al., 2005, 2007), no compound has yet been shown to have sufficient signaling bias to translate into meaningful pharmacological differences. Importantly, we believe that none of the known D₁-selective benzazepines is a highly biased D₁ ligand, contrary to what has been hypothesized. It is important that the field should focus on the substantial scientific issues that remain to be elucidated about D₁ function and their impact on drug discovery, and not be distracted by mechanisms that may be artificial.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: Lee, Yang, Mailman.

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