Diacylglycerol and ceramide formation induced by dopamine D2S receptors via Gβγ-subunits in Balb/c-3T3 cells

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Liu, Gele, Mohammad H. Gharremani, Behzad Banihashemi, and Paul R. Albert. Diacylglycerol and ceramide formation induced by dopamine D2S receptors via Gβγ-subunits in Balb/c-3T3 cells. Am J Physiol Cell Physiol 284: C640–C648, 2003.—Diacylglycerol (DAG) and ceramide are important second messengers affecting cell growth, differentiation, and apoptosis. Balb/c-3T3 fibroblast cells expressing dopamine-D2S (short) receptors (Balb-D2S cells) provide a model of G protein-mediated cell growth and transformation. In Balb-D2S cells, apomorphine (EC50 = 10 nM) stimulated DAG and ceramide formation by 5.6- and 4.3-fold, respectively, maximal at 1 h and persisting over 6 h. These actions were blocked by pretreatment with pertussis toxin (PTX), implicating G/i proteins. To address which G proteins are involved, Balb-D2S clones expressing individual PTX-insensitive Gαi3 proteins were treated with PTX and tested for apomorphine-induced responses. Neither PTX-insensitive Gαi3 nor Gα12 rescued D2S-induced DAG or ceramide formation. Both D2S-induced DAG and ceramide signals required Gβγ-subunits and were blocked by inhibitors of phospholipase C [1-6-(17β-3-methoxyestra-1,2,3[10]-tri-en-17yl)amino]hexyl)-1H-pyrrole-2,5-dione (U-73122) and partially by D609. The similar G protein specificity of D2S-induced calcium mobilization, DAG, and ceramide formation indicates a common Gβγ-dependent phospholipase C-mediated pathway. Both D2 agonists and ceramide specifically induced mitogen-activated protein kinase (ERK1/2), suggesting that ceramide mediates a novel pathway of D2S-induced ERK1/2 activation, leading to cell growth.

THE Dopamine-D2 receptor gene encodes two splice variants of the receptor, long and short forms (D2L and D2S), that are pharmacologically and functionally equivalent with both forms coupling equivalently to Gαi “inhibitory” proteins (10, 28). In pituitary cells, the dopamine D2S receptor inhibits ERK1/2 activation and cell proliferation (1, 13, 32, 37). Oppositely, in Balb/c-3T3, Chinese hamster ovarian (CHO), and C6 mesenchymal cells, the D2S receptor stimulates cell proliferation and induces tumor formation, involving calcium mobilization and ERK1/2 activation (8, 16, 20, 26, 31). These actions are blocked by pertussis toxin (PTX), implicating G/i proteins. However, the signaling pathways that mediate G/i-induced regulation of cell proliferation remain poorly defined.

Ceramide is a novel and important second messenger involved in a wide variety of signal transduction pathways that mediate cell-specific biological responses such as cell growth, differentiation, inflammation, and apoptosis (11, 12, 19, 22). Ceramide is produced from many sources, such as the action of sphingomyelinase (SMase) on sphingomyelin (SM) (33), de novo synthesis (27), or metabolism of other lipids (7, 17). There is also a close dynamic relationship between the biosynthetic pathways for diacylglycerol (DAG) and ceramide via SM synthase, which interconverts ceramide/phosphatidylcholine (PC) into SM/DAG (22). Thus this work focused on the relationship between DAG and ceramide levels and their regulation by D2S receptor activation in signaling to cell proliferation.

To address whether different G proteins mediate divergent D2S-induced responses, we have expressed antisense or PTX-insensitive mutant Gαi2/Gαi3 constructs in GH4 pituitary or Balb/c-3T3 fibroblast cells (15, 16, 23, 24). PTX-insensitive Gαi2 mutants were generated by a conservative Cys-to-Ser substitution and retain coupling to a variety of effectors (9, 16, 38), including Gαi-mediated inhibition of adenylyl cyclase, Gβγ-induced inhibition of calcium channels, and Gβγ-induced ERK1/2 activation. By pretreating cells with PTX to block endogenous Gαi3 proteins, the specific contribution of individual G proteins can be assessed in cells transfected with individual PTX-resistant Gαi3 proteins. In Balb-D2S cells (Balb/c-3T3 cells transfected with D2S receptor cDNA), Gαi2/Gβγ mediated D2S-induced ERK1/2 activation and DNA synthesis, and Gαi3 was required for D2S-induced transformation (16). These results indicate that whereas ERK1/2 activation is linked to D2S-mediated DNA synthesis, Gα3-dependent signaling to transformation did not require ERK1/2 and utilized unknown messengers. We therefore addressed the question of whether D2S receptor activation regulates other second messengers, such as DAG or ceramide, and which G proteins are required.

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MATERIALS AND METHODS

Materials. Apomorphine, dopamine, Staphylococcus aureus SMase, PTX, 1,2-diacyl-sn-glycero-3-phosphoethanolamine (C18:1[cis]-9), DAG, U-73122 and all other drugs, standards, and salts were purchased from Sigma. Escherichia coli pBR322 was obtained from Life Technologies. Thin-layer chromatography (TLC) plates (0.25 mm) were from Whatman. Solvents were supplied by BDH. Phospho-SAPK/JNK Tyr183/Tyr185) G9 monoclonal antibody, phospho-p38 (Tyr180/Tyr182) monoclonal antibody, and the phosphoplossus p44/42 MAP kinase antibody kit were purchased from New England Biolabs (Mississauga, Ontario, Canada).

Cell culture and transfection. Balb-D2S cells and derivative clones were cultured in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS). Balb-D2S (clone 11) cells were generated as described, and specific D2S receptor density was 143.6 ± 35.9 fmol/mg protein by saturation binding analysis with [3H]spiperone (16). PTX-insensitive mutant Goα/Gαs subunit constructs (Gια/PTX and Gαs-PTX) and GRK-ct were constructed previously (15) and transfected individually (30 μg) into Balb-D2S cells (clone 11), and the cells were selected in medium containing G418 (700 μg/ml) for 14 days. Antibiotic-resistant clones of each transfection were picked (24 clones/transfection) and tested for expression of the corresponding Goα/Gαs proteins using Northern blot and Western blot analyses.

Treatment and lipid extraction. Equivalent numbers of cells were cultured on 10 x 10 cm plates with DMEM plus 10% FBS in a humidified atmosphere of 5% CO2 at 37°C, growing to 80–90% confluence. Before experiments, the cells were cultured in serum-free medium for 16 h. For PTX treatment, the cells were treated with 50 ng/ml PTX for 16 h before experimentation. Cells were treated with apomorphine at the designed concentration and time and with S. aureus SMase (0.1 U/ml) for 30 min. After treatment, twice washing with ice-cold phosphate-buffered saline (PBS) terminated the reaction. The lipid extraction method was based on that of Bligh and Dyer (5). After centrifugation at 500 g for 1 min at 4°C, the supernatants were aspirated and the cells lysed with 0.5 ml of chloroform-methanol-HCl (20:40:1 vol/vol/vol). Extracts were sonicated each 5 s and centrifuged, and 40 μl of the supernatant was analyzed by TLC for phosphatidic acid, 0.2 ml of chloroform, and 0.2 ml water. The resultant organic phase was dried under N2 and reconstituted in 25 μl of chloroform/ methanol (95:5 vol/vol). The samples were spotted onto a Silica Gel 60 TLC plate being heat activated and developed in a solvent mixture of chloroform-acetone-methanol-acetic acid-water (10:4:3:2:1 vol/vol/vol/vol/vol). Because DAG kinase can use ceramide or DAG as a substrate, [32P]Jceramide-phosphate represented ceramide production and [32P]Jphosphatidic acid represented DAG production (34). The TLC plates were exposed to phosphor screens for 18 h, and [32P]Jceramide-phosphate and [32P]Jphosphatidic acid were quantified using the Molecular Dynamics System ImageQuant NT software. Results are expressed as percentage of control.

Western blot analysis. Cells were cultured in serum-free medium for 16 h and then treated with experimental compounds for 30 min. The cells were washed twice with ice-cold PBS and extracted with 100 μl of RIPA-L buffer [10 mM Tris (pH 8), 1.5 mM MgCl2, 5 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, and 5 μg/ml leupeptin]. Samples were frozen on dry ice/ethanol and stored at −80°C. Samples were sonicated 10–15 s, heated at 95°C for 5 min, centrifuged, and 40 μl/sample were loaded onto SDS-PAGE gel and electrotransferred to nitrocellulose membrane. The membrane was blocked (1 h at room temperature) and probed with primary antibody (1:1,000 overnight at 4°C). It was washed in TBST, incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000) and HRP-conjugated anti-biotin antibody (1:1,000) to detect biotinylated protein markers (2 h at room temperature), washed, incubated with Lumiglo (1 min), and exposed to X-ray film. Densitometric quantitation was done using the UN-SCAN-IT program (Silk Scientific, UT), and data were normalized to control.

Statistical analysis. The data were analyzed by repeated measure using ANOVA for each set of experiments. The data are presented as means ± SE. of at least three independent experiments. Differences of P < 0.05 were considered statistically significant. The percent inhibition data was analyzed with repeated measure using ANOVA, and the data from Goα-PTX expressing clones were compared with Balb-D2S cell (wild type) using Bonferroni multiple comparison posttest.

RESULTS

Apomorphine-induce DAG and ceramide production in Balb-D2S cells. In Balb/c-3T3 cells stably transfected with the dopamine-D2S receptor (Balb-D2S cells), DAG and ceramide production was induced by apomorphine (dopamine receptor agonist) in a concentration-dependent manner with Emax of 10−8 M (Fig. 1). Within 30 min, apomorphine (10−5 M) increased DAG and ceramide compared with control levels by 400% (500 pmol/107 cells) and 300% (430 pmol/107 cells), respectively. No response to apomorphine was observed in Balb/c-3T3 cells (data not shown), which do not express dopamine receptors. Exogenous SMase, used as a positive control, hydrolyzed endogenous SM

25°C for 30 min, and the reaction was terminated by addition of 0.5 ml of ice-cold chloroform-methanol (1:2 vol/vol). The lipids were extracted by the addition of 0.5 ml chloroform and 0.5 ml 1 M NaCl. The mixture was spun at 14,000g for 3 min, and the upper aqueous phase was discarded. The lower organic phase was sequentially washed with 0.5 ml of 1% perchloric acid, 0.3 ml chloroform-methanol (1:2 vol/vol), 0.2 ml chloroform, and 0.2 ml water. The resultant organic phase was dried under N2 and reconstituted in 25 μl of chloroform/methanol (95:5 vol/vol). The samples were spotted onto a Silica Gel 60 TLC plate being heat activated and developed in a solvent mixture of chloroform-acetone-methanol-acetic acid-water (10:4:3:2:1 vol/vol/vol/vol/vol). Because DAG kinase can use ceramide or DAG as a substrate, [32P]Jceramide-phosphate represented ceramide production and [32P]Jphosphatidic acid represented DAG production (34). The TLC plates were exposed to phosphor screens for 18 h, and [32P]Jceramide-phosphate and [32P]Jphosphatidic acid were quantified using the Molecular Dynamics System ImageQuant NT software. Results are expressed as percentage of control.

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to form ceramide and also increased DAG formation, suggesting conversion of ceramide to DAG. The bands of radioactive DAG and ceramide comigrated with DAG and ceramide standards and were quantified in the concentration range from 50–1,000 pmol. Apomorphine-induced DAG and ceramide production was time dependent (Fig. 2) and maximal at 1 and 6 h, respectively. Ceramide production returned to basal at 24 h, whereas DAG was elevated at 30 h. Apomorphine-induced DAG paralleled and was greater than the increase in ceramide production. Pretreatment with PTX blocked DAG and ceramide formation induced by 10–6 M apomorphine (Fig. 3), thus implicating Gᵢₒ proteins in D2S receptor action. As a positive control, exogenous SMase increased the levels of ceramide equally in cells treated or not treated with PTX (Fig. 3B), demonstrating G protein-independent hydrolysis of endogenous SM to form ceramide.

Gᵢₒ and Gᵢ₃-PTX fail to rescue apomorphine-induced DAG and ceramide formation. Balb-D2S cells express all Gᵢ/o/subunits, although Gᵢ₂ and Gᵢ₃ appear to be the most abundant based on densitometric analysis (16). To address the G protein specificity of D2S signaling to ceramide production, PTX-insensitive point mutants of Gᵢ₂ and Gᵢ₃ (Gᵢ₂-PTX and Gᵢ₃-PTX) were transfected into Balb-D2S cells to form Gᵢ₂-PTX (BDi2–22) and Gᵢ₃-PTX (BDi3–3) cells. The transfectant cell lines expressed twofold more Gᵢ protein than the corresponding endogenous Ga-subunit in parental Balb-D2S (16), suggesting that approximately equal amounts of mutant and wild-type proteins were produced in the transfected cell lines. In Gᵢ₂-PTX (BDi2–22) and Gᵢ₃-PTX (BDi3–3) cells, apomorphine-induced DAG and ceramide formation

![Fig. 1. Apomorphine induces diacylglycerol (DAG) and ceramide formation in a concentration-dependent manner in Balb/c-3T3 cells. Balb-D2S cells (Balb/c-3T3 cells stably transfected with dopamine D2S receptor cDNA) were treated with apomorphine (dopamine receptor agonist) from 10⁻⁹ to 10⁻⁷ M for 30 min as indicated. Lipids were extracted from cells, and [³²P]ceramide-phosphate and [³²P]phosphatidic acid were resolved by thin layer chromatography (TLC) as a measure of DAG and ceramide content, respectively (see MATERIALS AND METHODS). Top: a representative image of [³²P]ceramide-phosphate and [³²P]phosphatidic acid is shown here; the double bands for ceramide represent 2 isoforms of ceramide. The amounts of DAG and ceramide standards are as indicated. B, background (no DAG kinase); S, cells treated with exogenous sphingomyelinase (SMase; 0.1 unit/ml for 30 min); C, control untreated cells. Bottom: the relative levels (% of control) of DAG and ceramide were quantified by phosphorimager scan, and the data are expressed as a function of apomorphine concentration as means ± SE from 3 independent experiments. *P < 0.05; **P < 0.01.

![Fig. 2. Time course of apomorphine-induced increase in DAG and ceramide formation. Balb-D2S cells were treated with apomorphine (10⁻⁶ M) from 5–60 min and from 3–30 h. Lipids were extracted from cells, and [³²P]ceramide-phosphate and [³²P]phosphatidic acid were separated by TLC. A representative image of [³²P]ceramide-phosphate and [³²P]phosphatidic acid is shown. Standards for ceramide only were used at the indicated amounts. The relative levels (% control) of DAG and ceramide were quantified and plotted as a function of time. Data are expressed as means ± SE from 3 independent experiments. *P < 0.05; **P < 0.03; ***P < 0.01. Cer, ceramide.
was blocked after pretreatment with PTX (Figs. 4 and 5), suggesting that \( G_{12} \) or \( G_{13} \) alone do not mediate D2S-induced ceramide production. By contrast, apomorphine-induced inhibition of cAMP formation was rescued in both \( G_{12} \)- and \( G_{13} \)-PTX cells (16). As a positive control, exogenous SMase (0.1 U/ml) induced ceramide production. Hence, neither \( G_{12} \)-PTX or \( G_{13} \)-PTX rescued D2S receptor coupling to DAG and ceramide production after PTX treatment.

**GRK-ct blocks apomorphine-induced DAG and ceramide formation.** We examined whether \( G_{13} \)-subunits are involved in D2S-induced DAG and ceramide production in stably transfection of Balb-D2S cells with GRK-ct, a scavenger protein that binds to \( G_{13} \)-subunits to prevent their action. In these cells, apomorphine did not alter ceramide production, but exogenous SMase (0.1 U/ml) did induce ceramide production (Fig. 6). Importantly, other D2S-induced actions, such as inhibition of forskolin-induced cAMP accumulation, were not affected by expression of GRK-ct in these cells (16), indicating that functional D2S receptors were present in these clones. This result suggests a crucial role for mobilization of \( G_{13} \)-subunits in the D2S-mediated ceramide signal.

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**Fig. 3.** Pertussis toxin (PTX) blocks DAG and ceramide production induced by dopamine-D2S receptor activation. **Left:** Balb-D2S cells were treated with \( 10^{-6} \) M apomorphine for 30 min with or without pretreatment using 50 ng/ml PTX for 16 h. **Right:** wild-type LD2S Balb/c-3T3 cells were treated with SMase (0.1 unit/ml for 30 min) as a positive control. A representative image of \(^{32}P\)ceramide-phosphate and \(^{32}P\)phosphatidic acid is shown above. Data are quantified and expressed as means \( \pm \) SE from 3 independent experiments. * \( P < 0.05 \). B, blank; A, apomorphine; P, PTX; A+P, apomorphine + PTX; S, SMase; S+P, SMase + PTX; Std., standard.

**Fig. 4.** \( G_{13} \)-PTX fails to rescue block of apomorphine-induced DAG and ceramide production by PTX. Balb-D2S cells expressing \( G_{13} \)-PTX (BDi2–22 cells), the PTX-insensitive point mutant of \( G_{13} \), were treated with apomorphine (\( 10^{-6} \) M) for 30 min with or without pretreatment with 50 ng/ml PTX for 16 h. Cells were treated with SMase (0.1 unit/ml for 30 min) as a positive control. A representative image of \(^{32}P\)ceramide-phosphate and \(^{32}P\)phosphatidic acid is presented above. At bottom, the data are expressed as means \( \pm \) SE from 3 independent experiments. * \( P < 0.03 \).
Role of phospholipase C in D2S-induced formation of DAG/ceramide. According to the results above, we further investigated upstream of DAG/ceramide formation by the action of apomorphine in Balb-D2S cells (Fig. 7). Phospholipase C inhibitors U-73122 (10 μM, U) for phosphatidylinositol (PI)-PLC and D609 (10 μM, D) for PC-PLC were applied at maximal concentrations (29, 39). U-73122 completely blocked apomorphine-induced DAG/ceramide formation, whereas D609 partially inhibited this action by 50% or 60%, respectively. The PI-PLC inhibitor U-73122 reduced DAG and ceramide formation in parallel with IC50 of 1 μM (consistent with PLC inhibitory concentration), whereas inactive analog 1-(6-[(17β)-3-methoxyestr-1,2,3-10]-tri-en-17yl)amino]hexyl)-2,5-pyrrolidinidione (U-73343) was without effect up to 10 μM (Fig. 8). These results are consistent with a specific role for PLC activation in D2S-induced DAG and ceramide formation.

Regulation of ERK1/2 by ceramide. The possible downstream actions of ceramide were examined by comparing the actions of apomorphine, ceramide analog C2-ceramide, and SMase on mitogen-activated protein kinases in Balb-D2S cells (Fig. 9). Antibodies specific for phosphorylated forms that represent activated proteins were used in Western blot analysis. In other cell types, ceramide activates stress-activated protein kinases, such as p38 or JNK, to mediate apoptosis (2, 6, 41), so we examined these pathways. Neither dopamine agonist (apomorphine), C2-ceramide, nor SMase altered levels of phospho-p38 (Fig. 9), phospho-STAT3, or phospho-STAT5 (not shown) compared with control as assessed by densitometry. As previously reported (16), apomorphine induced a 2.2-fold increase in phospho-ERK1/2. In addition, C2-ceramide and SMase also induced a 2.1- and 2.0-fold, respectively, increase in phospho-ERK1/2. By contrast, whereas apomorphine induced a small (1.5-fold) increase in phospho-JNK, this was not mimicked by C2-ceramide or SMase treatment. Thus ceramide appears to specifically regulate ERK1/2 phosphorylation in Balb-D2S cells, providing a novel potential mechanism for dopamine D2S-induced ERK1/2 activation.

DISCUSSION

D2S-induced DAG and ceramide formation. In Balb/c-3T3 cells expressing D2S receptors, apomorphine in-
duced DAG and ceramide formation in a concentration- and time-dependent manner and was blocked by PTX, implicating G\textsubscript{i}/G\textsubscript{o} proteins. The D2S receptor provides a novel and interesting example of a G\textsubscript{i}/G\textsubscript{o}-coupled receptor that induces DAG and ceramide formation with the same G protein specificity: these actions were dependent on G\textsubscript{i2}-subunits but were not rescued by individual G\textsubscript{ai2}- or G\textsubscript{ai3}-PTX subunits (see model, Fig. 10). Other receptor subtypes such as the angiotensin II type 2 (AT2) receptor (21) or interferon-\gamma receptor (42) mediate PTX-sensitive ceramide production, but their G protein specificity has not been examined. The G\textsubscript{i}-coupled cannabinoid CB1 receptor also induces ceramide formation to inhibit tumor

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**Fig. 7.** Involvement of phospholipase C in D2S-induced DAG/ceramide generation. Balb-D2S cells were treated without (control) or with apomorphine (10^{-6} M), PC-PLC inhibitor D609 (10^{-5} M; D), PI-PLC inhibitor 1-(6-[17β]-3-methoxyestra-1,2,3\textsubscript{-10\textsuperscript{-trien-17yl]amino}[hexyl]-1H-pyrrole-2,5-dione (U-73122; 10^{-5} M; U), or SMase (0.1 U/ml) for 30 min. A representative image of \[^{32}P\]ceramide-phosphate and \[^{32}P\]phosphatidic acid is shown, and data are expressed as means ± SE from 3 independent experiments. *P < 0.03; **P < 0.01.

**Fig. 8.** Concentration dependence of inhibition of D2S-induced DAG/ceramide formation by U-73122 or 1-(6-[17β]-3-methoxyestra-1,2,3\textsuperscript{-10\textsuperscript{-trien-17yl]amino}[hexyl]-2,5-pyrrolinedione (U-73343). Balb-D2S cells were treated with apomorphine (10^{-6} M) without or with pretreatment U-73122 or inactive analog U-73343 for 3 h at the indicated concentration. A representative image of \[^{32}P\]ceramide-phosphate and \[^{32}P\]phosphatidic acid is shown, and at bottom quantified data are expressed as means ± SE from 3 independent experiments. *P < 0.03; **P < 0.01.
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growth (14), but it is unclear whether this is a Gαi3/Gβγ-mediated action.

The matching apomorphine concentration dependencies (EC50 = 10⁻⁸ M), PLC inhibitor concentration dependencies, G protein subunit dependencies, and the close temporal association between D2S-induced DAG and ceramide formation suggest that DAG formed by D2S-induced PLC activation is converted to ceramide as shown in Fig. 10. The differential action PLC inhibitor (U73,122 selective for PI-PLC stronger than D609 for PC-PLC) further indicates the primary role of PI-PLC in D2S-induced DAG and ceramide generation. Interconversion of DAG and ceramide could be catalyzed by SM synthase, which converts ceramide and PC into SM and DAG (22). In cells, this reaction is bidirectional and can convert DAG into ceramide, leading to depletion of SM (25, 40). Importantly, DAG inhibits the forward reaction to favor conversion to ceramide (18, 43). Ceramide inhibits de novo synthesis of PC (43), again favoring the reverse reaction to form ceramide from DAG. D609, a compound thought to be specific for PC-PLC but that also inhibits SM synthase (25), displayed slightly greater inhibition of ceramide than of DAG (60 vs. 50%), suggesting at best a partial role for SMase in DAG-ceramide conversion. Alternately, DAG can activate acidic SMase to generate ceramide (35, 36). For example, tumor necrosis factor couples to PC-PLC to increase production of DAG, which activates acidic SMase to induce ceramide production. The conversion of DAG to ceramide or DAG-induced ceramide formation would account for the identical Gαi2 and Gβγ dependencies of D2S-mediated DAG and ceramide formation.

G protein specificity of D2S responses. There are striking similarities between the pattern of G protein subunits required for D2S-induced DAG/ceramide formation and calcium mobilization in Balb-D2S or L-D2S cells: Gβγ-subunits were required and no individual or pair of Ga-PTX proteins reconstituted D2S-induced calcium mobilization (15, 16). Receptor-mediated activation of PI-PLC is known to generate IP3, which mediates calcium mobilization and DAG (3, 4), an important second messenger that activates PKC (30). Thus the D2S receptor mediates Gβγ-dependent calcium mobilization and DAG formation, possibly by activation of PI-PLC-β2 or -β3, which are Gβγ sensitive. D2S-induced DAG appears to be rapidly converted to increase ceramide levels (Fig. 10). The lack of rescue
amide formation mediates in part ERK1/2 phosphorylation, suggesting that D2S-induced ceramide generation, implicating Gαq, Gβγ, and ERK1/2 in cell growth (16). In the present study, D2S-induced ERK1/2 activation was mimicked by ceramide analog or SMase-mediated ceramide generation, suggesting that D2S-induced ceramide formation mediates in part ERK1/2 phosphorylation. Although G12-PTX did rescue D2S-induced ERK1/2 activation and cell growth, the rescue was only partial (16), suggesting that Gα2-dependent and -independent pathways may contribute. One Gα2-independent, Gβγ-dependent pathway activated by D2S receptors may involve ceramide formation. It is possible that Gα11 or Gαo couple D2S receptors to DAG or ceramide formation. Gα11 appears to couple to growth regulatory signaling, because Gα11-PTX clones became spontaneously transformed but were therefore weakly responsive to apomorphine and were not examined. These studies illustrate the utility of PTX-insensitive G proteins as a molecular approach to map G protein-mediated pathways and to place novel second messengers such as ceramide within these pathways.

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