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. Ghabremani, 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. First published November 13, 2002; 10.1152/ajpcell.00190.2002.—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 Gi/Go proteins. However, the signaling pathways that mediate Gi/Go-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 Gαi/Gαo constructs in GH4 pituitary or Balb/c-3T3 fibroblast cells (15, 16, 23, 24). PTX-insensitive Gαi 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 adenyl cyclase, Gβγ-induced inhibition of calcium channels, and Gγ-induced ERK1/2 activation. By pretreating cells with PTX to block endogenous Gαo proteins, the specific contribution of individual G proteins can be assessed in cells transfected with individual PTX-resistant Gαi proteins. In Balb-D2S cells (Balb/c-3T3 cells transfected with D2S receptor cDNA), Gα12/Gβ2 mediated D2S-induced ERK1/2 activation and DAG synthesis, and Gα13 was required for D2S-induced transformation (16). These results indicate that whereas ERK1/2 activation is linked to D2S-mediated DNA synthesis, Gα13-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-dioleoyl-rac-glycerol (C18:1cis-9), DAG, U-73122 and all other drugs, standards, and salts were purchased from Sigma. Escherichia coli DAG kinase (13 U/mg protein) and D609 were purchased from Calbiochem. [γ-32P]ATP (specific activity: >3,500 Ci/mmol) was supplied by Amersham. [α-32P]dCTP was from Amersham Sera. Media and geneticin (G418) were obtained from Life Technologies. Thin-layer chromatography (TLC) plates (0.25 mm) were from Whatman. Solvents were supplied by BDH. Phospho-SAPK/JNK (Tyr185/tyr186) G9 monoclonal antibody, phospho-p38 (Tyr185/tyr182) monoclonal antibody, and the phosphoplus 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 Gaq/Gao subunit constructs (Gaq-PTX and Gao-PTX) and GRK-ct were constructed previously (15) and were modiﬁed Eagle’s 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 gels 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 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 using the Molecular Dynamics System ImageQuaNT computer software. Results are expressed as percentage of control.

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 EC50 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 14,000 g 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). Because DAG kinase can use ceramide or DAG as a substrate, [32P]ceramide-phosphate represented ceramide production and [32P]phosphatidic acid represented DAG production (34). The TLC plates were exposed to phosphor screens for 18 h, and [32P]ceramide-phosphate and [32P]phosphatidic acid were quantified using the Molecular Dynamics System ImageQuant computer 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 gels 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 using repeated measure using ANOVA, and the data from Gaq-PTX expressing clones were compared with Balb-D2S cell (wild type) using Bonferroni multiple comparison posttest.
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 Gᵢ/Go 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.

Gᵢ₂- 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 10⁻⁵ 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.](http://ajpcell.physiology.org/)
was blocked after pretreatment with PTX (Figs. 4 and 5), suggesting that \( G_{i2} \) or \( G_{i3} \) alone do not mediate D2S-induced ceramide production. By contrast, apomorphine-induced inhibition of cAMP formation was rescued in both \( G_{\alpha i2}^{-} \) and \( G_{\alpha i3}^{-} \)-PTX cells (16). As a positive control, exogenous SMase (0.1 U/ml) induced ceramide production. Hence, neither \( G_{\alpha i2}^{-}\)PTX or \( G_{\alpha i3}^{-}\)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 \( G_{\beta \gamma} \)-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 \( G_{\beta \gamma} \)-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 \( G_{\beta \gamma} \)-subunits in the D2S-mediated ceramide signal.
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 (10⁻⁵ M, U) for phosphatidylinositol (PI)-PLC and D609 (10⁻⁵ M, 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-methoxyestra-1,2,3-10-trien-17yl]amino]hexyl)-2,5-pyrrolidinedione (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-

Fig. 5. Gα₁₃-PTX fails to rescue block of apomorphine-induced DAG and ceramide production by PTX. BD13–3 cells were treated with 10⁻⁶ M apomorphine for 30 min with or without pretreatment 50 ng/ml PTX for 16 h. Lipids were extracted from cells, and [³²P]ceramide-phosphate and [³²P]phosphatidic acid were separated and quantitated. At top is shown a representative image of [³²P]ceramide-phosphate and [³²P]phosphatidic acid. At bottom, the data are expressed as means ± SE from 3 independent experiments. *P < 0.03.
duced DAG and ceramide formation in a concentration- and time-dependent manner and was blocked by PTX, implicating G\(i/G\alpha\) proteins. The D2S receptor provides a novel and interesting example of a G\(i/G\alpha\)-coupled receptor that induces DAG and ceramide formation with the same G protein specificity: these actions were dependent on G\(\beta\gamma\)-subunits but were not rescued by individual G\(\alpha_{i2}\)- or G\(\alpha_{i3}\)-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\(i\)-coupled cannabinoid CB1 receptor also induces ceramide formation to inhibit tumor

![Graph 1](image1)

![Graph 2](image2)

![Graph 3](image3)

![Graph 4](image4)

![Graph 5](image5)

![Graph 6](image6)

![Graph 7](image7)

![Graph 8](image8)

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\(^{-6}\)M; D), PI-PLC inhibitor 1-(6-[(17β)-3-methoxyestra-1,2,3(10)-trien-17-yl]amino)hexyl)-1H-pyrrole-2,5-dione (U-73122; 10\(^{-6}\)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.

![Graph 9](image9)

Fig. 8. Concentration dependence of inhibition of D2S-induced DAG/ceramide formation by U-73122 or 1-(6-[(17β)-3-methoxyestra-1,2,3(10)-trien-17-yl]amino)hexyl)-2,5-pyridoline dione (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 Gia/Gsb-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 Ga 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
of these responses by individual (or paired) Ga-PTX proteins could indicate that activation of Ga11, Gaα, or multiple G proteins is required to mobilize sufficient Gβγ-subunits. However, the possibility remains that PTX-insensitive Go-subunits may couple inefficiently to this response because of the Cys-to-Ser change. By contrast, D2S-induced inhibition of forskolin-stimulated cAMP formation was rescued by Go12- or Go13-PTX, indicating their functionality in these cells (16).

Role of D2S-induced DAG or ceramide formation in ERK1/2 activation. As illustrated in Fig. 10, in Balb-D2 cells apomorphine-induced ERK1/2 activation and DNA synthesis are blocked by PTX and GRK-ct and are rescued by Go12-PTX but not Go13- or Goα-PTX, implicating Go12, 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 Go12-PTX did rescue D2S-induced ERK1/2 activation and cell growth, the rescue was only partial (16), suggesting that Go2-dependent and -independent pathways may contribute. One Go2-independent, Gβγ-dependent pathway activated by D2S receptors may involve ceramide formation. It is possible that Ga11 or Gaα couple D2S receptors to DAG or ceramide formation. Ga11 appears to couple to growth regulatory signaling, because Ga11-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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