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The D2s Dopamine Receptor Stimulates Phospholipase D Activity: A Novel Signaling Pathway for Dopamine

SUSAN E. SENOGLES

Department of Biochemistry, College of Medicine, University of Tennessee, Memphis, Tennessee

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ABSTRACT

The D2 dopamine receptor isoforms signal to a variety of cellular effectors in both the central nervous system and periphery. Two alternative splice forms of the D2 dopamine receptor exist, the D2s (short) and D2l (long), which has an insertion of 29 amino acids in the third intracellular loop (Dal Taso et al., 1989). In cells of the anterior lobe of the pituitary, D2 dopamine receptors (both forms) are present on lactotroph cells coupled to the inhibition of adenylyl cyclase, activation of voltage-gated calcium channels, and inhibition of potassium channels. We describe here a novel signaling pathway for the D2s, which is the activation of phospholipase D (PLD). GH4C1 cells, a clonal line derived from a rat pituitary tumor, were stably transfected with the gene encoding the D2s, generating GH4-121 cells. Treatment of GH4-121 cells with a dopaminergic agonist resulted in activation of PLD in both a dose-dependent and time-dependent manner. This signaling pathway was not inhibited by prior treatment of cells with pertussis toxin at concentrations that ablate other D2s receptor signaling in this cell line. The stimulation of PLD activity by D2s appeared to correlate with the presence of a specific protein kinase C isoform, PKCϵ. The D2s stimulation of PLD activity was blocked by preincubation of cells with C3 exoenzyme, indicating that the stimulation of PLD may involve Rho family members. The stimulation of PLD by dopaminergic agonists took place in the absence of any detectable stimulation of phosphoinositide metabolism.

Dopamine acts through several classes of membrane receptors to effect cellular responses (Gingrich and Caron, 1993; Missale et al., 1996). The D2 dopamine receptor family has two isoforms, long and short, which arise from alternative splicing of mRNA (Dal Toso et al., 1989). The two forms of the D2 dopamine receptor are pleiotropic in terms of transmembrane signaling because activation of these receptors inhibits adenylyl cyclase (McDonald et al., 1982) and results in inhibition of voltage-gated calcium currents (Lledo et al., 1992) and activation of potassium conductance (Einhorn et al., 1991). It has been shown previously that these processes are mediated through the G_i/G_o family of G proteins because the signaling through these pathways can be ablated by the treatment with pertussis toxin (PTX). A well studied system for D2 dopamine receptor function is the lactotroph cells of the anterior lobe of the pituitary, where activation of the D2 dopamine receptors results in inhibition of prolactin release. A clonal cell line conveniently used for study of lactotrophs is the GH4C1 cell line, cloned from a radiation-induced rat pituitary tumor (Tashjian et al., 1968). These cells secrete both prolactin and growth hormone basally and in response to secretagogues, as do primary cultures of isolated lactotroph. However, these clonal cells do not respond to dopamine or express high affinity D2 dopamine receptors (Malarkey et al., 1977; Cronin et al., 1980). When stably transfected with the cDNA encoding the D2s, the response of these cells to dopamine is very similar to that of normal lactotrophs; dopamine inhibits forskolin-stimulated adenylyl cyclase and prolactin release (Albert et al., 1990).

Phosphatidylcholine-specific phospholipase D (PLD) enzymes have been implicated in many regulatory roles in cells, including mitogenesis, oncogenesis, and regulation of metabolism (Exton, 1997). PLD catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline. PA and the diacylglycerol produced from dephosphorylation of PA by phosphatidate phosphohydrolase are active signaling molecules, implicated in a variety of cellular signaling pathways. Several mammalian forms of PLD have recently been cloned: PLD1 a and b (Hammond et al., 1995, 1997) and PLD2 (Kodaki and Yamashita, 1997). PLD1 isoform activity is reported to be dependent on phosphatidylinositol 4,5-bisphosphate and stimulated by Rho and ARF-1 small GTP family proteins and protein kinase C (PKC)-α (Hammond et

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ABBREVIATIONS: PTX, pertussis toxin; PLD, phospholipase D; PKC, protein kinase C; PLC, phospholipase C; D2s, D2 dopamine receptor, short form; PtdEtOH, phosphatidylethanol; PC, phosphatidylcholine; PA, phosphatidic acid; TRH, thyrotropin-releasing hormone; 4αPDD, 4α-phorbol 12,13-didecanoate; 4βPDD, 4β-phorbol 12,13-didecanoate; PMA, phorbol 12-myristate 13-acetate; DAG, diacylglycerol; NPA, *N*-propylnorapomorphine; C3 exoenzyme, C3 exoenzyme of *C. botulinum*; PAGE, polyacrylamide gel electrophoresis; IP, inositol phosphate.

al., 1995, 1997). The PLD2 enzyme appears to be dependent on phosphatidylinositol 4,5-bisphosphate but is not further stimulated by Rho, ARF-1, or PKCα in vitro (Kodaki and Yamashita, 1997).

The mechanisms by which G protein-coupled receptors stimulate PLD are not completely understood, although there appears to be a PKC-dependent pathway that commonly involves activation of phospholipase C (PLC), and a PKC-independent pathway. Prolonged treatment with phorbol esters will ablate the ability of many G protein-coupled receptors mediated to stimulate PLD (for review, see Exton, 1997), including the D2s (Senogles, 1994a). This suggests that receptors which signal through the PKC-dependent pathways activate PLC, generating diacylglycerol (DAG) and IP3, which in turn activates PKC. Indeed, this PKC-dependent activation has been correlated with a concomitant stimulation of PLC because many receptors can stimulate both PLD and PLC activities, such as the muscarinic M1-M4 receptors and endothelin receptors (Sandman et al., 1991; Ambar and Sokolovsky, 1993; Bocchino and Exton, 1996). Few G protein-coupled receptors have been reported to stimulate PLD without a concomitant increase in PLC, as described for the D2s in this study and for the α_2-adrenergic receptor (MacNulty et al., 1992). This study describes the activation of PLD by the D2s, without accompanying PLC activity and in a manner that correlates with the presence of a specific PKC isoform.

Previously, my laboratory demonstrated that the inhibition of [^{3}H]thymidine incorporation mediated by D2s involves PKC because phorbol ester down-regulation and PKC inhibitors blunt the ability of dopamine to inhibit [^{3}H]thymidine incorporation (Senogles, 1994a). Further investigation revealed that PKCϵ is involved because selective removal of PKCϵ by long-term treatment with thyrotropin releasing hormone (TRH) had the same effect as treatment with phorbol esters. Because previous data had suggested the involvement of PKC in the growth inhibition by dopamine, we were interested in potential sources of activators of PKC. Many previous investigators had shown that the activation of D2 dopamine receptors in lactotrophs did not result in the stimulation of polyphosphoinositide metabolism (Vallar et al., 1990). To identify other potential sources of DAG, we investigated the ability of dopamine to stimulate PLD activity in GH4-121 cells. As reported here, agonist activation of D2s receptors results in a stimulation of PLD activity in GH4-121 cells, with no concomitant activation of PLC. The ability of dopamine agonists to stimulate PLD activity is not PTX-sensitive and is correlated with the presence of PKCϵ. Selective down-regulation of PKCϵ abolishes the ability of dopamine agonists to stimulate PLD. This down-regulation by TRH is selective for PKCϵ because the other isoforms of PKC were unaffected by treatment as determined by Western blotting analysis with isoform-specific antibodies. In addition, incubation with C3 exoenzyme ablates the ability of dopaminergic agonists to stimulate PLD, implicating Rho in this signaling cascade. Thus, the D2s-mediated stimulation of PLD activity appears to be independent of PLC activity but may be dependent on Rho. These data suggest a novel signaling pathway for the D2s.

Experimental Procedures

Materials

Ham's F10, fetal calf serum (certified), and G418 sulfate were all obtained from Life Technologies (Gaithersburg, MD). [^{3}H]Thymidine (50–80 Ci/mmol) and [^{3}H]myristic and oleic acids (20–40 Ci/mmol), *myo*-[^{3}H]inositol, and [^{32}P]NAD (3300 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA). All of the ligands, including spiperone and *N*-propylnorapomorphine (NPA), were obtained from Research Biochemicals Inc. (Natick, MA). All other chemicals were obtained from Sigma Chemical Corp. (St. Louis, MO). PTX was obtained from List Laboratories (Campbell, CA). Silica gel G uniplates with preabsorbant zones were purchased from Analtech (Newark, DE). Phosphatidylethanol (PtdEtOH) and egg phosphatidylcholine were obtained from Avanti Polar Lipids (Alabaster, AL). Isoform-specific anti-PKC antibodies and rabbit anti-goat conjugated horseradish peroxidase were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). C3 exoenzyme was purchased from Sigma Chemical Co.

Methods

Cell Culture. GH4C1 cells were cultured in Ham's F10 medium, supplemented with 7.5% heat-inactivated fetal calf serum and 2.5% heat-inactivated horse serum with 50 μg/ml gentamicin, and grown in a 5% CO_2 environment at a constant 37°C. Stable transfectants of GH4C1 were generated and characterized as described previously (Senogles, 1994b). The cells were routinely grown in medium containing 400 μg/ml G418 (205 μg of active compound).

PLD Assays. PLD assays were performed by the method of Sandman et al. (1994) with some minor modifications. Cells were plated in 12-well cluster dishes at a density of approximately 250,000 cells/well and labeled for 24 to 48 h before assay by addition of 10 μCi/well of [^{3}H]myristic acid. For the assay, the cells were washed twice with PBS (25°C) to remove serum and were incubated in 2 ml of Dulbecco's modified Eagle's medium in a shallow water bath at a temper-

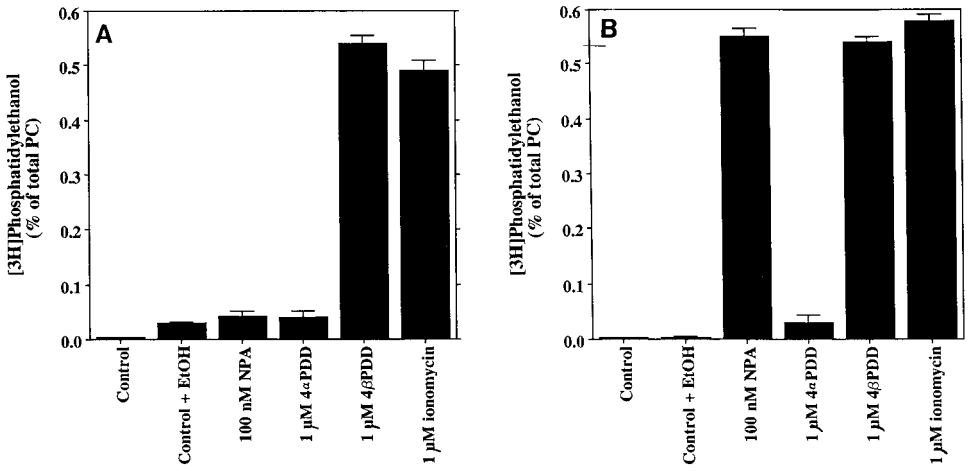

Fig. 1. PLD activity in GH4C1 and GH4-121 cells. The PLD activity of GH4C1 cells is shown in panel A, and the PLD activity of GH4-121 cells is shown in panel B. The cells were plated in 12-well cluster dishes and were labeled with 10 μCi of [^{3}H]myristic acid for 48 h before assay. PLD activity was measured by the transphosphatidylation assay carried out in Ham's F10 medium. The control condition is assayed without ethanol, and all other determinations are in the presence of 0.5% ethanol and agents as shown for 30 min at 37°C. Total cellular lipids were extracted and separated as described under *Methods*. The data represent the mean and S.D. for four independent experiments.

ature of 37°C. The agonists or drugs of interest were added to the cells, along with 0.5% ethanol as appropriate. The assay was allowed to incubate for 30 min at 37°C and was terminated by removal of the assay medium and addition of 1 ml of methanol, 2 M HCl (9:1 v/v). Cells were scraped and the wells were rinsed with an additional 1 ml of 0.25 M HCl, which was combined with the original cell scraping. The combined samples were extracted with 1 ml of chloroform, and the chloroform layer was removed and dried by vacuum evaporation. The dried samples were resuspended in a total volume of 50 μl with $CHCl_3$/methanol (9:1), and 5 μl of PtdEtOH (0.5 mg/ml) was added to each sample as an internal standard. The samples were spotted on silica G plates, along with standards for the phosphatidylcholine, PA, and PtdEtOH, and developed in a solvent containing $CHCl_3$/acetone/methanol/acetic acid/water (100:40:25:20:10 v/v). The phospholipid bands were visualized by staining with elemental iodine, and the bands corresponding to PA, phosphatidylcholine, and PtdEtOH were scraped and quantified by liquid scintillation counting. The data were normalized by expressing the PtdEtOH as a percentage of the total cellular phosphatidylcholine.

Inositol Phosphate Accumulation. GH4-121 cells were labeled for 2 days with 5 μCi/ml [^{3}H]inositol. The cells were washed with prewarmed Krebs buffer and incubated with 10 mM lithium chloride in the presence of agonist for 10 min or as designated at 37°C. The reactions were stopped by the addition of 1 ml of MeOH, 2 M HCl (9:1) and placed on ice. The cells were scraped, and the plates were washed with an additional 0.5 ml of water that was added to the initial cell homogenate. The samples were centrifuged, and the supernatants extracted with $CHCl_3$. The aqueous phase was subjected to Dowex chromatography to fractionate the inositol phosphates as described (Berridge et al., 1983).

PTX Treatment. GH4-121 cells were treated with PTX as follows. GH4-121 cells were seeded at the usual density in 24-well cluster dishes in Ham's F10 medium with the usual serum supplementation. PTX treatment of the GH4-121 cells was performed for 12 to 16 h at 37°C, using a concentration of PTX (20 ng/ml), which has been shown previously to fully ADP-ribosylate the G_i/G_o family of proteins in these cells (Senogles, 1994b) .

C3 Exoenzyme Treatment. GH4-121 cells were washed with PBS and then placed in a buffer containing 114 mM KCl, 15 mM NaCl, 5.5 mM $MgCl_2$, and 10 mM Tris-HCl. C3 exoenzyme (final concentration, 25 μg/ml) was added to the cells. The cells were scraped as described (Malcolm et al., 1996), plated into 6-well cluster dishes, and allowed to recover for 48 h. The viability of scrape-loaded cells, as assessed by exclusion of Trypan Blue, was quite variable, ranging from 50 to 80%.

ADP-Ribosylation Using [^{32}P]NAD. To test for the effectiveness of C3 exoenzyme treatment, cells were prepared by scrape-loading and allowed to culture for 48 h. Membranes from GH4-121 cells were prepared as described (Senogles, 1994b), and ADP-ribosylation of the membranes using [^{32}P]NAD was performed as previously described (Malcolm et al., 1994). The samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and the gel was visualized by autoradiography.

Western Blotting. The cells were washed with PBS, scraped into microfuge tubes, and centrifuged at 13,000*g* for 15 min. The crude pellet was resuspended in Laemmli sample buffer containing 5% SDS. The samples were subjected to SDS-PAGE and the gels were blotted for 1 h at 100 V onto nitrocellulose using 192 mM glycine, 25 mM Tris + 20% methanol. The blots were blocked by 1 h of incubation with PBS containing 3% nonfat dry milk + 0.05% Tween 20 and incubated overnight with the primary antibody at 4°C. The next morning, the blots were washed with PBS + 0.3% Tween 20 for 15 min, followed by two washes with PBS. The second antibody, goat anti-rabbit-conjugated horseradish peroxidase, was incubated for 1 h

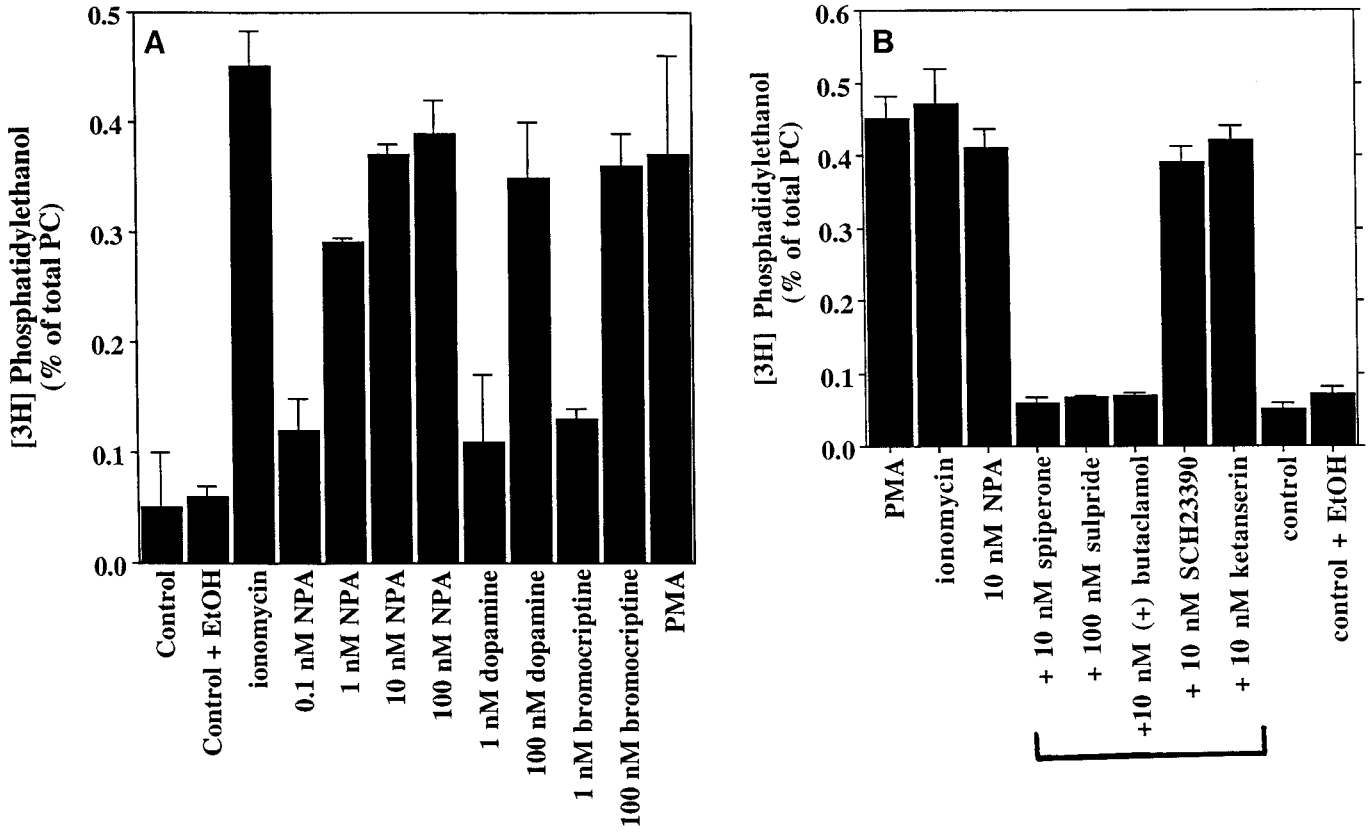

Fig. 2. Effect of D2 dopaminergic agonists and antagonists on PLD activity in GH4-121 cells. The effect of dopaminergic agonists on PLD activity is shown in panel A, and the antagonist blockade of PLD activity is shown in panel B. For the antagonist blockade, the cells were incubated with 10 nM NPA in addition to the various antagonists (indicated by the black line). When present, the concentration of both PMA and ionomycin was 1 μM. These data represent the mean and S.D. for four independent experiments.

at ambient temperature, and the blots were washed with two changes of PBS. The blot was visualized by using Opti4CN substrate (Bio-Rad, Hercules, CA) following the manufacturer's protocol.

Results

D2s Receptors Stimulate PLD Activity in GH4-121 Cells. PLD catalyzes the hydrolysis of phospholipids, resulting in the generation of PA and the release of the polar head group (for review, see Exton, 1998, 1999; Nishizuka, 1992). To date, many G protein-coupled receptors have been shown to stimulate PLD activity, such as m1-m4 muscarinic, α_2-adrenergic and endothelin receptors (Exton, 1994). PLD has been shown to be stimulated by the addition of phorbol esters or the calcium ionophore, ionomycin (Exton, 1994). As shown in Fig. 1 (panel A), the PLD activity of GH4C1 cells (the parent cell line of GH4-121 cells) is stimulated 15.4- and 13.7-fold over basal activity by these agents, respectively. The active phorbol ester, 4β-phorbol 12,13-didecanoate (4βPDD) stimulates PLD activity, whereas the inactive isomer, 4α-phorbol 12,13-didecanoate (4αPDD), is ineffective. Treatment of cells with NPA (D2 agonist) had no effect on PLD stimulation. Stable transfection of the D2s into GH4C1 yields GH4-121 cells (21). This cell line has the same re-

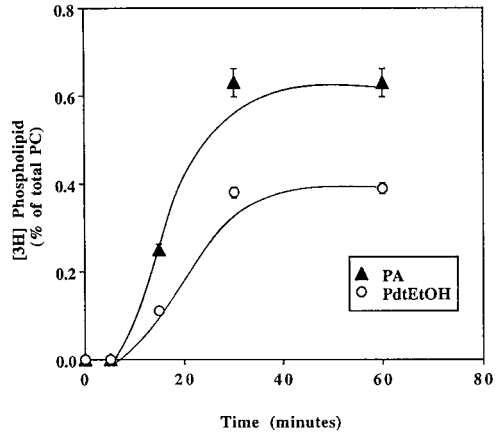

Fig. 3. Time course of D2s-stimulated PLD activity. The time course of PLD activity was measured by labeling with [^{3}H]myristic acid and assaying PLD activity at the time points shown after addition of agonist, 10 nM NPA, at time zero. Duplicate assays were performed in the presence (PtdEtOH) or absence (PA) of ethanol, and the cellular lipids were extracted and separated as described under *Methods*. These data represent the mean and S.D. of three independent experiments.

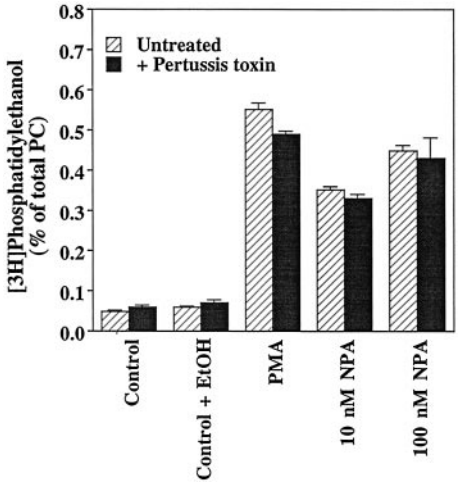

Fig. 4. Effect of PTX on D2s-stimulated PLD. The PLD assay was performed by labeling cells with [^{3}H]myristic acid for 48 h. Half of the cells were treated 24 h before assay with 20 ng/ml PTX, and the other half were untreated cells. These data represent the mean and S.D. of six independent experiments.

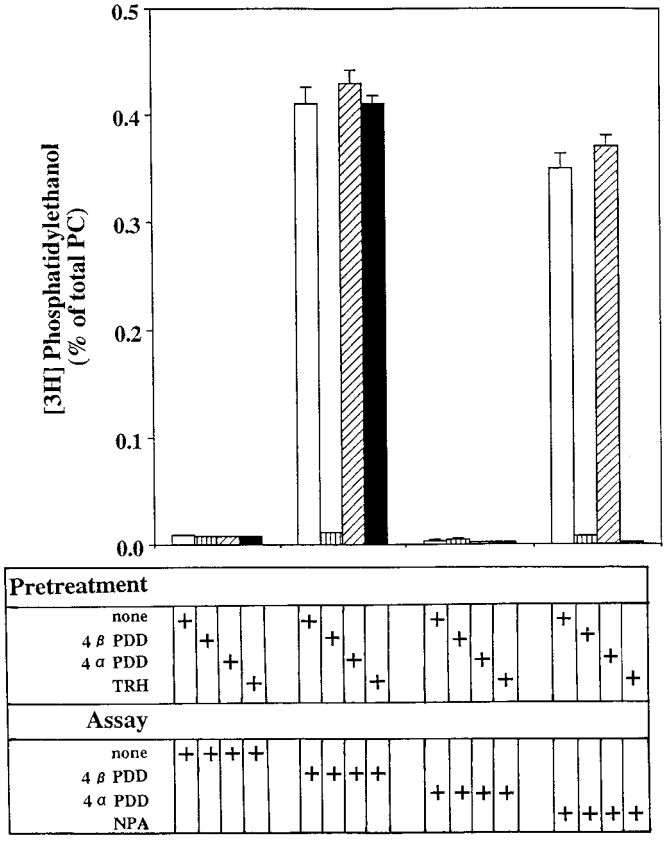

Fig. 5. Effect of down-regulation of PKC on D2s-stimulated PLD activation. Cells were pretreated with: 1) control (vehicle); 2) 10 nM 4βPDD; 3) 10 nM 4αPDD; or 4) 10 nM TRH for 18 h before assay of PLD. The cells were labeled with [^{3}H]myristic acid for 30 h before addition of the agents shown above. Shown below the figure are the assay conditions for each pretreatment protocol. These data are representative of five independent experiments.

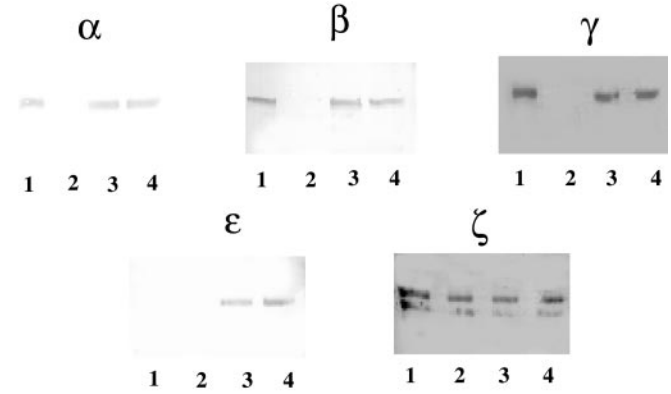

Fig. 6. Effect of down-regulation of PKC on PKC isozyme expression. Cells were pretreated with the following conditions: 1) 10 nM TRH, 2) 10 nM 4βPDD, 3) 10 nM 4αPDD, and 4) control for 18 h. The cells were prepared for SDS-PAGE and Western blotting as described under *Methods*. The primary antisera were PKC isoform-specific antisera, and the blot was visualized by incubation with anti-goat-conjugated horseradish peroxidase using Opti-4CN substrate. This blot is representative of three independent experiments.

sponse to phorbol ester and ionomycin as the parent cell line (Fig. 1, panel B). In addition, activation of the D2s by agonist results in a stimulation of PLD activity (Fig. 1, panel B). The PLD activity of GH4-121 cells is stimulated in a dose-dependent manner by addition of D2s agonists: NPA, bromocriptine, and dopamine (Fig. 2, panel A). The EC_{50} for activation of PLD by NPA is approximately 300 pM (data not shown). The activation of PLD by dopamine agonists is completely blocked by inclusion of antagonists for the D2s, such as (+) butaclamol and spiperone (Fig. 2, panel B) but not by either a D1 dopamine receptor antagonist (SCH 23390) or a serotonin antagonist (ketanserin). These data indicate that the stimulation of PLD activity is a result of a receptor-mediated signaling event in GH4-121 cells.

Time Course for D2s-Stimulated PLD Activity. The time course for D2s-stimulated PLD activity is shown in Fig. 3. The GH4-121 cells labeled with [^{3}H]myristic acid were assayed for the production of both PA and PtdEtOH as a function of time, with agonist added at zero time. As shown, the production of PA and PtdEtOH was not detected until 5 min after addition of agonist. The stimulation of PLD by agonist is maximal at approximately 30 to 40 min.

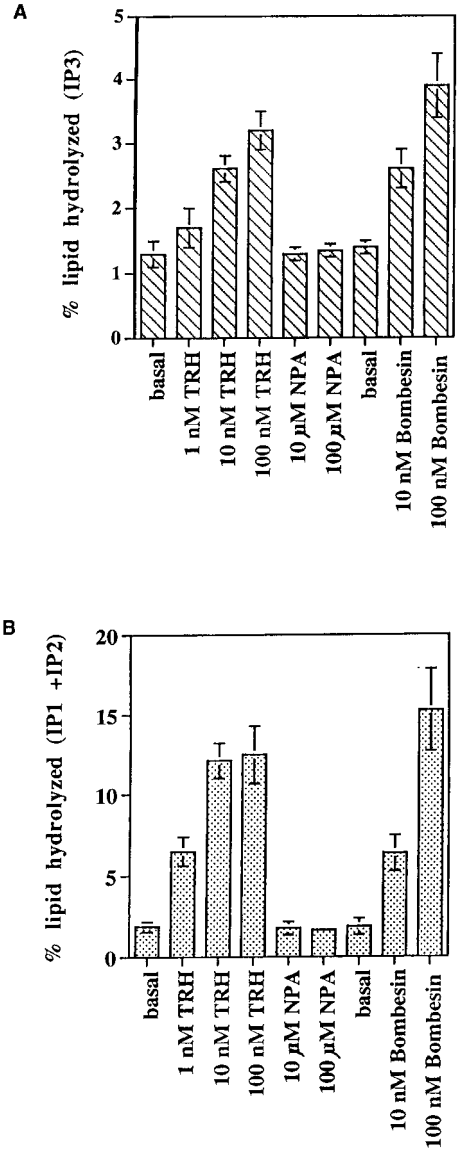

Fig. 7. Effect of NPA on inositol phosphate accumulation in GH4-121 cells. Cells were labeled with [^{3}H]inositol as described under *Methods*. The cells were incubated for 10 min with NPA, TRH, and bombesin before isolation and fractionation of IPs. Shown are the results for IP3 (panel A) and IP1 + IP2 (panel B). These data are the mean and S.D. of three independent experiments.

PTX Sensitivity of D2s-Stimulated PLD Activity. As stated previously, the activation of the D2s has been shown to inhibit forskolin-stimulated adenylyl cyclase, activate K^+ channels, and inhibit voltage-sensitive Ca^{2+} channels. All of these signaling events are sensitive to treatment with PTX, which will covalently modify members of the G_i/G_o family of G proteins by ADP ribosylation. In contrast, the ability of D2 dopamine agonists to inhibit growth in GH4ZR7 (another cell line derived from GH4C1 stably transfected with D2s) cells as assessed by [^{3}H]thymidine incorporation is not sensitive to the actions of PTX (Senogles, 1994a). As shown in Fig. 4, the ability of D2s to stimulate PLD is not blocked by PTX treatment. The stimulation of PLD activity by both 10 and 100 nM NPA was unaffected by pretreatment of the cells with 20 or 50 ng/ml PTX (data not shown). The ability of phorbol 12-myristate 13-acetate (PMA) to stimulate PLD was not affected by treatment of the cells with PTX. An experiment was performed in parallel, assessing the dopamine receptor-mediated inhibition of forskolin-stimulated adenylyl cyclase (data not shown). This experiment showed that pretreatment with 20 ng/ml PTX fully ablated the ability of dopamine to inhibit forskolin-stimulated adenylyl cyclase as previously reported (Senogles, 1994b). It should be noted that overnight treatment with 20 ng/ml PTX has been previously shown to modify >98% of the G_i/G_o proteins in this cell line (Senogles, 1994b).

Effect of Phorbol Ester Treatment on D2s Stimulation of PLD Activity. Previous work (Senogles, 1994a) has shown that the inhibition of [^{3}H]thymidine incorporation mediated by dopamine could be blunted by down-regulation of PKC using phorbol esters or by the use of PKC inhibitors. Moreover, the involvement of PKCε was determined by specific down-regulation of PKCε using treatment with TRH. Because phorbol esters were shown to stimulate PLD activity in GH4-121 cells (Fig. 1), we chose to investigate the effect of down-regulation of PKC on PLD activity using both phorbol ester and TRH pretreatment. Shown in Fig. 5 are four pretreatment conditions: 1) control (vehicle); 2) 100 nM 4βPDD for 18 h; 3) 100 nM 4αPDD for 18 h; and 4) 10 nM TRH for 18 h before assay of PLD. After the pretreatment protocols, the GH4-121 cells were assayed for both NPA and phorbol ester-stimulated PLD activity. Down-regulation with 4βPDD, the active phorbol ester, abolishes both the NPA and phorbol ester activation of PLD. In contrast, down-regulation with the 4αPDD (the inactive isomer) has no effect on subsequent activation of PLD by either NPA or 4βPDD. Long-term treatment with TRH (>18 h), shown previously to ablate the ability of dopamine to inhibit growth and [^{3}H]thymidine incorporation, also blunted the stimulation of PLD evoked by NPA but not by phorbol esters.

Effect of Phorbol Ester and TRH Treatment on the PKC Isoforms in GH4-121 Cells. GH4C1 cells contain several isoforms of PKC: α, β, γ, ε, and ζ (Kiley et al., 1990, 1991). The PKC isoforms α, β, and γ are conventional, Ca^{2+}-dependent enzymes, which can be down-regulated by treatment with phorbol ester (for review, see Nishizuka, 1992). PKCε is a novel isoform not dependent on calcium but down-

regulated with phorbol ester treatment. PKCζ is an atypical isoform and is not regulated by either calcium or phorbol ester treatment. The GH4-121 cells were treated with 10 nM 4αPDD, 10 nM 4βPDD, or 10 nM TRH for 18 h before cell lysis. Western blot analysis with isoform-specific antibodies was performed to determine the effect of various agents on PKC isoform expression. As shown in Fig. 6, PKCα, -β, -γ, and -ϵ were effectively down-regulated by prolonged exposure to 4βPDD (lane 2), compared with the control (lane 4), but exposure to 4αPDD (lane 3) had no effect. In contrast, only PKCϵ expression was affected by prolonged TRH treatment (lane 1). PKCζ expression was unaffected by any of the treatments.

Effect of Dopaminergic Agonists on Phosphoinositide Metabolism. GH4-121 cells were assayed for the ability of dopaminergic agonists to stimulate the production of inositol phosphates. Cells were incubated with TRH, bombesin, and NPA for 10 min, and the inositol phosphates were isolated. As shown in Fig. 7, TRH and bombesin stimulated the accumulation of inositol phosphates at 10′. In contrast, NPA had no effect on accumulation of either IP3 or IP1 + IP2. Incubation times of 30 min gave the same results, with no detectable inositol phosphates generated by NPA (data not shown).

Effect of C3 Exoenzyme on D2s Stimulation of PLD. Previous work has shown that Rho, a member of the small G protein family, can be inactivated by C3 exoenzyme, a toxin from *Clostridium botulinum* by covalent modification. Because Rho family members have been implicated in regulation of PLD (for review, see Exton, 1998, 1999), it was of interest to test the effects of C3 treatment on the D2s stimulation of PLD activity. C3 exoenzyme was scrape-loaded into GH4-121 cells, and the effect of toxin treatment on D2s stimulation of PLD was monitored. As shown in Fig. 8A, C3 exoenzyme treatment blocks >95% of NPA stimulation of PLD activity. The C3 exoenzyme treatment also decreases both 4βPDD and ionomycin stimulation by approximately 50%. Shown in panel B, is the autoradiogram of the SDS-PAGE of the in vitro ADP ribosylation of GH4-121 cells using C3 and [^{32}P]NAD before and after scrape-loading with C3 exoenzyme. As shown, cells that were not treated with C3 exoenzyme could be labeled in vitro with C3 exoenzyme and [^{32}P]NAD, whereas the pretreated cells did not incorporate significant amounts of label. The data suggest that the C3 exoenzyme was indeed loaded into the GH4-121 cells and was able to modify the Rho proteins. Taken together, these data suggest a role for Rho in the D2s signaling to PLD in these cells.

Discussion

The data presented in this study suggest D2 dopamine agonist activation of PLD activity in GH4-121 cells. The activation of PLD activity was dose-dependent and pharmacologically selective. The ability of agonists to stimulate PLD activity and of antagonists to block activation has a selectivity and potency that is appropriate for the D2s. The parent cell line, GH4C1, does not show a PLD response to dopaminergic agonists, which underscores that the effect observed is through activation of the D2s receptor. These combined data suggest that the stimulation of PLD activity is due to a receptor-mediated event.

PLD has been shown to be activated by a number of G protein-coupled receptors, including the m1-m4 muscarinic (Sandman et al., 1991), endothelin (Ambar and Sokolovsky, 1993), α_2-C10 adrenergic receptors (MacNulty et al., 1992), and many others (Bocchino and Exton, 1996). For most of these receptors, activation of PLD is concomitant with activation of PLC. Thus, these receptors can bring about activation of PLD by a PKC-dependent mechanism. DAG generated from the PLC appears to activate PKC, which either directly acts on PLD or acts in concert with small G proteins to

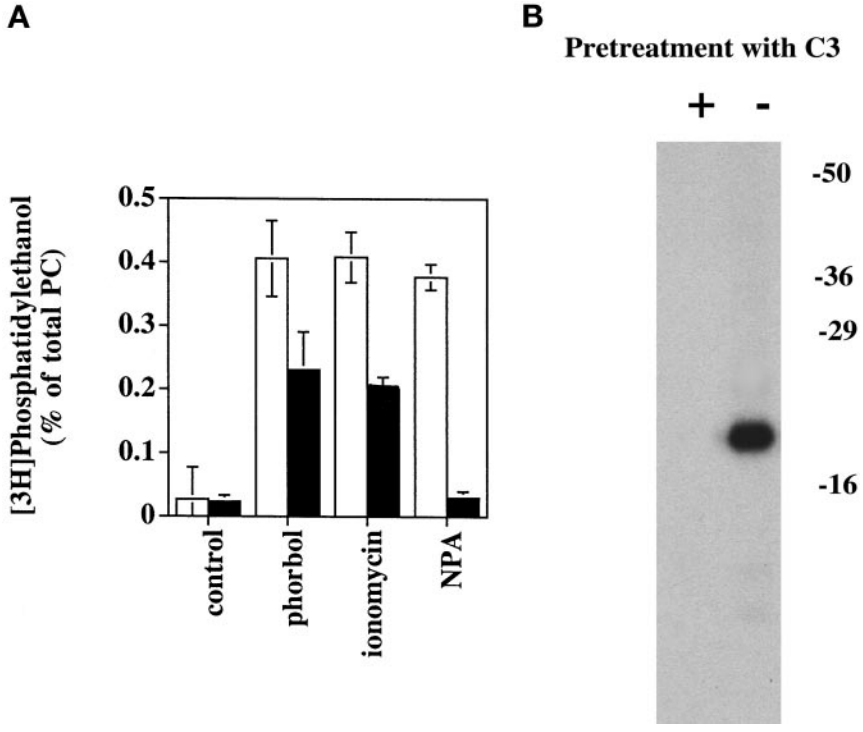

Fig. 8. Effect of C3 exoenzyme treatment on PLD activity. Cells were treated by scrape-loading GH4-121 cells in the presence or absence of 20 μg/ml C3 exoenzyme (□, control; ■, + C3 exoenzyme). The cells were labeled and assayed for PLD activity (panel A). These data represent the mean and S.D. of four independent experiments. Membranes were prepared from GH4-121 cells and GH4-121 cells that had been scrape-loaded with C3 exoenzyme. These membranes were ADP-ribosylated in vitro using [^{32}P]NAD and C3 exoenzyme as described under *Experimental Procedures*. The samples were subjected to SDS-PAGE and the gels were visualized by autoradiography. A scan of the gel is shown in panel B with the position of the molecular weight markers indicated.

activate PLD (Exton, 1998, 1999). In contrast, the studies presented here for the D2s indicate a novel pathway for the stimulation of PLD, because concomitant activation of PLC is not involved.

Similar to the D2s effects described in this study, the α_2-adrenergic receptor has been shown to stimulate PLD without a concomitant stimulation by PLC (MacNulty et al., 1992). The stimulation of PLD by the α_2-adrenergic receptor was suggested to be inappropriate receptor coupling due to overexpression of the receptor in a heterologous cell line. Heterologous expression of the D2s has been shown to lead to aberrant signaling, such as that observed in Ltk cells (Vallar et al., 1990). However, the parent cell line used for this study is the GH4 clonal line, derived from a pituitary tumor. Transfection of the D2s into GH4 cell lines has resulted in cell lines that respond to dopamine agonists similar to lactotroph cells in culture (Albert et al., 1990), in terms of signaling and cellular response, such as prolactin release. Also, the expression of D2s receptor in this cell line is quite low, only 300 fmol/mg of protein as assessed by [^{3}H]spiperone binding (S. Senogles, unpublished data). The EC_{50} for NPA activation of PLD is approximately 300 pM (data not shown). This is comparable with the EC_{50} value of 500 pM obtained for NPA-mediated inhibition of forskolin-stimulated adenylyl cyclase (Senogles, 1994b). These combined data argue against the stimulation of PLD observed in GH4-121 cells as being due to overexpression of receptor, however transfection artifacts cannot be completely ruled out.

PLD has been shown to be regulated by the actions of several isoforms of PKC. For example, several studies have shown that overexpression of PKCα and PKCβ results in stimulated PLD (Pai et al., 1991; Pachter et al., 1992; Eldar et al., 1993; Conricode et al., 1994). In addition, PKCϵ has been shown to mediate the stimulation of PLD activity in rat mesangial cells (Pfeilschifter and Merriweather, 1993). In studies of the cloned and expressed PLD isoforms, PKCα stimulated the activity of the PLD1 isoform in vitro (Hammond et al., 1995, 1997). Our studies have shown a correlation between the presence of PKCϵ and D2s-mediated stimulation of PLD. The down-regulation of PKCϵ, selectively by treatment with TRH, ablated the ability of dopaminergic agonists to stimulate PLD. Western blot analysis of cells treated with TRH indicated that only the PKCϵ isoform was affected by this treatment, whereas phorbol ester treatment resulted in down-regulation of PKCα, -β, -γ, and -ϵ. However, a direct role for PKCϵ in the D2s stimulation of PLD has not been shown, and the role of PKCϵ in this pathway remains unclear. The long-term treatment with TRH needed to down-regulate PKCϵ may impact cellular signaling in a myriad of ways. For example, long-term stimulation with TRH could deplete various lipid pools or many other cofactors and cause a loss of PLD stimulation.

The mechanism by which the D2s stimulate PLD activity is still undefined. The activation of PLD by dopaminergic agonists is insensitive to the actions of PTX and would suggest that the G_i/G_o family of proteins is not involved in this pathway, as they are in other signaling pathways stimulated by the D2s. In this study, C3 exoenzyme has been shown to ablate D2s stimulation of PLD. One explanation for the stimulation of PLD by D2s is that there is a direct interaction of the D2s with Rho family small G proteins. Previous work has suggested that the motif NPXXY in the seventh transmembrane spanning domain of G protein-coupled receptors can predict the ability of the receptor to interact directly with Rho and ARF to stimulate PLD (Mitchell et al., 1998). The D2s contains this motif (Bunzow et al., 1988), and one hypothesis to explain these data may be the direct interaction of D2s with small G to activate PLD. The details of the mechanism by which D2s stimulates PLD activity remain unclear and will be the focus of future investigations.

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References

Albert PR, Neve KA, Bunzow JR and Civelli O (1990) Coupling of a cloned rat dopamine D2 receptor to inhibition of adenylyl cyclase and prolactin secretion. *J Biol Chem* **265:**2098–2104

Ambar I and Sokolovsky M (1993) Endothelin receptors stimulate both phospholipase C and D activities in different cell lines. *Eur J Pharmacol* **245:**31–41.

Berridge MJ, Dawson MC, Downes CP, Heslop JP and Irvine RFK (1983) Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phospholipids. *Biochem J* **212:**473–482.

Bocchino SB and Exton JH (1996) *Handbook of Lipid Research* pp 75–123, Plenum Publishing Co., New York.

Bunzow JR, Van Tol HHM, Grandy DK, Albert P, Salon M, Christie M, Machida CA, Neve KA and Civelli O (1988) Cloning and expression of a rat D2 dopamine receptor cDNA. *Nature (Lond)* **336:**783–786.

Conricode KM, Smith JL, Burns DL and Exton JH (1994) Phospholipase D activation in fibroblast membranes by the α and β isoforms of protein kinase C. *FEBS Lett* **342:**149–153.

Cronin MJ, Faure N, Martial JA and Weiner RI (1980) Absence of high affinity dopamine receptors in GH3 cells: A prolactin-secreting clone resistance to the inhibitory action of dopamine. *Endocrinology* **106:**718–723.

Dal Toso R, Sommer B, Ewert M, Herb A, Pritchett DB, Bach A, Shivers BD and Seeburg PH (1989) The dopamine D2 receptor: Two molecular forms generated by alternative splicing. *EMBO J* **13:**4025–4034.

Einhorn LC, Gregerson KA and Oxford GS (1991) D2 dopamine receptor activation of potassium channels in identified rat lactotrophs. *J Neurosci* **11:**3727–3737.

Eldar H, Ben-Av P, Schmidt U-S, Livneh E and Liscovitch M (1993) Up regulation of phospholipase D activity induced by overexpression of protein kinase Cα. *J Biol Chem* **268:**12560–12564.

Exton J (1994) Phosphatidylcholine breakdown and signal transduction. *Biochim Biophys Acta* **1212:**26–42.

Exton J (1997) Phospholipase D: Enzymology, mechanisms of regulation, and function. *Physiol Rev* **77:**303–320.

Exton J (1998) Phospholipase D. *Biochim Biophys Acta* **1436:**105–115.

Exton J (1999) Regulation of Phospholipase D. *Biochim Biophys Acta* **1439:**121–133.

Gingrich JA and Caron MG (1993) Recent advances in the molecular biology of dopamine receptors. *Annu Rev Neurosci* **16:**299–321.

Hammond SM, Altshuller YM, Sung TC, Rudge SA, Rose K, Engebrecht J, Morris AJ and Frohman MA (1995) Human ADP-ribosylation factor-activated phoshatidylcholine-specific phospholipase D defines a new and highly conserved gene family. *J Biol Chem* **270:**29640–29643.

Hammond SM, Jenco JM, Nakashima S, Cadwallader K, Gu Q-m, Cook S, Nozawa Y, Prestwich GD, Frohman MA and Morris AJ (1997) Characterization of two alternately spliced forms of phospholipase D1. *J Biol Chem* **272:**3860–3868.

Kiley S, Parker P, Fabbro D and Jaken S (1991) Differential regulation of protein kinase C isozymes by thyrotrophin-releasing hormone in GH4C1 cells. *J Biol Chem* **266:**23761–23768.

Kiley S, Schaap D, Parker P, Hsieh LL and Jaken S (1990) Protein kinase C heterogeneity in GH4C1 rat pituitary cells. *J Biol Chem* **265:**15704–15712.

Kodaki T and Yamashita S (1997) Cloning, expression and characterization of a novel phospholipase D complementary DNA from rat brain. *J Biol Chem* **272:** 11408–11413.

Lledo PM, Homburger V, Bockaert J and Vincent JD (1992) Differential G protein coupling of D2 dopamine receptors to K+ and Ca2+ currents in rat anterior pituitary cells. *Neuron* **8:**455–463.

MacNulty EE, McClue SJ, Carr IC, Jess T, Wakelam MJO and Milligan G (1992) α2 C10 adrenergic receptors expressed in rat 1 fibroblasts can regulate both adenylyl cyclase and phospholipase D mediated hydrolysis of phophatidylcholine by interacting with pertussis toxin sensitive guanine nucleotide binding proteins. *J Biol Chem* **267:**2149–2156.

Malarkey WB, Groshong JC and Milo GE (1977) Defective dopaminergic regulation of prolactin secretion in a rat pituitary tumor cell line. *Nature (Lond)* **266:**640–641.

Malcolm KC, Elliot CM and Exton JH (1996) Evidence for rho-mediated agonist stimulation of phospholipase D in rat 1 fibroblasts. *J Biol Chem* **271:**13135–13139.

Malcolm KC, Rose AH, Qiu R-G, Symons M and Exton JH (1994) Activation of rat liver phospholipase D by the small GTP-binding protein RhoA. *J Biol Chem* **269:**25951–25954.

McDonald WM, Sibley DR, Kilpatrick BF and Caron MG (1982) Dopaminergic

inhibition of adenylate cyclase correlates with high affinity agonist binding to anterior pituitary D2 dopamine receptors. *Mol Cell Endocrinol* **36:**201–209.
Missale C, Nash SR, Robinson SW, Jaber M and Caron MG (1996) Dopamine receptors: From structure to function. *Physiol Rev* **78:**189–225.
Mitchell R, McCulloch D, Lutz E, Johnson M, MacKensie C, Fennell M, Fink G, Zhou W and Sealfon SC (1998) Rhodopsin-family receptors associate with small G-proteins to activate phospholipase D. *Nature (Lond)* **392:**411–413.
Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science (Wash DC)* **258:**607–614.
Pachter JA, Pai J-K, Mayer-Ezell R, Petrin JM, Dobek E and Bishop WR (1992) Differential regulation of phosphoinositol and phosphatidylcholine hydrolysis by protein kinase Cβ1 overexpression. *J Biol Chem* **267:**9826–9830.
Pai J-K, Pachter JA, Weinstein IB and Bishop WR(1991) Overexpression of protein kinase Cβ1 enhances phospholipase D activity and diacylglycerol formation in phorbol ester-stimulated rat fibroblasts. *Proc Natl Acad Sci USA* **88:**598–602.
Pfeilschifter J and Merriweather C (1993) Extracellular ATP and UTP activation of phospholipase D is mediated by protein kinase Cϵ in rat renal mesangial cells. *Br J Pharmacol* **110:**847–853.
Sandman J, Peralta EG and Wurtman RJ (1991) Coupling of transfected muscarinic acetylcholine receptor subtypes to phospholipase D. *J Biol Chem* **266:**6031–6034.
Senogles SE (1994a) The D2 dopamine receptor mediates inhibition of growth in GH4ZR7 cells: Involvement of protein kinase Cϵ. *Endocrinology* **134:**783–789.
Senogles SE (1994b) The D2 dopamine receptor isoforms signal through distinct Gi proteins to inhibit adenylyl cyclase. *J. Biol. Chem.* **269:**23120–23127.
Tashjian AH, Yasamura Y, Levine L, Sato GH and Parker ML (1968) Establishment of clonal strains of rat pituitary tumors cells that secrete growth hormone. *Endocrinology* **82:**342–352.
Vallar L, Muca C, Magni M, Albert P, Bunzow J, Meldolesi J and Civelli O (1990) Differential coupling of dopaminergic D2 receptors expressed in different cell types. *J Biol Chem* **265:**10320–10326.

Send reprint requests to: Dr. Susan E. Senogles, University of Tennessee, 858 Madison Ave. G01, Memphis, TN 28163. E-mail: ssenogles@utmem.edu