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Phospholipase D Activation by Endogenous 5-Hydroxytryptamine 2C Receptors Is Mediated by $G\alpha_{13}$ and Pertussis Toxin-Insensitive $G\beta\gamma$ Subunits

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ABSTRACT

Phospholipase D activation was measured in primary cultures of rat choroid plexus epithelial cells, which endogenously express the 5-hydroxytryptamine (5-HT) 2C receptor, as well as a heterologous cell line expressing the cloned receptor. In both systems, serotonin stimulation of the 5-HT_{2C} receptor activates phospholipase D in addition to phospholipase C, the traditional effector. Specific inhibitors and membrane permeable blocking peptides were used to determine which heterotrimeric G-proteins were involved. Results suggest that both α and free $\beta\gamma$ subunits from G_{13} heterotrimers are responsible for phospholipase D activation.

The serotonin 5-HT_{2C} receptor belongs to the superfamily of G-protein-coupled receptors (GPCRs). Like many other GPCRs, the $5HT_{2C}$ receptor activates multiple intracellular signaling cascades. Many studies of GPCR signaling, including $5HT_{2C}$ receptor signaling, have used cultured cells heterologously expressing cloned receptors; however, it is important to understand how these various signals contribute to cellular functions of endogenous GPCRs. The targeted disruption of protein-protein interactions with synthetic peptides allows a dissection of endogenous signal transduction pathways (Hamm and Rarick, 1994; Taylor and Neubig, 1994). Our laboratory used this strategy to show that the $5HT_{2C}$ receptor couples to both G_q and G_{13} proteins with the G_q protein mediating activation of PLC in choroid plexus (Chang et al., 2000; Price et al., 2001). Herein, we show that endogenous $5HT_{2C}$ receptors also activate phospholipase D (PLD) and have evaluated the role of G-proteins in this signal.

Materials and Methods

Materials. The design of the membrane permeable peptides and method of synthesis were as described previously (Chang et al., 2000; Price et al., 2001). Most peptides were synthesized in our laboratory (Chang et al., 2000); $G\alpha_q$ blocking peptide (PLCβ1M) was also synthesized by Biopeptide LLC (San Diego, CA) and G_{13}CT peptide (H-Cys-Leu-His-Asp-Asn-Leu-Lys-Gln-Leu-Met-Leu-Gln-OH) was purchased from Calbiochem (San Diego, CA). This peptide is based on the last 11 amino acids in the C-terminal tail of the rat G_{13} G-protein. These peptides have been extensively characterized to document specificity for G-protein heterotrimers as well as broad $G_{\beta\gamma}$ sequestering applications (Chang et al., 2000).

Cell Culture. Primary cultures of choroid plexus epithelial (CPE) cells were prepared as described previously (Barker and Sanders-Bush, 1993). Briefly, choroid plexi removed from 20-day-old Harlan Sprague-Dawley rats were treated with Pronase (333 μg/ml) containing DNase (7.5 μg/ml) in Hank's balanced salt solution (without Ca^{2+}/Mg^{2+}) (HBSS) for 10 min at 37°C to dissociate cells. Dissociated cells were resuspended in 10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM) plus D-Val (Invitrogen, Carlsbad, CA) Cells were plated in six-well (PLD) or 48-well (PLC) plates for 3 days.

3T3–2C cells were prepared and maintained as in (Backstrom et al., 2000). Briefly, NIH3T3 fibroblasts stably expressing the rat INI-isoform of the 5-HT_{2C} receptor were plated into 24-well (PLD) or 48-well (PLC) plates for 2 to 3 days (70 to 80% confluence). Cells were grown in DMEM containing 9% bovine calf serum, 0.5 mg/ml G418, 5 units/ml penicillin, and 5 μg/ml streptomycin.

Phospholipase D Assay. This method is adapted from Hess et al. (1997). Two days after plating, the medium was replaced with DMEM supplemented with 0.5% fatty-acid free bovine serum albumin and 2 μCi/ml [9,10-^{3}H]myristic acid; 16 to 20 h later, cells were washed two times with DMEM, once with HBSS, and then incubated with peptides solubilized in HBSS at 37°C for 30 min. Cells were

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; GPCR, G-protein-coupled receptor; PLD, phospholipase D; PLC, phospholipase C; CPE, choroid plexus epithelial (cells); HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PTX, pertussis toxin; SB206553, 5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-*f*]indole; MDL100907, (*R*)-(+)-α(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol; GST, glutathione *S*-transferase.

then treated with 0.3% 1-butanol, 10 μM pargyline, and 1 μM citalopram for 10 min before stimulation with 5-HT for 15 min. Incubations were terminated by removing the medium, washing once with ice-cold phosphate-buffered saline, and adding ice-cold methanol. Cells were scraped off the plates, and the lipids were extracted and separated with methanol/chloroform/0.1 N HCl (1:1:1). The lower phase was dried under N_2, resuspended in 30 μl of chloroform/methanol (2:1) and spotted onto silica gel 60A thin-layer chromatography plates (Whatman, Clifton, NJ). The plates were developed in the upper phase of the solvent system of ethyl acetate/iso-octane/H_2O/acetic acid (55:25:50:10) and then stained with iodine. A phosphatidylbutanol standard was used to locate the bands, which were scraped into scintillation vials containing 0.5 ml of methanol and 7.5 ml of Ready Organic scintillation mixture (Bio-Rad, Hercules, CA).

Phosphoinositide Hydrolysis Assay. Cells were plated into 48-well plates. Two days after plating, the medium was replaced with DMEM (minus inositol) containing 2 μCi/ml [*myo*-^{3}H]inositol and incubation continued for 16 to 20 h. At the start of the experiment, cells were washed two times with 0.25 ml of HBSS/well and then incubated with peptides solubilized in HBSS at 37°C for 30 min. Subsequently, 10 mM lithium chloride, 1 μM citalopram, and 10 μM pargyline were added to the cells 10 min before 5-HT activation for 30 min. Incubations were terminated by removing the medium, and fixing in 25 μl of methanol/well. [^{3}H]Inositol monophosphates were isolated as described previously (Barker et al., 1994).

ADP-Ribosylation Assay. ADP-ribosylation was performed as described previously (Grotewiel et al., 1994). Briefly, CPE cells were plated in six-well plates and cultured for 2 days in DMEM supplemented with 10% FBS. Then cells were incubated with 500 ng/ml pertussis toxin (PTX) for 16 h in the absence of serum. Membranes, suspended in 50 mM Tris, pH 8.0, containing 5 mM $MgCl_2$ and 1 mM EDTA buffer, were subjected to ADP ribosylation at 30°C for 1 h in a 50-μl reaction containing 100 μg of membrane protein, 1 mM ATP, 20 mM arginine, 20 mM thymidine, 100 mM NaCl, 0.25% lubrol, 5 mM dithiothreitol, 1 μg/ml PTX, and 2.5 μCi of [^{32}P]nicotinamide adenine dinucleotide. The reaction was terminated by adding 1.2 ml of 20 mM HEPES, pH 8.0. Membranes were pelleted and separated by polyacrylamide gel electrophoresis and ribosylated proteins were visualized using a PhosphoImager system (Amersham Biosciences, Piscataway, NJ).

Rhotekin Assay for Active Rho. This assay was adapted from Reid et al. (1996) with the kind permission of Shuh Narumiya (Kyoto, Japan). Briefly, a fusion protein composed of amino acids 7 to 113 of the Rhotekin protein fused to GST has been demonstrated to bind selectively to GTP-bound Rho A (Reid, et al., 1996) and was used to isolate active Rho A. 3T3–2C cells were cultured in 100 mm dishes, and were serum-starved overnight before assay. Cells were treated with 10 nM 5-HT or 1% FBS for 5 min. Cells were then lysed in 40 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% Nonidet P40, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Cell lysates were cleared of insoluble material by centrifugation at 12,000*g* for 15 min and then incubated with the Rhotekin-GST fusion protein bound to glutathione agarose beads, with rocking for 30 min at 4°C. The beads were collected by centrifugation and washed three times. Protein was eluted by boiling the beads in sample buffer with 5% β-mercaptoethanol, separated using SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with an antibody for Rho A (Santa Cruz). A horseradish peroxidase-conjugated secondary antibody and ECL detection substrate (Pierce Super Signal Dura) were used to detect the Rho proteins. A 25-μl sample of cell lysate was run for comparison (total Rho).

Results

5-HT Acting via Endogenous 5-HT$_{2C}$ Receptors Stimulates PLD Activity. Cultured CPE cells were used to determine whether 5-HT$_{2C}$ receptors have the ability to activate PLD in an endogenous system. In these cells, 5-HT produced a robust increase in PLD activity, with a relative maximum that was as large as the PLC signal (Table 1). The addition of the 5-HT$_{2C}$ antagonist SB206553, but not the 5-HT$_{2A}$ antagonist MDL100907, blocked 5-HT stimulation of PLD activation to basal levels (Fig. 1A), consistent with 5-HT$_{2C}$ receptors mediating activation of PLD.

Role of G-Proteins in the 5-HT$_{2C}$ Receptor-Mediated PLD Signal. To determine the role of heterotrimeric G-proteins in mediating the endogenous signal, specific membrane permeable peptides that mimic the C-terminal tail of Gα subunits were used to block receptor activation of individual G-protein heterotrimers. As shown previously (Chang et al., 2000), the PLC signal is completely blocked by preincubation with the G_q blocking peptide (Table 1), whereas the PLD signal is left intact. On the other hand, the G_{13} blocking peptide completely blocks PLD activation by 5-HT but does not modify PLC activation. As an additional index of the specificity of the C-terminal blocking peptides, we showed that a peptide targeting the G_s protein, which blocks adenylyl cyclase activation by β-adrenergic receptors (Chang et al., 2000), has no effect on 5-HT$_{2C}$ receptor-mediated PLD or PLC activation (Table 1). These results demonstrate the specificity of the blocking peptides and suggest that the PLD activation is mediated by interaction of the 5-HT$_{2C}$ receptor with G_{13} protein. This conclusion was confirmed in NIH3T3 cells stably expressing 5-HT$_{2C}$ receptors (3T3–2C), where 5-HT produces a robust, dose-dependent increase in PLD activity (Fig. 1B) with an EC_{50} of 10 nM. This effect was blocked by mianserin (data not shown), confirming that PLD is a downstream consequence of 5-HT$_{2C}$ receptor interaction with the G_{13} protein. The specificity of G_{13} blocking peptide for blocking PLD, but not PLC, was reproduced in transfected fibroblasts (data not shown). In addition, treatment with a maximal concentration of 5-HT (1 μM) was able to overcome the peptide and stimulate PLD activity, showing that the peptide blockade is reversible (data not shown).

5-HT Stimulated PLD Activation, but Not PLC Activation, was Blocked by G$\beta\gamma$ Sequestering Peptides. Two different peptides that sequester free G$\beta\gamma$ subunits (a phosducin-like peptide and a peptide that mimics the interacting domain of PLCβ2) were used to characterize further the endogenous PLD and PLC signal transduction pathways. Although these peptides differ significantly in length and sequence, both inhibited 5-HT-stimulated PLD activation in CPE cells (Fig. 2A), but had no effect on 5-HT stimulated PLC activity in the same cells (Fig. 2B), demonstrating that the effect is specific. Similar data were obtained in 3T3–2C

TABLE 1

Gα_{13} C-terminal blocking peptide, but not Gα_q peptide, prevents PLD activation

PLD and PLC activity were measured as described under *Materials and Methods*. 5-HT (30 nM) was added with and without pretreatment with 100 μM G_{13}CT or PLCβ 1MC or with 50 μM G_sCT.

Treatment	PLD Activity	PLC Activity
	-fold basal	
Basal	1.00 ± 0.13	1.00 ± 0.05
Control (5-HT alone)	3.69 ± 0.70	2.67 ± 0.28
G_{13}CT	1.31 ± 0.09*	2.20 ± 0.42
G_q blocking	3.94 ± 1.08	0.52 ± 0.15*
G_sCT	3.49 ± 0.44	2.52 ± 0.57

*, $P < 0.05$ compared with control (5-HT alone); $n = 3$.

cells (Fig. 3), documenting that transfected fibroblasts are an appropriate model system for studying 5-HT$_{2C}$ receptor signal transduction pathways.

Role of $G_{i/o}$ G-Proteins in 5-HT–Stimulated PLC and PLD Activation. Neither PLD (Fig. 2A) nor PLC (Fig. 2B) activation in CPE is sensitive to PTX treatment, suggesting that the free G$\beta\gamma$ subunits involved in mediating 5-HT–stimulated PLD activation do not come from $G_{i/o}$ heterotrim-

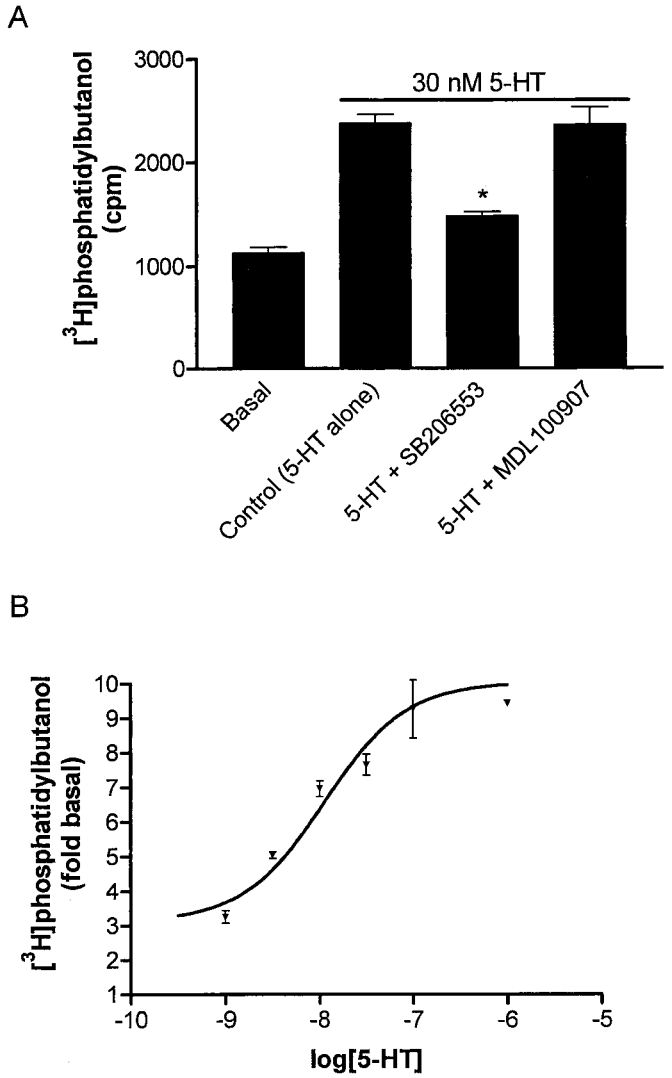

Fig. 1. PLD Activity in CPE (A) and 3T3–2C (B) cells. A, CPE cells were incubated with DMEM supplemented with 0.5% fatty-acid free BSA and 2 μCi/ml [9,10-^{3}H]myristic acid for 16 h, washed three times, and then incubated in DMEM for 50 min. Cells were then treated with 0.3% 1-butanol and 1 μM MDL100907 or SB206553 where indicated for 10 min before stimulation with 30 nM 5-HT for 15 min. [^{3}H]Phosphatidylbutanol was isolated using thin-layer chromatography and subjected to scintillation counting. The addition of the 5-HT$_{2C}$ antagonist SB206553, but not the 5-HT$_{2A}$ antagonist MDL100907, blocked 5-HT–stimulated PLD activation to basal levels. This is consistent with 5-HT activation of 5-HT$_{2C}$ receptors mediating the PLD signal. B, NIH3T3 cells, stably transfected with the INI isoform of the 5-HT$_{2C}$ receptor, were incubated with DMEM supplemented with 0.5% fatty-acid free BSA and 2 μCi/ml [9,10-^{3}H]-myristic acid for 16 h, washed three times, and then incubated in DMEM for 50 min. Cells were then treated with 0.3% 1-butanol for 10 min before stimulation with the indicated concentration of 5-HT for 15 min. [^{3}H]Phosphatidylbutanol was isolated using thin-layer chromatography and subjected to scintillation counting. The addition of 5-HT produced a dose-dependent increase in PLD activation. Representative of four experiments.

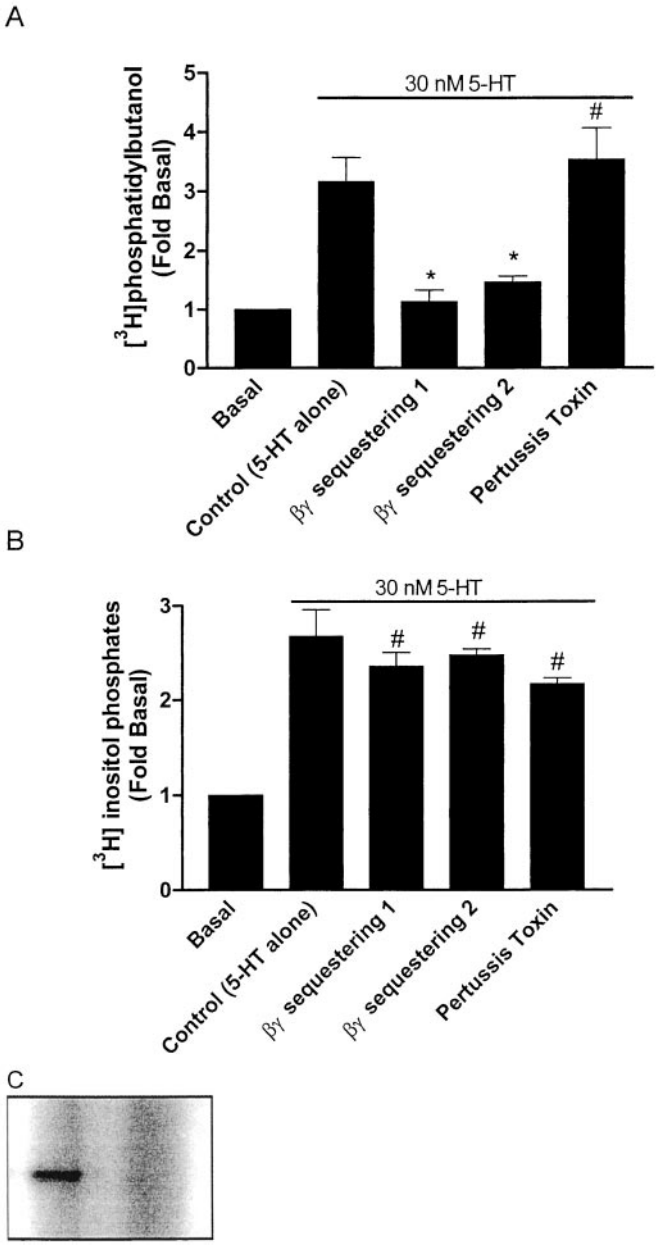

Fig. 2. Peptide blockade of PLD and PLC Activity in CPE cells. A, CPE cells were incubated with DMEM supplemented with 0.5% fatty-acid free BSA and 2 μCi/ml [9,10-^{3}H]myristic acid for 16 h, washed three times, and then incubated with peptides solubilized in HBSS for 30 min. 1-Butanol (0.3%) was added for 10 min before stimulation with 30 nM 5-HT for 15 min. [^{3}H]Phosphatidylbutanol was isolated and subjected to scintillation counting. Pretreatment with $\beta\gamma$-sequestering peptides blocks 5-HT–mediated PLD stimulation to basal levels. *, $p < 0.01$ compared with control (5-HT alone); $p > 0.05$ compared with basal. #, $p < 0.001$ compared with basal; $p > 0.05$ compared with control (5-HT alone). $n = 3$. B, CPE cells were incubated with DMEM (minus inositol) and 2 μCi/ml [^{3}H]inositol for 16 h, then incubated with peptides solubilized in HBSS for 30 min before activation with 30 nM 5-HT for 30 min. [^{3}H]Inositol monophosphate was isolated and subjected to scintillation counting. The addition of G$\beta\gamma$ sequestering peptides had no effect. *, $p < 0.001$ compared with control (5-HT alone), $p > 0.05$ compared with basal. #, $p < 0.01$ compared with basal, $p > 0.05$ compared with control (5-HT alone). $n = 3$. C, pertussis toxin (500 ng/ml) was added to labeling medium so that CPE cells were treated for 16 h. Membranes from treated and untreated cells were subjected to in vitro ADP ribosylation as described under *Materials and Methods*. Membranes were pelleted and separated by SDS-polyacrylamide gel electrophoresis and ribosylated proteins were visualized using an Amersham PhosphorImager system. The absence of ADP-ribosylated Gi/o in the PTX-treated lane indicated that the pertussis toxin treatment was effective.

ers. Similarly, PLD activation in 3T3–2C cells was insensitive to PTX (Fig. 3). As a control for PTX activity, cells were treated overnight with PTX using conditions identical to those in the functional studies and then subjected to an in vitro ADP-ribosylation assay. Cells treated overnight with PTX were not ADP-ribosylated by PTX added in vitro, whereas non–PTX-treated cells were ADP-ribosylated, as illustrated in Fig. 2C for CPE cells.

5-HT–Mediated Stimulation of 5-HT$_{2C}$ Receptors Activates Rho GTPases. Because G_{13} G-proteins have been demonstrated to activate specific Rho GTP exchange factors (Hart et al., 1998; Kozasa et al., 1998), resulting in activation of Rho proteins, and because Rho activation has been demonstrated to stimulate PLD activity (Malcolm et al., 1994; Hess et al., 1997), the role of Rho activation was determined. Pretreatment of 3T3–2C cells with C3 exoenzyme from *Clostridia botulinum,* which inactivates Rho by ADP ribosylation (Majumdar, 1999; Borbiev et al., 2000), abolishes the PLD signal (Fig. 3) but does not block serotonin stimulated PLC activity (data not shown).

Reid et al. (1996) isolated and characterized a protein, called Rhotekin, that interacts with GTP-bound Rho A. Using a Rhotekin-GST fusion protein, GTP-bound (active) Rho A can be isolated from cell lysates. This assay allowed us to document Rho A activation in response to stimulation with 5-HT as illustrated in Fig. 4. This activation is blocked by pretreatment with G_{13}CT peptide (Fig. 4), consistent with G_{13} activation of Rho proteins after 5-HT stimulation. Figure 4 also shows that G$\beta\gamma$-sequestering peptides do not block Rho activation. These findings are consistent with a bifurcating pathway in which Gα_{13} subunits mediate Rho activation, whereas G$\beta\gamma$ subunits stimulate PLD activity by a different mechanism.

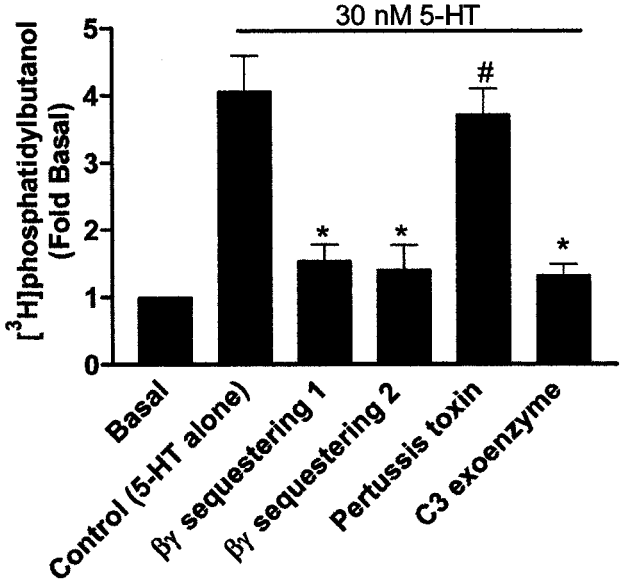

Fig. 3. Peptide blockade of PLD Activity in 3T3–2C cells. NIH3T3 cells stably expressing 5-HT$_{2C}$ receptor were incubated with DMEM supplemented with 0.5% fatty-acid free BSA, 2 μCi/ml [9,10-^{3}H]myristic acid, 30 μg/ml LipofectAMINE, and 5 μg/ml C3 exoenzyme where indicated for 16 h, washed three times, and then incubated with peptides solubilized in HBSS for 30 min. 1-Butanol (0.3%) was added for 10 min before stimulation with 10 nM 5-HT for 15 min. [^{3}H]Phosphatidylbutanol was isolated using thin-layer chromatography and subjected to scintillation counting. The addition of the G$\beta\gamma$-sequestering peptides or the C3 exoenzyme blocked 5-HT–stimulated PLD activation to basal levels, whereas pertussis toxin had no effect. *, $p < 0.01$ compared with control (5-HT alone), $p > 0.05$ compared with basal. $n = 3$ to 5.

Discussion

5-HT$_{2C}$ receptors activate PLC through PTX-insensitive G-proteins (Conn et al., 1986; Conn and Sanders-Bush, 1986a,b) and recently this activation has been demonstrated to be mediated by Gα_q subunits (Chang et al., 2000). The current study represents the first demonstration of PLD activation by the 5HT$_{2C}$ receptor. PLD activation has been suggested as a mediator of stress fiber formation (Gohla et al., 1999) and has also been linked to vesicle trafficking (Brown et al., 1993; Malcolm et al., 1994), the formation of oxygen radicals (Grewal et al., 1999) and cell cycle control (for reviews, see Houle and Bourgoin, 1996; Exton, 1999). Recently, regulators of G-protein signaling proteins have been used to demonstrate that transfected M_3 muscarinic acetylcholine receptors can couple to PLD through the $G_{12/13}$ family of G-proteins when transfected into human embryonic kidney 293 cells (Rumenapp et al., 2001). The current studies extend that conclusion to show that G_{13} protein mediates PLD activation by an endogenous GPCR, the 5-HT$_{2C}$ receptor in choroid plexus, and further suggests dual, converging pathways involving both Gα and G$\beta\gamma$ subunits. Results were verified in NIH3T3 cells that stably express 5-HT$_{2C}$ recep-

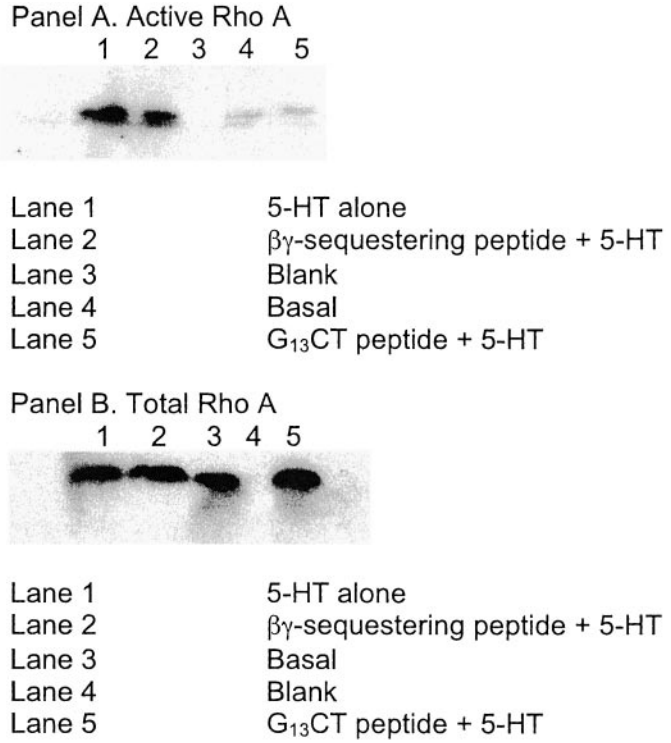

Fig. 4. 5-HT–mediated activation of Rho GTPase in 3T3–2C cells. NIH3T3 cells stably expressing the rat INI isoform of the 5-HT$_{2C}$ receptor were washed three times, incubated with DMEM supplemented with 0.5% fatty-acid free BSA for 16 h, and then incubated as indicated with peptides solubilized in HBSS for 30 min. Cells were treated with 10 nM 5-HT for 5 min where indicated. Cell lysates were prepared and Rho activation was analyzed as described under *Materials and Methods*. A, active Rho. 5-HT produces an increase in active Rho, compared with basal, which is blocked by the G_{13}CT peptide, but not by G$\beta\gamma$ sequestering peptides. B, total Rho. 25 μl of cell lysate was run for comparison. All samples show comparable levels of Rho protein.

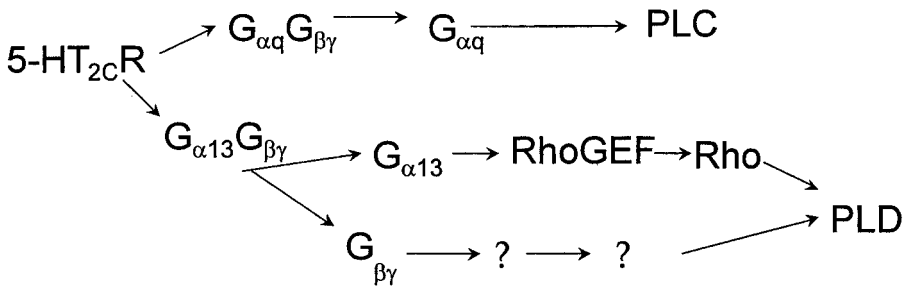

Fig. 5. Pathway for activation of PLD by 5HT$_{2C}$ receptors.

tors, thus providing a model system for studying the mechanism of 5-HT$_{2C}$ receptor signal transduction.

Although 5-HT$_{2C}$ receptors produce robust PLC and PLD signals, the evidence suggests that PLD activation is not downstream of PLC. Thus, the G$_q$ blocking peptide, a peptide that targets and presumably interrupts G$_q$/PLC interaction, and direct PLC inhibitors (data not shown), completely block PLC activation without altering PLD activation. Conversely, the G$_{13}$ blocking peptide and inhibitors that block PLD activity do not inhibit PLC activation. We therefore propose that the endogenous 5HT$_{2C}$R activates PLC and PLD independently, by coupling to two different G-proteins, G$_q$ and G$_{13}$, respectively.

As illustrated in Fig. 5, our data also suggest that activation of PLD involves both Gα_{13} and free G$\beta\gamma$ subunits. Furthermore, the small G-protein Rho seems to be an essential mediator in the Gα_{13} signal. 5-HT activates Rho and this activation is prevented by addition of the G$_{13}$ blocking peptide. In addition, the C3 exoenzyme, which inhibits Rho signaling, prevents PLD activation by 5-HT. These data suggests that the 5HT$_{2C}$ receptor couples to G$_{13}$ protein with subsequent activation of Rho, which in turn activates PLD. A second, still undefined pathway mediates the PLD signal that is generated by G$\beta\gamma$ subunits as evidenced by our finding that although G$\beta\gamma$ sequestering peptides block the PLD signal, they do not block Rho activation by the 5HT$_{2C}$ receptor. G$\beta\gamma$ subunits from G$_{i/o}$ heterotrimers have previously been demonstrated to activate effectors, including PLCβ2, and mitogen activated protein kinase (Katz et al., 1992; Luttrell et al., 1995; Murthy et al., 1995). However, we show here that the G$\beta\gamma$ subunits involved in PLD activation are not generated from G$_{i/o}$ heterotrimers, but rather seem to be derived from G$_{13}$ heterotrimers. This mechanism represents a unique signal transduction pathway in which PTX-insensitive G$\beta\gamma$ subunits mediate an endogenous cellular signal.

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