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RNA Editing of the Human Serotonin 5-HT_{2C} Receptor Disrupts Transactivation of the Small G-Protein RhoA

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ABSTRACT

The human serotonin 5-HT_{2C} receptor undergoes adenosine-to-inosine RNA editing at five positions, generating multiple receptor isoforms with altered G-protein coupling properties. In the current study, we demonstrate that RNA editing regulates the pattern of intracellular signaling. The non-edited human 5-HT_{2C} receptor isoform INI activates phospholipase D via the G_{13} heterotrimer G-protein. We present evidence that transactivation of the small G-protein RhoA is required for phospholipase D activation. In contrast, neither transactivation of RhoA nor phospholipase D activation was detected in cells expressing the fully edited VGV isoform. The ability to activate phospholipase C is also reduced in VGV-expressing cells, but not to the extent found for the phospholipase D signal. We conclude that RNA editing represents a novel mechanism for regulating 5-HT_{2C} receptor signaling to pathways linked to actin cytoskeletal organization and regulated exocytosis.

The serotonin 5-HT_{2C} receptor subtype signals through the heterotrimeric G-protein G_q to activate phospholipase C (PLC) (Chang et al., 2000a), leading to the intracellular accumulation of inositol trisphosphate and subsequent calcium release. RNA transcripts encoding the human 5-HT_{2C} receptor undergo RNA editing events at five positions, A, B, C, D, and E (Fig. 1A) (Burns et al., 1997; Niswender et al., 1998), altering the amino acid sequence in the putative second intracellular loop of the protein, a region important for receptor/G-protein coupling (Arora et al., 1995, 1997; Blin et al., 1995; Gomeza et al., 1996; Liu and Wess, 1996; Iida-Klein et al., 1997; Verrall et al., 1997; Ballesteros et al., 1998; Burstein et al., 1998). We and others have demonstrated a decrease in agonist potency, ligand binding affinity, and constitutive activity at the human edited VSV and VGV isoforms (named for the amino acids at positions 156, 158, and 160), compared with the non-edited INI isoform (Fitzgerald et al., 1999; Herrick-Davis et al., 1999; Niswender et al., 1999; Price and Sanders-Bush, 2000).

Although these changes were originally proposed to simply result from a reduced G_q-protein coupling efficiency, we have recently shown that editing also alters the profile of activation of heterotrimeric G-proteins (Price et al., 2001). Specifically, the VGV isoform has no, or greatly attenuated, ability to functionally couple to G_{13} compared with the INI isoform. We therefore examined whether two human receptor isoforms (the nonedited INI isoform and fully edited VGV isoform) differed in their capacity to modulate downstream effectors of G_{13}.

Materials and Methods

Peptide Design and Synthesis. The G_{13} carboxyl-terminal peptide (G13) was designed based on the last 10 amino acids of the carboxyl terminus (aa 367-377), a region of G_α subunits identified as a site of interaction between G-proteins and receptors (Hamm and Rarick, 1994; Taylor and Neubig, 1994). This peptide was attached to a hydrophobic membrane-permeable sequence as described previously (Chang et al., 2000b). The membrane-permeable sequence is AAVALLPAVLLALLAK-S, and the G13-mimicking peptide sequence is CLHDNLKQLME. The Gq-blocking peptide sequence (QLKKLKEICEKEKKELKKKMDKKRQEKITEAK) is based on the PLCβ1 enzyme sequence that interacts with active $G_{q\alpha}$ subunits and hence blocks $G_{q\alpha}$ activation of PLC. Peptides were solubilized in HBSS at maximum concentration. Previous studies from our laboratory have demonstrated that these peptides block specific protein-

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ABBREVIATIONS: PLC, phospholipase C; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle's Medium; FBS, fetal bovine serum; DOI, (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane; ANOVA, analysis of variance; GST, glutathione *S*-transferase PLD, phospholipase D.

protein interactions in a competitive, reversible manner (Chang et al., 2000a; McGrew et al., 2002).

Cell Culture. NIH-3T3 cells stably expressing human INI and VGV isoforms of the 5-HT$_{2C}$ receptor were generated as described previously (Niswender et al., 1999); receptor densities were 2047 and 5375 fmol/mg of protein, respectively. Cells were grown in DMEM containing 10% FBS, 100 units ml/penicillin, and 100 μg/ml streptomycin under 5% CO_2 at 37°C.

Phospholipase D Assay. This method is adapted from Hess et al. (1997) as described in detail by McGrew et al. (2002). Two days after plating in 24-well plates, cells were labeled with 2 μCi/ml [9,10-^{3}H]myristic acid in DMEM supplemented with 0.5% (w/v) fatty acid free bovine serum albumin for 16 to 20 h. The C3 exoenzyme (5 μg/ml) was added where indicated during the labeling step. Labeled cells were washed two times with 0.5 ml of DMEM containing 0.5% fatty acid free bovine serum albumin and incubated at 37°C with peptides or vehicle for 30 min, then treated with 0.03% (v/v) 1-butanol for 10 min before stimulation with DOI. After an additional 15-min incubation with DOI, lipids were extracted using a methanol/chloroform/0.1 N HCl (1:1:1) mixture and spotted onto silica gel 60A thin-layer chromatography plates (Whatman, Clifton, NJ). The plates were developed in the upper phase of an ethyl acetate/isooctane/H_2O/acetic acid (55:25:50:10) solvent system and then stained with iodine. An unsaturated phosphatidylbutanol standard (Avanti Polar Lipids, Birmingham, AL) was used to locate the bands, which were then scraped into scintillation fluid and counted. Data were analyzed using one-way ANOVA with Tukey's multiple-comparison post hoc test.

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 HBSS/well and then incubated, where indicated, 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 DOI 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). Data were analyzed using one-way ANOVA with Tukey's multiple-comparison post hoc test.

Rhotekin Assay for Active Rho. This assay was adapted from Reid et al. (1996) with the kind permission of Shuh Narumiya (Kyoto, Japan) and uses a Rhotekin-GST fusion protein that selectively binds to active GTP-bound RhoA. Cells were cultured in 100-mm dishes and serum-starved overnight. After treating with 100 nM DOI or 1% FBS for 5 min, the cells were lysed in 40 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% Nonidet P-40, 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 for 30 min at 4°C. Beads were collected by centrifugation and washed three times with 1 ml of lysis buffer. 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 RhoA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). A horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and enhanced chemiluminescence detection substrate (Pierce Super Signal Dura; Pierce Biotechnology, Rockford, IL) were used to detect Rho proteins. A 25-μl sample of cell lysate was run for comparison. NIH Image software was used to determine relative densities for statistical analysis. Data were analyzed using one-way ANOVA with Tukey's multiple-comparison post hoc test.

Results

5-HT$_{2C}$ INI Receptors, but Not 5-HT$_{2C}$ VGV Receptors, Stimulate Rho GTPases. Earlier studies documented that agonist stimulation of the rat 5-HT$_{2C}$ receptor activates Rho proteins (Gohla et al., 1999; McGrew et al., 2002). The current experiments were designed to determine whether Rho activation was preserved in the human 5-HT$_{2C}$ receptor and whether the ability to activate Rho was altered by RNA editing. GTP-bound (active) Rho was isolated from cell lysates using a GST fusion protein with a fragment of Rhotekin that specifically binds active Rho (Reid et al., 1996). NIH-3T3 cells stably expressing the INI receptor isoform showed robust Rho activation in the presence of 100 nM DOI, a 5-HT$_{2C}$ receptor agonist (Fig. 1B). In contrast, cells stably transfected with the VGV isoform did not show Rho activation in response to DOI (Fig. 1B) despite normal Rho expression and serum-induced activation in these cells (Fig. 1B). These results demonstrate that Rho activation is a consequence of INI, but not VGV, receptor activation.

5-HT$_{2C}$ Receptor-Mediated Activation of PLD is Disrupted by RNA Editing. To elucidate the consequences of these alterations in Rho activity, we tested whether RNA editing altered stimulation of PLD, a downstream effector of Rho (Malcolm et al., 1994, 1996). DOI produced a robust increase in PLD activity in cells expressing INI receptors (EC_{50} = 3 nM; Fig. 2A). In contrast, DOI stimulation of VGV-expressing cells did not increase PLD activity above basal levels (Fig. 2A) even with micromolar concentrations. These data demonstrate that the human 5-HT$_{2C}$ INI receptor, but not the VGV isoform, produces a robust PLD signal. The VGV isoform of the 5-HT$_{2C}$ receptor is able to stimulate

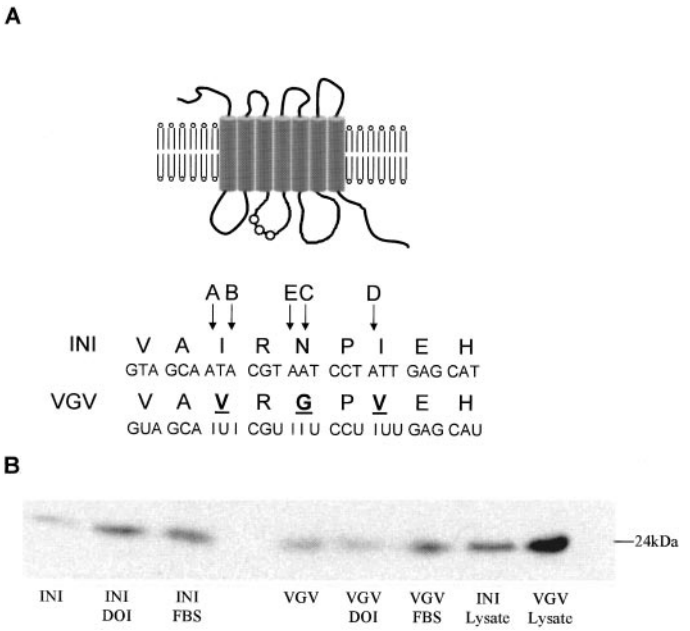

Fig. 1. The INI, but not VGV, isoform of the 5-HT$_{2C}$ receptor activates Rho GTPases. A, the positions of the editing sites within human 5-HT$_{2C}$ receptor RNA (top) and the predicted amino acid sequence (bottom) are shown for the non-edited INI isoform and the edited VGV isoform. B, DOI- and FBS-mediated activation of Rho GTPase in NIH-3T3 cells stably expressing either the INI or VGV isoform. Cell lysates were prepared and Rho activation was analyzed as described under *Materials and Methods*. VGV samples were run at higher protein levels to ensure detection of small signals, if present.

PLC activity; however, compared with the INI isoform, the potency and efficacy of DOI is greatly reduced (Fig. 2B).

PLD Activation Is Downstream of G_{13} Heterotrimeric G-Protein and the Small G-Protein, Rho. To evaluate the role of heterotrimeric G-proteins in mediating this signal, we used specific membrane-permeable peptides that mimic the C-terminal tail of G_{13} G_{α} subunits to block receptor activation of the G_{13} G-protein heterotrimers or those that block interaction of PLCβ isozymes with active $G_{q\alpha}$ subunits. As demonstrated in Fig. 3A, in which the addition of the G13 peptide produces a 10-fold rightward shift in the concentration-response curve (EC_{50} = 40 nM versus 3 nM for controls in Fig. 2A), but no decrease in maximum response for PLD activation, this blockade is a competitive, reversible interaction that is specific to the targeted G-protein. The Gq peptide, on the other hand, does not modify the PLD signal (EC_{50}, ~4 nM). The inset shows collective results for a single 10 nM concentration of DOI used for statistical analyses (n = 3); only the G13 peptide blocks PLD activation. Figure 3B shows that the Gq peptide produces a rightward shift in the PLC concentration-response curve (EC_{50}, ~100 nM) for PLC activity, whereas the G13 peptide has no effect (EC_{50}, ~1 nM). The inset showing mean data (n = 3) for a single submaximum concentration of DOI (10 nM) confirms that only the Gq-blocking peptide prevents PLC activation. These results demonstrate that PLD activation is not downstream of G_q-linked PLC activation, but rather is mediated by interaction of the 5-HT_{2C} receptor with G_{13}.

Because the non-edited 5-HT_{2C} receptor activates both Rho and PLD, we postulated that these two signals may be linked. The addition of C3 exoenzyme from *Clostridia botulinum*, which inactivates Rho by ADP-ribosylation of RhoGEFs (Majumdar et al., 1999; Borbiev et al., 2000), blocks the activation of PLD by DOI (Fig. 4A). In contrast, C3 exoenzyme does not reduce PLC activation by the 5-HT_{2C} receptor (Fig. 4B), providing evidence that the reduction in PLD activity is specific, not secondary, to cellular toxicity. Thus, we conclude that Rho activation by the 5-HT_{2C} receptor promotes the activation of PLD and, furthermore, that the extensively edited isoform, created by editing at five sites, has no, or greatly attenuated, ability to activate Rho and PLD.

Discussion

Previous studies have demonstrated that RNA editing of the 5-HT_{2C} receptor has profound functional consequences, including alterations in agonist potency and constitutive activity in the PLC pathway, and selectivity of G-protein coupling (Burns et al., 1997; Fitzgerald et al., 1999; Herrick-Davis et al., 1999; Niswender et al., 1999; Price et al., 2001).

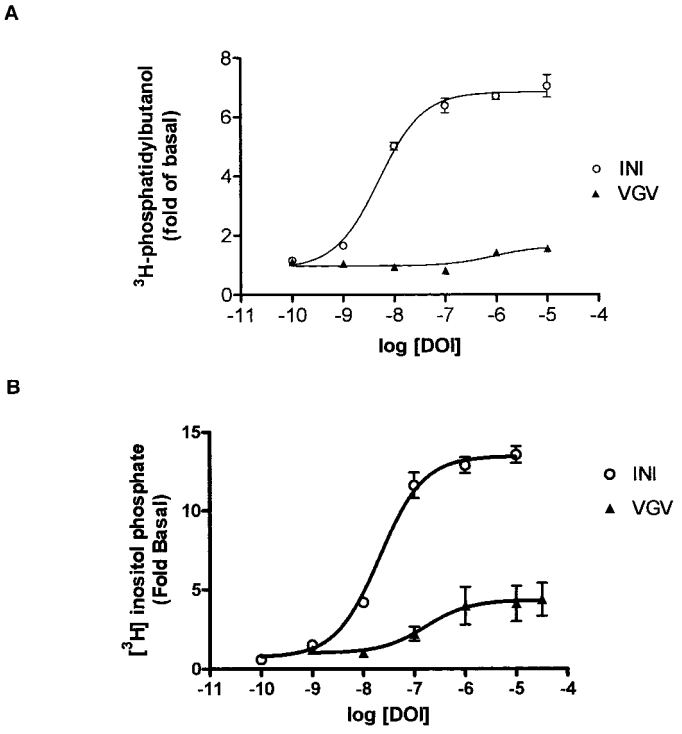

Fig. 2. 5-HT_{2C} receptor-mediated activation of PLD, but not PLC, is disrupted by RNA editing. A, representative agonist concentration-response curve for PLD activation in NIH-3T3 cells stably expressing the INI or VGV isoforms, as indicated (EC_{50} for 5-HT_{2C} INI, ~3 nM). The experiment was repeated three times with similar results. B, representative agonist concentration-response curve for PLC activation in NIH-3T3 cells stably expressing the INI or VGV isoforms, as indicated (EC_{50} for 5-HT_{2C} INI, ~3 nM; EC_{50} for 5-HT_{2C} VGV, ~750 nM).

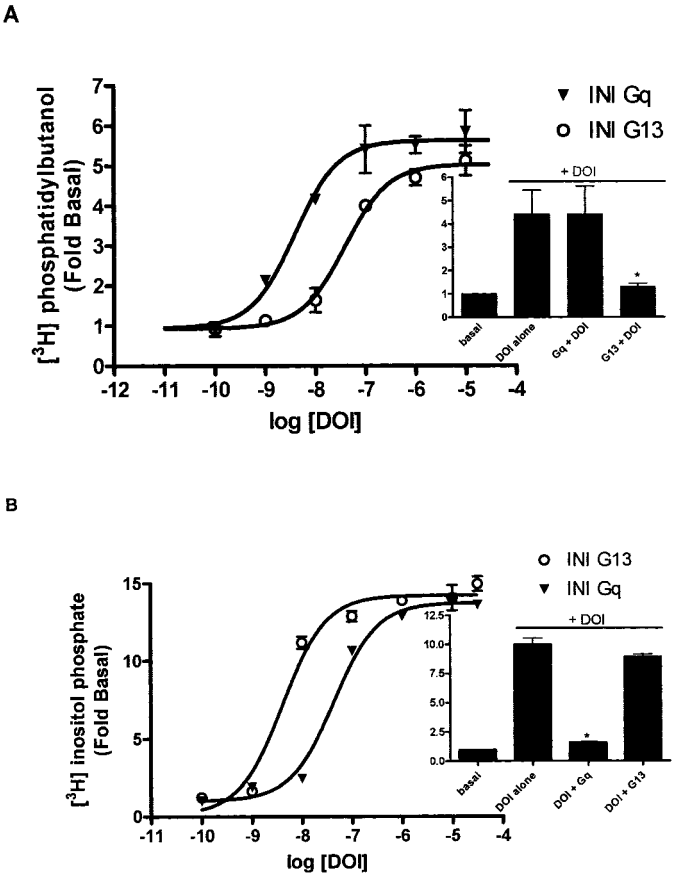

Fig. 3. PLD activation via the 5-HT_{2C} receptor occurs downstream of G13. A, stable INI cell lines were stimulated with increasing concentrations of DOI and assayed for PLD activation in the presence of a peptide (G13) designed to block receptor-mediated activation of G_{13}, or a peptide (Gq) designed to block interaction of $G_{q\alpha}$ subunits with PLCβ1 enzymes. The inset shows collective data for a single concentration of DOI (10 nM) (n = 3). B, stable INI cell lines were stimulated with increasing concentrations of DOI and assayed for PLC activation in the presence of a peptide (G13) designed to block receptor-mediated activation of G_{13}, or a peptide (Gq) designed to block interaction of $G_{q\alpha}$ subunits with PLCβ1 enzymes. The inset shows collective data for a single concentration of DOI (10 nM) (n = 3).

Furthermore, the region-specific generation of edited isoforms, together with evidence that RNA editing is altered by drugs and disease state, suggests that RNA editing contributes to in vivo differences in serotonergic signaling and therapeutic responses (Niswender et al., 2001; Sodhi et al., 2001; Gurevich et al., 2002a,b). The current study addresses the hypothesis that RNA editing is a mechanism for sculpting the intracellular signals produced by activation of the 5-HT$_{2C}$ receptor.

Receptors that couple to heterotrimeric G-proteins often elicit transactivation of low molecular weight G-proteins of the Rho subfamily. The 5-HT$_{2C}$ receptor has been shown to induce stress fiber formation via Rho-dependent mechanisms (Gohla et al., 1999). Furthermore, Rho activation is required for 5-HT$_{2C}$ receptor-mediated PLD activation (McGrew et al., 2002). The current study demonstrates that activation of Rho is regulated by RNA editing of the 5-HT$_{2C}$ receptor. The fully edited isoform of the human receptor, which has five amino acids changed in the second intracellular loop, is unable to promote Rho GTPase activity. Multiple mechanisms exist for PLD activation (reviewed in Exton, 1999, 2002), including the Gq-PLC pathway. However, our data suggest that PLD activation is not downstream of Gq-linked PLC activation but rather is mediated by interaction of the 5-HT$_{2C}$ receptor with G13. Furthermore, the activation of PLD, but not PLC, is completely prevented by pretreatment with C3 exoenzyme, which blocks Rho activation. Thus, we conclude that the loss of Rho activation as a result of RNA editing is accompanied by an inability, or greatly attenuated ability, to stimulate the PLD pathway.

The current results show that the PLD signal is undetectable in cells expressing the VGV isoform of the receptor. Although the PLC signal is also reduced (as reported previously by Niswender et al., 1999), there remains a residual PLC response. These experiments were performed using NIH-3T3 cells heterologously expressing a high density of a single isoform of the 5-HT$_{2C}$ receptor. It is possible that the differential extent of loss of PLC and PLD reflects stoichiometry or kinetics, rather than any preference that is physiologically relevant. Endogenous 5-HT$_{2C}$ receptors in primary cultures of choroid plexus epithelial cells have also been shown to stimulate PLD and PLC (McGrew et al., 2002), but multiple isoforms are expressed in these cells. Thus, it is not possible to tease out the relative contributions of individual receptor isoforms to these signals.

RNA editing of the 5-HT$_{2C}$ receptor is prominent in the central nervous system; indeed, most of the receptors in the brain exist as edited isoforms (Burns et al., 1997; Fitzgerald et al., 1999; Niswender et al., 2001). Because Rho and PLD are linked to a number of downstream responses, including actin cytoskeletal rearrangement and regulated exocytosis, the differential ability of the 5-HT$_{2C}$ receptor isoforms to engage Rho-dependent pathways has important implications. For example, RNA editing may alter the ability of the 5-HT$_{2C}$ receptor to regulate dopamine release (Millan et al., 1998; Porras et al., 2002). In addition, by restricting the ability of the 5-HT$_{2C}$ receptor to promote transactivation of Rho, RNA editing may regulate fundamental properties of cells, including migration, polarity, and transformation, hence explaining the emerging evidence for alterations in RNA editing of the 5-HT$_{2C}$ receptor in neurodevelopmental diseases such as schizophrenia.

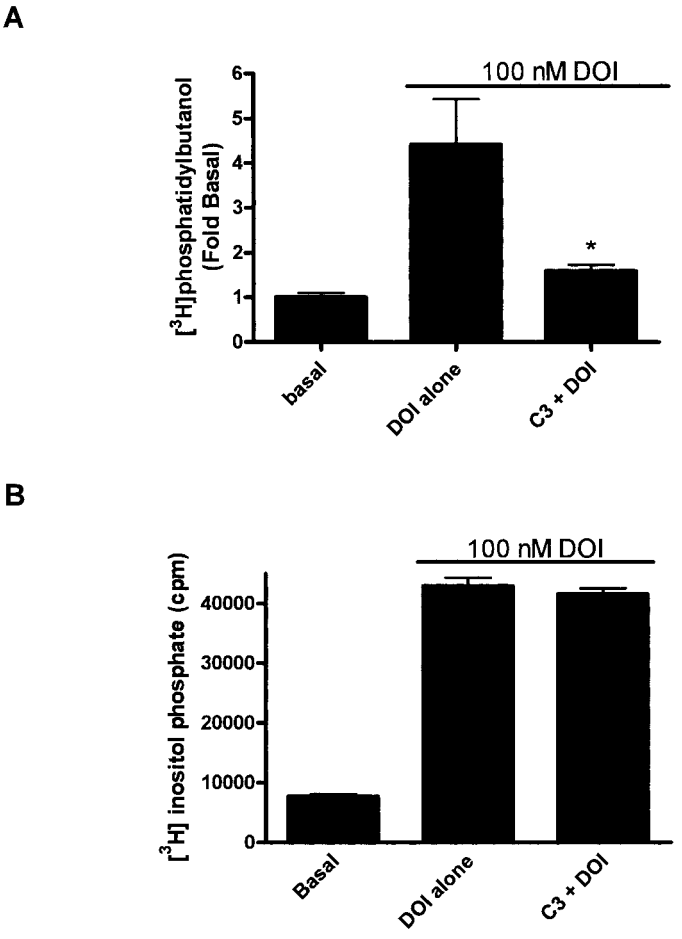

Fig. 4. PLD activation via the 5-HT$_{2C}$ receptor requires RhoA activation. A, stable INI cell lines were stimulated with 100 nM DOI and assayed for PLD activation in the absence or presence of C3 exoenzyme. Results are the mean of four independent experiments. *p*-values: basal versus DOI and DOI versus c3 + DOI, $p < 0.05$; basal versus c3 + DOI, $p > 0.05$. B, stable INI cell lines were stimulated with 100 nM DOI and assayed for PLC activation in the absence or presence of C3 exoenzyme.

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