G protein $\beta\gamma$ subunits directly interact with and activate phospholipase Cϵ

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Phospholipase C (PLC) enzymes hydrolyze membrane phosphatidylinositol 4,5 bisphosphate (PIP_2) and regulate Ca^{2+} and protein kinase signaling in virtually all mammalian cell types. Chronic activation of the PLCϵ isoform downstream of G protein–coupled receptors (GPCRs) contributes to the development of cardiac hypertrophy. We have previously shown that PLCϵ-catalyzed hydrolysis of Golgi-associated phosphatidylinositol 4-phosphate (PI4P) in cardiac myocytes depends on G protein $\beta\gamma$ subunits released upon stimulation with endothelin-1. PLCϵ binds and is directly activated by Ras family small GTPases, but whether they directly interact with G$\beta\gamma$ has not been demonstrated. To identify PLCϵ domains that interact with G$\beta\gamma$, here we designed various single substitutions and truncations of WT PLCϵ and tested them for activation by G$\beta\gamma$ in transfected COS-7 cells. Deletion of only a single domain in PLCϵ was not sufficient to completely block its activation by G$\beta\gamma$, but blocked activation by Ras. Simultaneous deletion of the C-terminal RA2 domain and the N-terminal CDC25 and cysteine-rich domains completely abrogated PLCϵ activation by G$\beta\gamma$, but activation by the GTPase Rho was retained. *In vitro* reconstitution experiments further revealed that purified G$\beta\gamma$ directly interacts with a purified fragment of PLCϵ (PLCϵ-PH-RA2) and increases PIP_2 hydrolysis. Deletion of the RA2 domain decreased G$\beta\gamma$ binding and eliminated G$\beta\gamma$ stimulation of PIP_2 hydrolysis. These results provide first evidence that G$\beta\gamma$ directly interacts with PLCϵ and yield insights into the mechanism by which $\beta\gamma$ subunits activate PLCϵ.

G protein–coupled receptors (GPCRs)[2] regulate a wide variety of cellular functions through stimulation of a few canonical signal transduction cascades. One major GPCR-stimulated pathway is the agonist-dependent hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) by phospholipase C (PLC) to regulate intracellular Ca^{2+} and protein kinase activation (1). PLC signaling is stimulated by both receptor tyrosine kinases (RTKs) and GPCRs and regulates multiple cellular functions in normal and disease states. Thirteen PLC isoforms have been identified with some common structural features (2–5). All PLC isoforms contain a pleckstrin homology (PH) domain, an EF-hand domain, a highly conserved catalytic core domain (composed of the X and Y domains separated by a linker region) and a phospholipid-binding C2 domain. The diversity in regulation of the PLCs arises in large part from regions outside the conserved core domains.

The PLCβ family of PLCs is directly activated by Gα_q or G$\beta\gamma$ subunits with varying efficacies and potencies (6–11). PLCϵ is a more recently discovered PLC isoform and is also downstream of GPCRs, as well as RTKs (12–15). PLCϵ binds directly to members of the Ras family of small GTPases including Ras, Rap, and Rho. Ras family members can be activated by both GPCRs and RTKs and subsequently activate PLCϵ (5). G protein $\beta\gamma$ subunits have also been shown to regulate PLCϵ in a COS-7 cell co-transfection system, but direct activation by G$\beta\gamma$ has not been demonstrated (16).

PLCϵ has a unique domain architecture where, apart from conserved core domains, it has N-terminal cysteine-rich (Cys) and CDC25 guanine nucleotide exchange factor (GEF) domains that activate Rap, and two C-terminal Ras-association domains (RA1 and RA2) (Fig. 1*A*). A putative PH domain was identified *in silico*, but its properties have not been studied biochemically (16). The RA2 domain binds directly to Ras and is required for activation by Ras (12). A domain required for activation by Rho is in the Y catalytic domain but a direct binding site for Rho has not been identified (17). Although G$\beta\gamma$ has been shown to activate PLCϵ, the domains on PLCϵ required for this activation have not been delineated (17).

We recently found that G$\beta\gamma$-dependent regulation of PLCϵ at the Golgi apparatus in cardiac myocytes regulates local PI4P hydrolysis, protein kinase D (PKD) activation, gene expression, and hypertrophy (18). Thus understanding how PLCϵ is regulated by G$\beta\gamma$ is important to fully understand this process. Here we identified two independent domains of PLCϵ that are involved in its regulation by G$\beta\gamma$ and demonstrate direct interactions between G$\beta\gamma$ and PLCϵ. These data place PLCϵ activation directly downstream of GPCRs.

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[2] The abbreviations used are: GPCRs, G protein–coupled receptors; PIP_2, phosphatidylinositol 4,5 bisphosphate; PLC, phospholipase C; PI4P, phosphatidylinositol 4-phosphate; RTKs, receptor tyrosine kinases; PH, pleckstrin homology; IP, inositol phosphates; buffer A, 20 mM HEPES, pH 8, 100 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mM EDTA, and 0.1 M EGTA; SEC buffer, 20 mM HEPES, pH 8, 200 mM NaCl, 2 mM DTT, 0.1 mM EDTA, and 0.1 M EGTA; CV, column volume; ANOVA, analysis of variance.

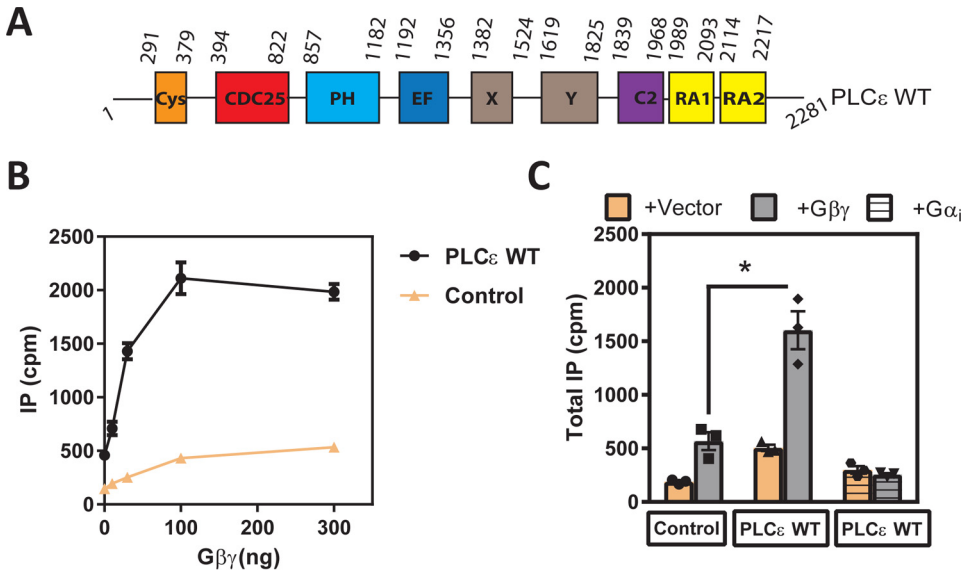

Figure 1. Regulation of PLCε by G proteins. *A*, PLCε WT and corresponding domain boundaries are illustrated. *B*, COS-7 cells were transfected with PLCε (300 ng) in the presence or absence of varying concentrations of $G\beta_1$ and $G\gamma_2$ plasmids. Total [^{3}H]inositol phosphate accumulation was quantified as described under "Experimental Procedures." The data shown are mean ± S.E. for triplicate samples. The experiment was done thrice with similar results. *C*, effect of $G\alpha_{i1}$ on PLCε activation by Gβγ. PLCε (300 ng) was co-transfected with $G\beta_1$ (200 ng) and $G\gamma_2$ (200 ng) in the presence or absence of $G\alpha_{i1}$ (200 ng). The data shown are mean ± S.E. for three independent experiments and analyzed by one-way ANOVA with Dunnett's post test. *, $p < 0.05$ *versus* Gβγ.

Results

Regulation of PLCε by Gβγ subunits

G protein–dependent activation of PLCε is difficult to reconstitute with purified G proteins (12, 17), so to understand the mechanism by which Gβγ regulates PLCε, we confirmed previous work showing that Gβγ co-transfected with PLCε increases accumulation of inositol phosphates (IP) in COS-7 cells labeled with [^{3}H]inositol (16). When Gβ and γ subunits are transfected in the absence of transfected PLC, increased IP accumulation is observed because of stimulation of endogenous PLCs present in COS-7 cells (Fig. 1, *B* and *C*) (19). However, cells co-transfected with Gβγ and PLCε markedly increased IP accumulation in a concentration-dependent manner (Fig. 1, *B* and *C*). To confirm that free Gβγ is necessary for activation of PLCε in COS-7 cells, we included $G\alpha_{i1}$ in the co-transfection to bind and sequester free Gβγ. In the presence of $G\alpha_{i1}$, Gβγ-dependent accumulation of IP was inhibited (Fig. 1*C*). These results indicate that activation of PLCε by Gβγ requires free Gβγ and confirms previously reported Gβγ-dependent activation of PLCε (16).

Effect of C-terminal domain mutation and deletion on activation of PLCε by Gβγ

Gβγ has been reported to activate Ras (20). Mutation of the lysine residues 2150 and 2152 in the PLCε RA2 domain to glutamate (Fig. 2*A*) inhibits activation of PLCε by Ras (12). Western blot analysis of the mutants showed similar expression of PLCε WT and Lys-Glu (Fig. 2*B*). To investigate whether activation of PLCε by Gβγ in COS-7 cells is mediated indirectly through endogenous activated Ras, we tested PLCε 2150/2152 Lys-Glu for activation by Gβγ and Rho to rule out indirect activation by endogenous Ras. Co-transfection of PLCε Lys-Glu with Gβγ or constitutively active Rho (RhoG14V) in COS-7 cells resulted in marked accumulation of IP (Fig. 2*C*, *left* and *middle panels*) in contrast to constitutively activated Ras (RasG12V) co-transfected cells (Fig. 2*C*, *right panel*). These results confirm previously published data indicating that activation of PLCε by Gβγ or Rho is independent of endogenous Ras activation in COS-7 cells (16).

To determine whether the RA2 domain of PLCε is required for its activation by Gβγ, we next deleted residues 2114–2281 composing the RA2 domain and tested for activation by Gβγ, Rho, and Ras (Fig. 2*A*). The unstimulated basal activity of PLCε-ΔRA2 when co-transfected with empty vector was significantly lower than PLCε WT despite similar levels of expression (Fig. 2, *B* and *D*). RasG12V was unable to activate this mutant as expected (Fig. 2*D*, *right panel*). Co-transfection of this mutant with RhoG14V caused significant accumulation of IP but not to the levels of PLCε WT indicating the enzyme is still active and can be activated (Fig. 2*D*, *middle panel*). Gβγ transfection resulted in a small but statistically significant increase in IP accumulation when PLCε-ΔRA2 was co-transfected compared to control with Gβγ only transfected (Fig. 2*D*, *left panel*). The reduced levels of IP accumulation may be because of the lower basal activity of PLCε-ΔRA2. However, because the Rho activation is relatively robust it suggests that the RA2 domain is important for Gβγ stimulation. This will be explored in more detail later.

Effect of N-terminal domain deletions on PLCε activation by Gβγ

To investigate the involvement of the N-terminal domains of PLCε in Gβγ-mediated activation, a series of N-terminal truncations of PLCε were created (Fig. 3*A*). It has been reported that the N-terminal domains are not essential for PLCε activation by Gβγ or Rho (17). The basal activity of PLCε-CDC25-RA2 was significantly higher than PLCε WT when co-transfected with empty vector (Fig. 3*C*), whereas PLCε-PH-RA2 and EF-RA2

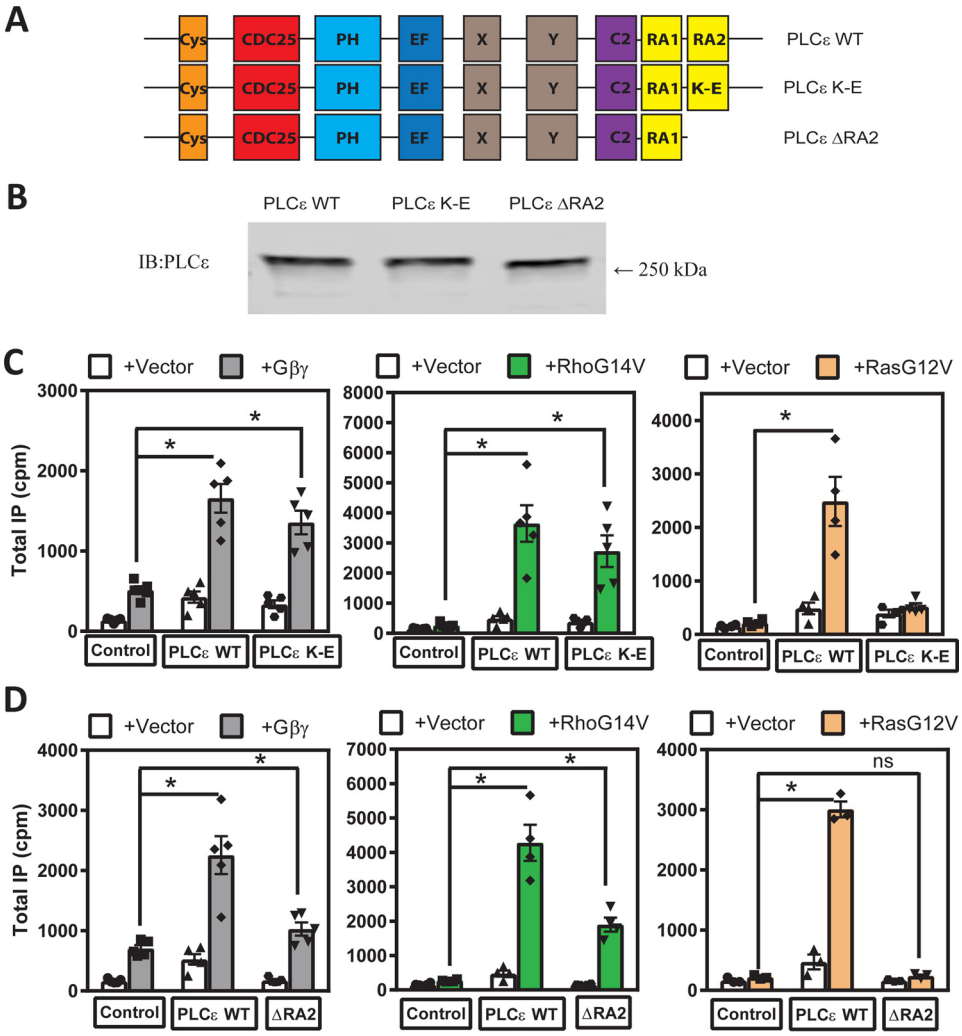

Figure 2. RA2 domain deletion and mutations does not completely inhibit PLCε activation by Gβγ. *A*, schematic of PLCε WT, PLCε Lys-Glu (K2150/2152E), and PLCε ΔRA2 (1–2113) constructs. *B*, a representative Western blot showing expression of the PLCε constructs. *C*, COS-7 cells were transfected with 300 ng PLCε WT or PLCε Lys-Glu in the presence or absence of 200 ng $G\beta_1$ and 200 ng $G\gamma_2$, or constitutively active small GTPases (RhoG14V (200 ng) and RasG12V (100 ng)) and total [^{3}H]inositol phosphate accumulation was measured. *D*, COS-7 cells were transfected with PLCε WT or PLCε ΔRA2 in the presence or absence of 200 ng $G\beta_1$ and 200 ng $G\gamma_2$ or constitutively active small GTPases (RhoG14V (200 ng) and RasG12V (100 ng)) and total [^{3}H]inositol phosphate accumulation was measured. The data shown are mean ± S.E. for at least three independent experiments and analyzed by one-way ANOVA with Dunnett's post test. *, $p < 0.05$ *versus* Gβγ; *ns*, not significant.

had decreased basal activity compared with PLCε WT (Fig. 3, *D* and *E*). There was no correlation between expression levels of the mutant PLCε proteins (Fig. 3*B*) and observed basal activities (Fig. 3, *C–E*). PLCε-PH-RA2 and EF-RA2 had significantly reduced capacity for activation by free Gβγ, whereas activation by RhoG14V was largely retained (Fig. 3, *C–E*). The reduced accumulation of IP in the presence of the mutants (PH-RA1 and EF-RA2) and Gβγ or RhoG14V may be because of their lower basal activity, however, Gβγ stimulation of the EF-RA2 PLCε was still minimal. Overall, our results indicate that deletion of the N-terminal domains is not sufficient to completely inhibit activation of PLCε by Gβγ but significantly reduces it.

Effect of simultaneous N-terminal and C-terminal domain deletions on PLCε activation by Gβγ

The results of the foregoing experiments could indicate that some PLCε activation by Gβγ requires interaction with only the catalytic core. An alternate hypothesis is that PLCε activation by Gβγ may involve multiple interaction sites at the C terminus and the N terminus. To test these hypotheses, we made a series of combined truncations of PLCε that lack both the N- and C-terminal domains as shown in Fig. 4*A*. COS-7 cells were transfected with either Gβγ or RhoG14V and one of the mutant PLCε constructs shown in Fig. 4*A*. A Western blot showing expression levels of these constructs is seen in Fig. 4*B*. Although the basal activity of CDC25-RA1 is significantly lower than PLCε WT as shown in Fig. 4*C*, co-transfection with Gβγ resulted in a small but statistically significant accumulation of IP relative to control without PLCε co-expression. RhoG14V was also able to activate this mutant. Deletion of the CDC25 domain completely eliminated Gβγ-dependent accumulation of IP above levels of cells transfected with Gβγ alone (Fig. 4*D*, *left panel*). In contrast, co-transfection of this PLCε mutant with RhoG14V resulted in some Rho-stimulated accumulation of IP, confirming that this construct can be activated to some level (Fig. 4*D*, *right panel*). EF-RA1 is also not activated upon

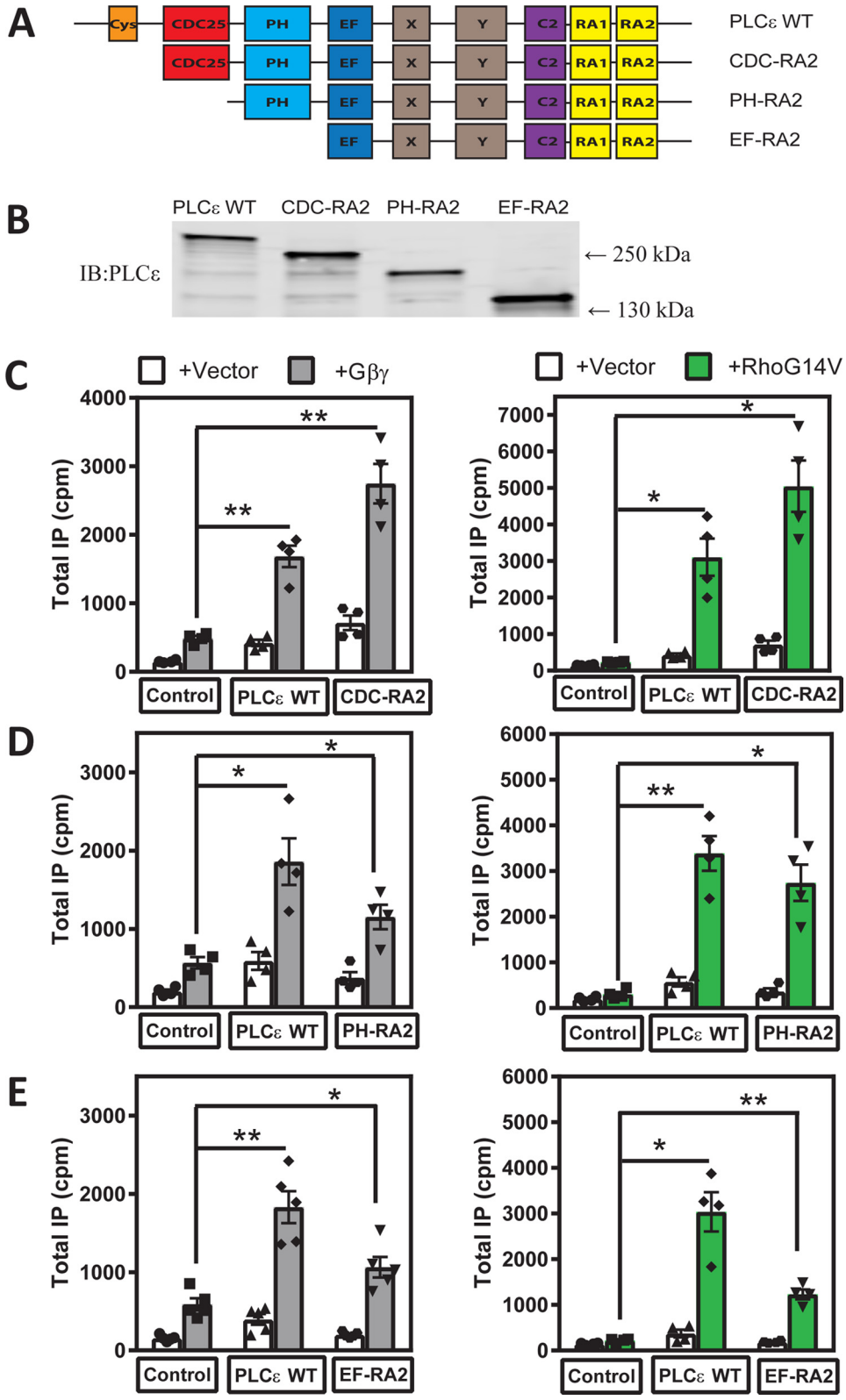

Figure 3. N-terminal domain deletions do not completely inhibit PLCε activation by Gβγ. *A*, schematic of PLCε WT, CDC25-RA2 (394–2281), PH-RA2 (837–2281), and EF-RA2 (1198–2281) constructs. *B*, a representative Western blot showing expression of the PLCε constructs. *C*, COS-7 cells were transfected with PLCε WT or CDC-RA2 (300 ng) in the presence or absence of 200 ng $G\beta_1$ and 200 ng $G\gamma_2$ or constitutively active Rho (RhoG14V) (200 ng) and total [^{3}H]inositol phosphate accumulation was measured. *D*, COS-7 cells were transfected with 300 ng WT PLCε or PH-RA2 in the presence or absence of 200 ng $G\beta_1$ and 200 ng $G\gamma_2$ or 200 ng RhoG14V and total [^{3}H]inositol phosphate accumulation was measured. *E*, COS-7 cells were transfected with PLCε WT or EF-RA2 (300 ng) in the presence or absence of 200 ng $G\beta_1$ and 200 ng $G\gamma_2$ or 200 ng RhoG14V and total [^{3}H]inositol phosphate accumulation was measured. The data shown are mean ± S.E. for at least three independent experiments and analyzed by one-way ANOVA with Dunnett's post test. **, $p < 0.01$; *, $p < 0.05$ *versus* Gβγ.

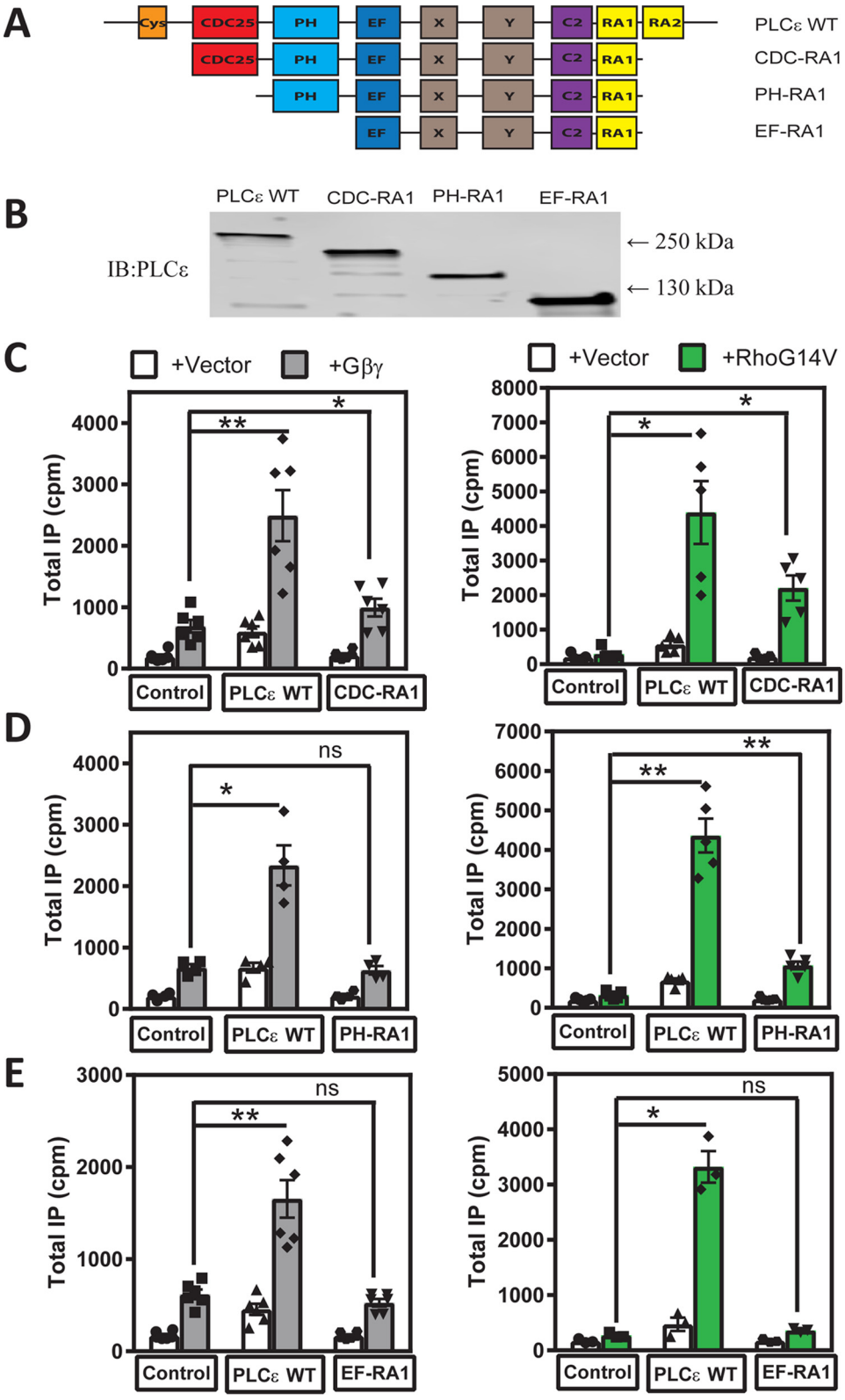

Figure 4. Simultaneous deletion of the N-terminal and the RA2 domains of PLCϵ blocks activation by Gβγ. *A*, schematic of PLCϵ WT, CDC25-RA1 (394–2113), PH-RA1 (837–2113), and EF-RA1 (1198–2113) constructs. *B*, a representative Western blot showing relative expression of the PLCϵ constructs. *C*, COS-7 cells were transfected with 300 ng PLCϵ WT or CDC25-RA1 in the presence or absence of 200 ng $G\beta_1$ and 200 ng $G\gamma_2$ or 200 ng RhoG14V and total [^{3}H]inositol phosphate accumulation was measured. *D*, COS-7 cells were transfected with 300 ng PLCϵ WT or PH-RA1 in the presence or absence of 200 ng $G\beta_1$ and 200 ng $G\gamma_2$ or 200 ng RhoG14V and total [^{3}H]inositol phosphate accumulation was measured. *E*, COS-7 cells were transfected with 300 ng PLCϵ WT or EF-RA1 in the presence or absence of 200 ng $G\beta_1$ and 200 ng $G\gamma_2$ or 200 ng RhoG14V and total [^{3}H]inositol phosphate accumulation was measured. The data shown are mean ± S.E. for at least three independent experiments and analyzed by one-way ANOVA with Dunnett's post test. **, $p < 0.01$; *, $p < 0.05$ *versus* Gβγ; *ns*, not significant.

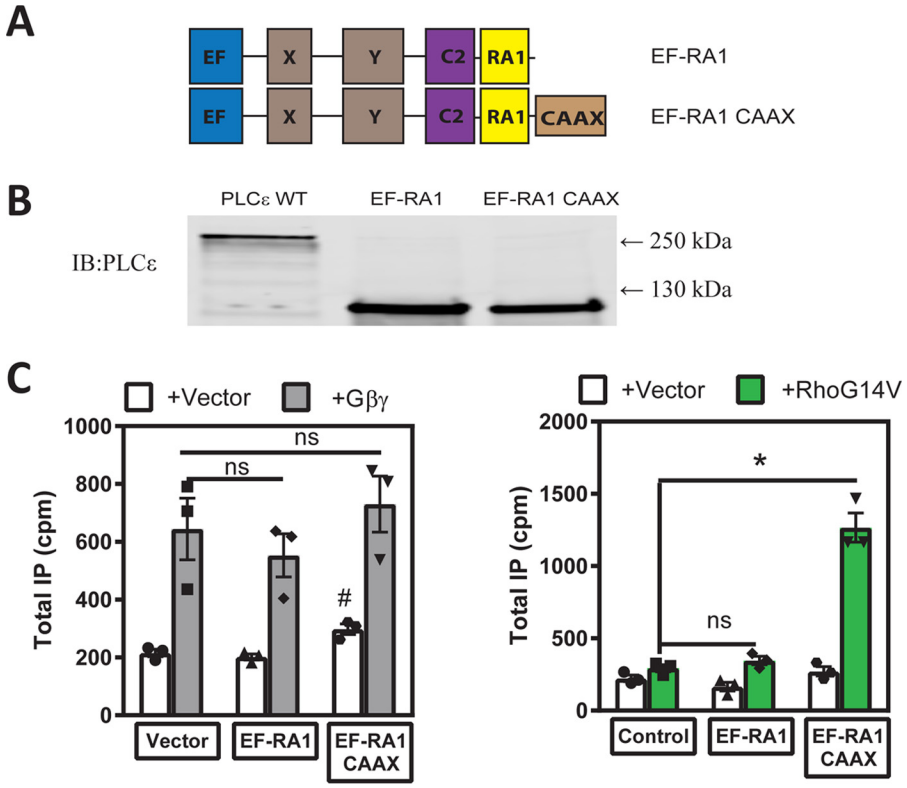

Figure 5. Constitutive membrane localization of PLCε EF-RA1 rescues activation by Rho but not Gβγ. *A*, schematic of EF-RA1 (1198–2113) and EF-RA1 CAA*X* (contains CAA*X* tag from Kras4B) constructs. *B*, a representative Western blot showing expression of the PLCε constructs. *C*, COS-7 cells were transfected with 300 ng EF-RA1 or EF-RA1 CAA*X* in the presence or absence of 200 ng $G\beta_1$ and 200 ng $G\gamma_2$ or 200 ng RhoG14V and total [^{3}H]inositol phosphate accumulation was measured. The data shown are mean ± S.E. for at least three independent experiments and analyzed by one-way ANOVA with Dunnett's post test. **, $p < 0.01$; *, $p < 0.05$ *versus* Gβγ; #, $p < 0.05$ verses EF-RA1; ns; not significant.

co-transfection with Gβγ. In this case co-transfection with RhoG14V also did not result in accumulation of IP above levels of cells transfected with Gβγ or Rho alone (Fig. 4*E*). These data together suggest that PLCε activation by Gβγ requires both the N (cysteine-rich and CDC25 domains) and C termini (RA2 domain) for full activation of PLCε.

Constitutive membrane localization of PLCε-EF-RA1 rescues Rho-dependent activation, but not Gβγ-dependent activation

Confounding interpretation of these experiments is the reduction in basal activity associated with many of the domain deletion mutations. In particular, the EF-RA1 construct shows little basal activity and no activation by either Rho or Gβγ. We hypothesized that this could be because of a loss in membrane association of the enzyme. To test this, we designed a EF-RA1 variant with a C-terminal CAA*X* box to allow for C-terminal prenylation and constitutive membrane localization as shown in previous studies (21) (Fig. 5*A*). Western blot analysis of the mutants showed similar levels of expression of EF-RA1 and EF-RA1 CAA*X*, which were slightly increased relative to PLCε WT expression (Fig. 5*B*). In contrast to EF-RA1 transfected cells, EF-RA1 CAA*X* transfected cells showed elevated basal activity above empty vector transfected cells, indicating that this construct was indeed active when localized to the plasma membrane (Fig. 5*C*). Interestingly, the EF-RA1 CAA*X* mutant rescued activation by Rho but not Gβγ (Fig. 5*C*). The data indicate that a PLCε mutant lacking the cysteine-rich, CDC25, and RA2 domains can be activated by Rho when targeted to the membrane, but cannot be stimulated by Gβγ. These data strongly support these domains as being required for Gβγ activation.

PLCε directly interacts with Gβγ and increases PIP_2 hydrolysis

To test whether Gβγ directly interacts with PLCε, PLCε truncations lacking the cysteine-rich and CDC25 domains (PH-RA2), or the cysteine-rich, CDC25, and RA2 domains (PH-RA1) were expressed and purified (Figs. 6*A* and 7*A*). To test for direct binding of Gβγ to PLCε, purified biotinylated Gβγ (bGβγ) was mixed with purified PLCε-PH-RA2 and precipitated with neutravidin beads. PLCε-PH-RA2 was readily detected associated with beads bound to bGβγ, but not beads alone (Fig. 6*B*). The two bands observed are likely the result of proteolysis that occurs during the incubation. To test the specificity of Gβγ interaction with PLCε, purified PLCε-PH-RA2 and bGβγ were incubated with or without $G\alpha_{i1}$-GDP to sequester free Gβγ. $G\alpha_{i1}$-GDP strongly inhibited the pulldown of PLCε-PH-RA2 by bGβγ (Fig. 6, *B* and *C*), demonstrating a direct and specific interaction between PLCε and Gβγ.

Unlike the PLCβ isoforms, reconstitution of PLCε activation with purified components has been difficult to demonstrate, and generally the amount of PLCε activation observed upon incubation with purified upstream activators such as Rho and Ras is minimal (17, 22). Nevertheless, to test the specificity of Gβγ activation of PLCε, purified PLCε-PH-RA2 and Gβγ were incubated with or without $G\alpha_{i1}$-GDP in reconstituted

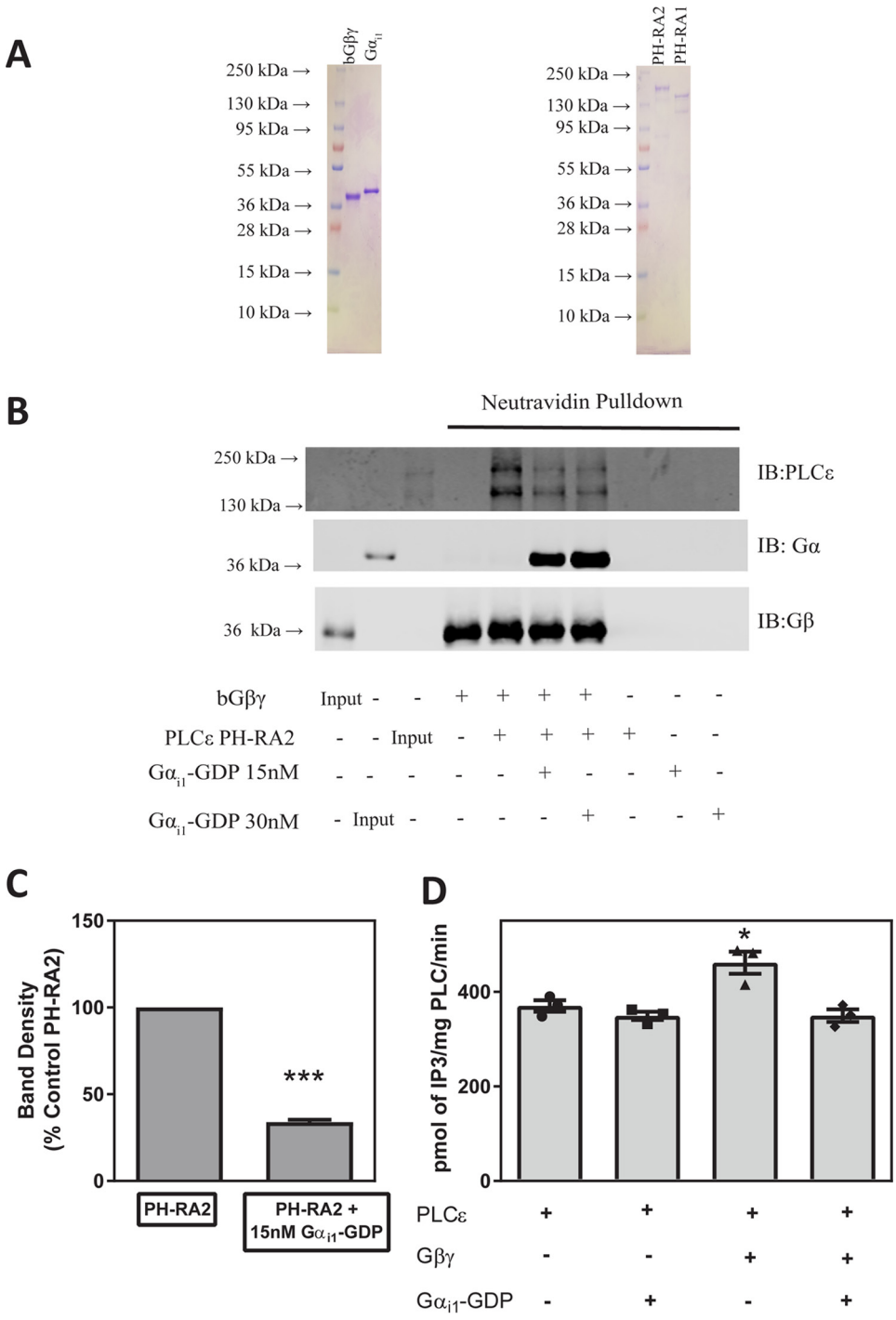

Figure 6. PLCε-PH-RA2 directly binds to Gβγ. *A*, Coomassie Blue–stained polyacrylamide gel showing purified proteins. *B*, PLCε-PH-RA2 binding to Gβγ. Biotinylated $G\beta_1\gamma_2$ was used to pull down PLCε mutants in the presence of neutravidin beads. Representative blot showing direct pulldown of PLCε-PH-RA2 with $G\beta_1\gamma_2$ and inhibition of this interaction by $G\alpha_{i1}$-GDP. 50 ng of purified PLCε-PH-RA2 was mixed with purified 15 nM biotinylated $G\beta_1\gamma_2$ in the presence or absence of $G\alpha_{i1}$-GDP for 2 h. 15 μl 50% magnetic neutravidin bead slurry was added and the mix was incubated for another 2 h. Pellet was washed thrice and subjected to gel electrophoresis and Western blotting. Input is 1/20 of supernatant. *C*, quantitation of the PLCε band densities from three experiments as in *B* (PH-RA2 binding was normalized to 100%). The data shown are mean ± S.E. and analyzed by Student's *t* test. ***, $p < 0.001$ *versus* PH-RA2 binding in the absence of $G\alpha_{i1}$-GDP. *D*, 300 nM $G\beta_1\gamma_2$ alone or with 600 nM $G\alpha_{i1}$-GDP was incubated with 10 ng PLCε PH-RA2. The effect on PLCε mutant activity is shown. Values are the mean ± S.E. of triplicate determinations and representative of three independent experiments and analyzed by one-way ANOVA with Dunnett's post test.

lipid vesicles containing inositol PIP_2, and the amount of $[^3H]IP_3$ produced was measured. Fig. 6*D* shows a small but statistically significant increase in PIP_2 hydrolysis by PLCε-PH-RA2 when Gβγ is added. This activation was inhibited by $G\alpha_{i1}$-GDP confirming that this increase is through direct interactions of PLCε with Gβγ. This is also the first direct demonstration of Gβγ-dependent activation of PLCε using reconstituted proteins.

Our data from the COS-7 cell co-transfection experiments indicate that PLCε activation by Gβγ requires both the N ter-

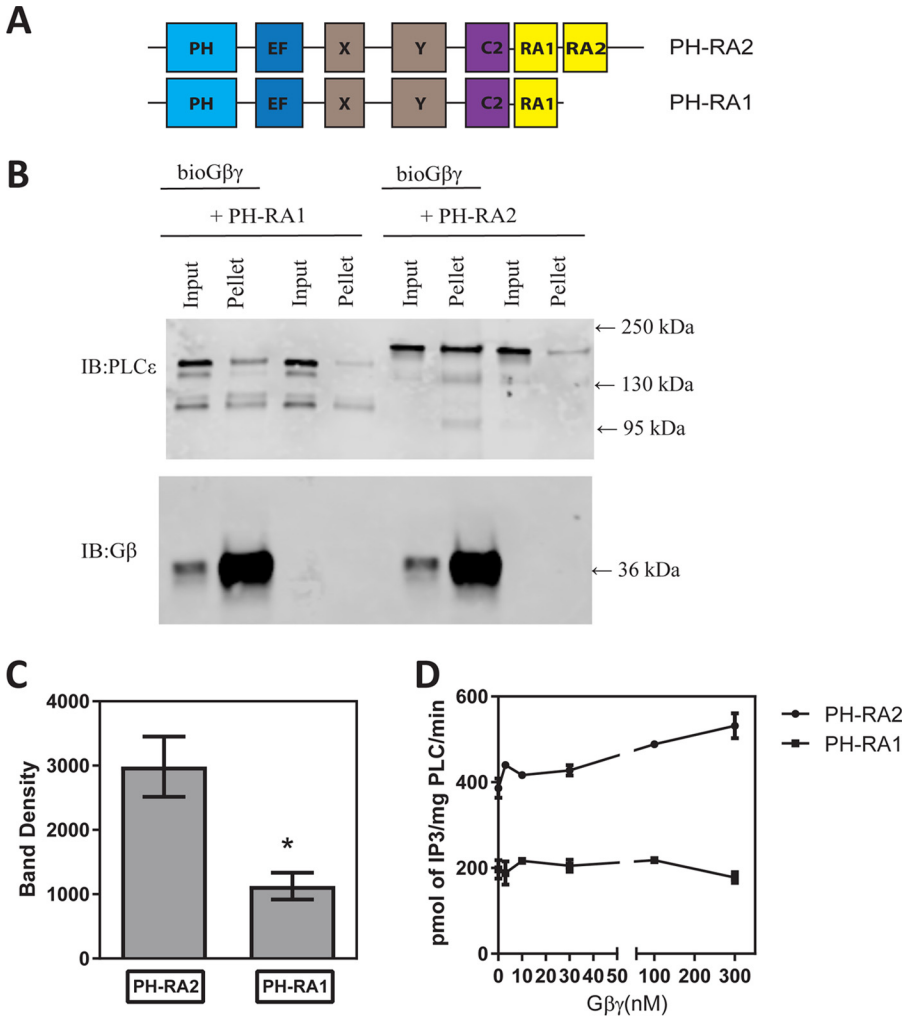

Figure 7. Deletion of the PLCε RA2 domain inhibits binding and activation by Gβγ. *A*, schematic of PLCε RA domain mutants. *B*, representative Western blot showing direct pulldown of PLCε-PH-RA2 but not PH-RA1 by biotinylated $G\beta_1\gamma_2$. Input is 1/10 of supernatant. *C*, PLCε band densities are quantitated from three independent experiments as in *B*. The data shown are mean ± S.E. *, $p < 0.05$ *versus* PH-RA2 and analyzed by Student's *t* test. *D*, representative assay of PLC enzymatic activity for the indicated purified PLCε protein fragments reconstituted with the indicated concentrations of purified Gβγ subunits performed in triplicate, repeated three times with similar results. The data shown are mean ± S.E. and analyzed by one-way ANOVA with Dunnett's post test. *, $p < 0.05$ *versus* basal PLCε activity.

minus (cysteine-rich and CDC25 domains) and C terminus (RA2 domain) that are potentially compensatory in the absence of one or the other. Therefore, we hypothesized that if PLCε is directly regulated by Gβγ binding to the RA2 domain, Gβγ will bind to PLCε-PH-RA2 but not PH-RA1. To test this, purified bGβγ and N- and C-terminally truncated PLCε mutants (PH-RA2 or PH-RA1) were incubated and then precipitated with neutravidin beads. As shown in Fig. 7, *B* and *C*, PLCε-PH-RA2 was strongly pulled down with bGβγ bound to beads. In contrast PLCε-PH-RA1 was only weakly pulled down with bGβγ.

We next tested the ability of purified Gβγ to activate purified PLCε-PH-RA2 or PH-RA1 in reconstituted lipid vesicles containing $[^3H]PIP_2$. PH-RA1 had significantly less basal activity than PH-RA2 (Fig. 7*D*). Gβγ also activated PLCε-PH-RA2, but not PH-RA1, in a concentration-dependent manner (Fig. 7*D*) consistent with the decreased binding of Gβγ to PH-RA1 (Fig. 7*B*) and the lack of activation of this construct in COS-7 cells (Fig. 4*D*). Overall, these results suggest that the activation of PLCε involves a direct interaction with Gβγ that requires the C terminus RA2 domain and the N-terminal cysteine-rich and CDC25 domains.

Discussion

In our previous work we showed that inhibiting Gβγ with the C terminus of G protein–coupled receptor kinase 2 (GRK2ct), or with gallein, inhibited PLCε-dependent PI4P hydrolysis in cardiac myocytes (18). Activation of PLCε can be mediated by small GTPases and/or Gβγ (12, 13, 16, 17). To date, direct regulation of PLCε by Gβγ has not been reported. If indeed free Gβγ subunits activate PLCε, $G\alpha_{i1}$-GDP should form an inactive heterotrimer with Gβγ, preventing PLCε activation. We confirmed that co-transfection of cells with cDNAs encoding Gβγ, PLCε, and $G\alpha_{i1}$ completely prevented activation of PLCε mediated by free Gβγ. Our results also demonstrate that Gβγ directly interacts with PLCε to increase PIP_2 hydrolysis. In addition, the results from domain deletion analysis of PLCε show an unexpected involvement of the RA2 and CDC25 domains in the activation of PLCε by Gβγ.

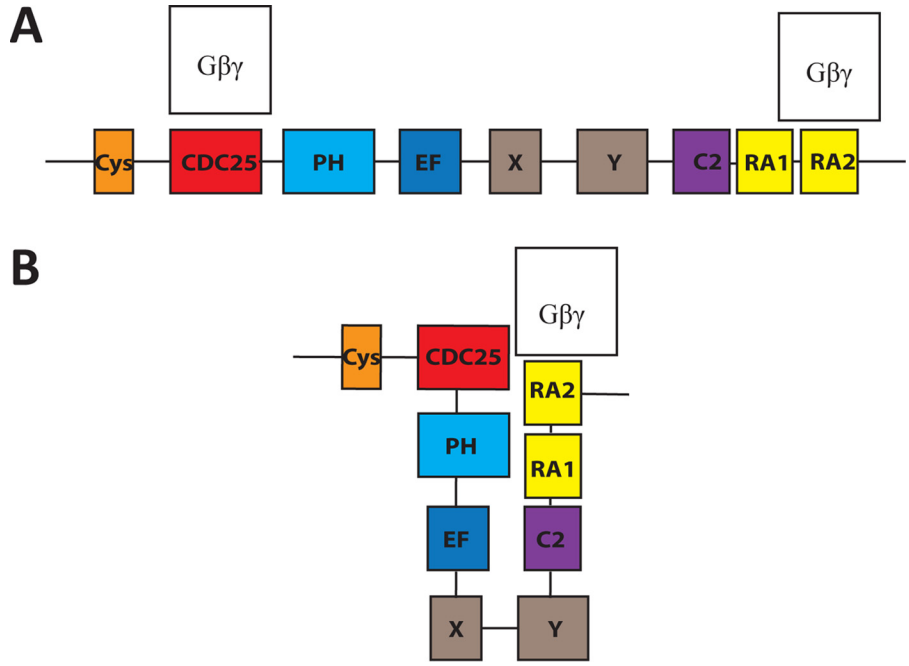

Figure 8. Model of PLCε activation by Gβγ. *A*, in the first scenario, free Gβγ interacts with and activates PLCε by binding to the N-terminal CDC25 domain and the C-terminal RA2 domain at two independent sites. Interaction with both sites is required for full activation. *B*, alternatively, the CDC25 and RA2 domains could be in close proximity to one another in the three-dimensional structure of PLCε, forming a single binding site for Gβγ.

Regulation of PLCε by Ras is through direct binding to the RA2 domain (12). The regulation of PLCε by Gβγ is not understood. It was previously reported that PLCε lacking the N-terminal domains retained activation by Gβγ subunits, Ras, and Rho, suggesting activation was not dependent upon the PLCε N-terminal domains (17). In addition, it was also reported that PLCε-CDC25-C2 can be stimulated by Gβγ subunits, but not by Ras, suggesting that Gβγ activation was independent of the RA2 domain (17). Our results generally support these findings.

The most compelling data indicating that Gβγ may be interacting with both the CDC25 domain and the RA2 domain is that simultaneous deletion of the N-terminal cysteine-rich and CDC25 domains and the RA2 C-terminal domain resulted in complete loss of activation by free Gβγ, but not Rho (Fig. 4*D*). Further evidence that the CDC25 and RA2 domains interact with Gβγ is that simultaneous deletion of the N-terminal cysteine-rich, CDC25, and PH domains and the RA2 C-terminal domain led to loss of PLCε activation. Addition of a CAA*X* sequence to target this mutant to the membrane rescued Rho activation but not Gβγ activation (Figs. 4 and 5). Because deletion of the cysteine-rich domain alone has no effect on Gβγ regulation, these data support a role for both the N-terminal CDC25 domain and the C-terminal RA2 domain in Gβγ-dependent activation of PLCε.

The inability of Gβγ to activate the PLCε-PH-RA1 mutant in COS-7 cells implies that this mutant lacks a binding site for Gβγ. The purified PLCε-PH-RA1 protein had enzymatic activity but was not activated by Gβγ and only weakly bound to Gβγ in pulldown assays for protein binding supporting the COS-7 cell data. Purified PLCε-PH-RA2, which retains one of the two Gβγ-binding sites (RA2) implicated in the transfection experiments, bound Gβγ more strongly than PLCε-PH-RA1, and was activated by Gβγ, implicating the RA2 domain as a binding site for Gβγ. These results demonstrate for the first time that PLCε is directly stimulated by G protein βγ subunits and a requirement for the RA2 domain. The activation of PLCε-PH-RA2 by Gβγ *in vitro* may be weak because it is missing the important CDC25 domain–binding site, or because as has been raised previously, PLCε activation is notoriously difficult to activate *in vitro*. We have been unable to purify full-length PLCε to discriminate between these possibilities.

In summary we have demonstrated that PLCε is directly regulated by Gβγ through binding to the RA2 domain with additional contributions from the CDC25 domain. Potential mechanisms for regulation of PLCε by Gβγ are shown in Fig. 8. In the first scenario, free Gβγ interacts with and activates PLCε by binding to the N-terminal CDC25 domain and to the C-terminal RA2 domain through two independent sites, and interaction with both is required for full activation. An alternative model is that, in the three-dimensional structure of PLCε, the RA2 and CDC25 domains are in close proximity and form a single Gβγ-binding site.

Our previous observation using a neonatal rat ventricular myocyte (NRVM) model of cardiac hypertrophy showed that PLCε-mediated PI4P hydrolysis at the Golgi is Gβγ-dependent (18). Furthermore, PLCε is also implicated in the development of cardiac hypertrophy and has been shown to localize to a subcellular compartment in neonatal rat ventricular myocytes (23). Our studies indicate that Gβγ activation of PLCε is direct and likely occurs at the Golgi apparatus in cardiac myocytes. These studies extend our knowledge of the regulation of PLCε by Gβγ and could result in novel strategies for targeting PLCε regulation in hypertrophy, heart failure, and other diseases linked to PLCε activation.

Experimental procedures

Materials and plasmids

Full-length WT rat PLCε pCMV-scriptPLCε-FLAG (a kind gift of Dr. Grant Kelley) was used in most experiments. AVI-$G\beta_1$, $G\gamma_2$, and enhanced YFP (EYFP) were in pCI-neo vector. HA-tagged HRasG12V was in pDCR. RhoG14V was in 3× HA pcDNA3.1+ vector. $G\alpha_{i1}$ was in pcDNA3.1+. Rabbit anti-PLCε (2163) was used as described previously (24).

Construction of PLCε domain deletions and mutations

Variations of the WT rat PLCε pCMV-scriptPLCε-FLAG were generated using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) and manufacturer's protocol followed. The following constructs were verified by sequencing: pCMV-scriptPLCε Lys-Glu–FLAG (lysine residues 2150 and 2152 in the RA2 domain changed to glutamate), pCMV-scriptPLCε-CDC-RA2-FLAG (Δ2–393), pCMV-scriptPLCε-PH-RA2-FLAG (Δ2–836), pCMV-scriptPLCε-EF-RA2-FLAG (Δ2–1197), pCMV-scriptPLCε-CDC25-RA1-FLAG (394–2113), pCMV-scriptPLCε-PH-RA1-FLAG (837–2113), pCMV-scriptPLCε-EF-RA1-FLAG (1198–2113). pCMV-scriptPLCε-EF-RA1-CAA*X* (C-terminal amino acids 175–188 of KRAS4B (KKKKKKSKTKCVIM) was made using a modified Quik Change Lightning Site-Directed Mutagenesis protocol. Protein expression was confirmed by Western blotting using anti-PLCε 2163 antibody.

The cDNAs PLCε-PH-RA2 (837–2281) and PH-RA1 (837–2098) variants used for *in vitro* assays were subcloned into pFastBac HTA to yield N-terminal His-tagged protein. Baculoviruses were generated using a recombinant baculovirus system (Invitrogen/Thermo Fisher Scientific) Sf9 (*Spodoptera frugiperda*) insect cells (Invitrogen or Expression Systems) were used for virus production and protein expression. Cells were infected with baculovirus at a multiplicity of infection of 1, harvested at 48–72 h post infection, and flash frozen in liquid nitrogen.

Transfection of COS-7 cells and quantitation of phospholipase C activity

All cell culture reagents were obtained from Invitrogen. COS-7 cells were obtained from ATCC. COS-7 cells were seeded in 12-well culture dishes at a density of 2×10^5cells per well and maintained in high-glucose Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C. The following day, indicated vectors were transfected using Lipofectamine 2000 (Invitrogen) transfection reagent (2 μl Lipofectamine per 1 μg of DNA). Total DNA varied from 700 to 900 ng per well and included EYFP control vector as necessary to maintain equal amount of DNA per well within individual experiments. Approximately 24 h after transfection, the culture medium was changed to low-inositol Ham's F-10 medium (Gibco) containing 1.5 μCi/well Myo-[2-^{3}H(N)]inositol (Perkin Elmer) for 12–16 h. Accumulation of [^{3}H]inositol phosphate was quantitated after the addition of 10 mM LiCl for 1 h to inhibit inositol monophosphate phosphatases. Media were aspirated and cells washed with 1× PBS, followed by the addition of ice-cold 50 mM formic acid to lyse cells. Soluble cell lysates containing [^{3}H]inositol phosphate were transferred onto Dowex AGX8 chromatography columns to separate total IP by anion exchange chromatography. Columns were washed with 50 mM and eluted with 100 mM formic acid into scintillation vials containing scintillation fluid and counted.

Purification of free biotinylated Gβγ

Purification of *in vivo* biotinylated Gβγ (bGβγ) was performed by co-expressing Gβγ with His_6 $G\alpha_{i1}$ in High Five insect cells and nickel-agarose chromatography as described previously (25, 26).

Purification of His_6 PLCε-PH-RA2 and PH-RA1

Baculovirus-infected Sf9 cells expressing PLCε-PH-RA2 or PH-RA1 were lysed by Dounce on ice in lysis buffer containing 20 mM HEPES, pH 8, 50 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mM EDTA, 0.1 M EGTA, and two Roche EDTA-free protease inhibitor tablets at one-third strength. The lysate was centrifuged at 100,000 × *g*, and the supernatant was loaded onto a Ni-NTA column pre-equilibrated with buffer A (20 mM HEPES, pH 8, 100 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mM EDTA, and 0.1 M EGTA). The column was washed with 3 column volumes (CVs) of buffer A, followed by 3 CVs of buffer A supplemented with 300 mM NaCl and 10 mM imidazole. The protein was eluted from the column with 3–10 CVs of buffer A supplemented with 200 mM imidazole. Proteins were concentrated and loaded onto tandem Superdex 200 Increase columns (10/300 GL; GE Healthcare) equilibrated with SEC buffer (20 mM HEPES, pH 8, 200 mM NaCl, 2 mM DTT, 0.1 mM EDTA, and 0.1 M EGTA). Fractions of interest were confirmed by SDS-PAGE, pooled, concentrated, and flash frozen in liquid nitrogen.

Free biotinylated Gβγ pulldown assay

Biotinylated Gβγ pulldown assay was performed as described previously (27), with minor modifications. Briefly, 50 ng purified PLCε-PH-RA2 or PH-RA1 was mixed with purified 15 nM bGβγ in the presence or absence of $G\alpha_{i1}$-GDP for 2 h at 4 °C in a rotator. 15 μl 50% magnetic neutravidin bead (GE Healthcare) slurry was added to the mix and it was further incubated for 2 h. Pellet was washed thrice and subjected to gel electrophoresis and Western blot analysis.

Phospholipase C activity assay

The assay was performed as described previously (28), with minor modification. Briefly, lipids containing ~5000 cpm of [^{3}H-inositol]PIP_2, 37.5 μM PIP_2, and 150 μM phosphatidylethanolamine were mixed with 10 ng PLCε. Varying concentrations of Gβγ (with or without preincubation with $G_{\alpha i1}$-GDP (1:2 ratio)) were added to the PLCε and the lipid mixture. The reaction was set at 30 °C for 30 min and quenched using 5% BSA and 10% TCA. Released soluble [^{3}H]IP_3 was measured by liquid scintillation counting.

Statistical analysis

Data were analyzed using Prism 7 (GraphPad Software). Mean values and standard errors were calculated, and the statistical significance established using either Student's *t* test or

one-way analysis of variance (ANOVA), as appropriate. All experiments were repeated at least three times.

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References

1. Berridge, M. J. (1984) Inositol triphosphate and diacylglycerol as second messengers. *Biochem. J.* **220,** 345–360 CrossRef Medline
2. Harden, T. K., Waldo, G. L., Hicks, S. N., and Sondek, J. (2011) Mechanism of Activation and inactivation of Gq/phospholipase C-β signaling nodes. *Chem. Rev.* **111,** 6120–6129 CrossRef Medline
3. Kadamur, G., and Ross, E. M. (2013) Mammalian phospholipase C. *Annu. Rev. Physiol.* **75,** 127–154 CrossRef Medline
4. Singer, W. D., Brown, H. A., and Sternweis, P. C. (1997) Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. *Ann. Rev. Biochem.* **66,** 475–509 CrossRef Medline
5. Smrcka, A. V., Brown, J. H., and Holz, G. G. (2012) Role of phospholipase Cε in physiological phosphoinositide signaling networks. *Cell. Signal.* **24,** 1333–1343 CrossRef Medline
6. Smrcka, A. V., Hepler, J. R., Brown, K. O., and Sternweis, P. C. (1991) Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. *Science* **251,** 804–807 CrossRef Medline
7. Smrcka, A. V., and Sternweis, P. C. (1993) Regulation of purified subtypes of phosphatidylinositol specific phospholipase Cβ by G protein α and βγ subunits. *J. Biol. Chem.* **268,** 9667–9674 Medline
8. Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P. J., and Gierschik, P. (1992) Isozyme-selective stimulation of phospholipase Cβ2 by G protein βγ-subunits. *Nature* **360,** 684–686 CrossRef Medline
9. Boyer, J. L., Waldo, G. L., and Harden, T. K. (1992) βγ-Subunit activation of G-protein-regulated phospholipase C. *J. Biol. Chem.* **267,** 25451–25456 Medline
10. Wu, D. Q., Lee, C. H., Rhee, S. G., and Simon, M. I. (1992) Activation of phospholipase C by the α subunits of the Gq and G11 proteins in transfected Cos-7 cells. *J. Biol. Chem.* **267,** 1811–1817 Medline
11. Taylor, S. J., Chae, H. Z., Rhee, S. G., and Exton, J. H. (1991) Activation of the β1 isozyme of phospholipase C by α subunits of the Gq class of G proteins. *Nature* **350,** 516–518 CrossRef Medline
12. Kelley, G. G., Reks, S. E., Ondrako, J. M., and Smrcka, A. V. (2001) Phospholipase Cε: a novel Ras effector. *EMBO J.* **20,** 743–754 CrossRef Medline
13. Kelley, G. G., Reks, S. E., and Smrcka, A. V. (2004) Hormonal regulation of phospholipase Cε through distinct and overlapping pathways involving G12 and ras family proteins. *Biochem. J.* **378,** 129–139 CrossRef Medline
14. Song, C., Hu, C. D., Masago, M., Kariyai, K., Yamawaki-Kataoka, Y., Shibatohge, M., Wu, D., Satoh, T., and Kataoka, T. (2001) Regulation of a novel human phospholipase C, PLCε, through membrane targeting by Ras. *J. Biol. Chem.* **276,** 2752–2757 CrossRef Medline
15. Song, C., Satoh, T., Edamatsu, H., Wu, D., Tadano, M., Gao, X., and Kataoka, T. (2002) Differential roles of Ras and Rap1 in growth factor-dependent activation of phospholipase Cε. *Oncogene* **21,** 8105–8113 CrossRef Medline
16. Wing, M. R., Houston, D., Kelley, G. G., Der, C. J., Siderovski, D. P., and Harden, T. K. (2001) Activation of phospholipase C-ε by heterotrimeric G protein βγ-subunits. *J. Biol. Chem.* **276,** 48257–48261 CrossRef Medline
17. Wing, M. R., Snyder, J. T., Sondek, J., and Harden, T. K. (2003) Direct activation of phospholipase C-ε by Rho. *J. Biol. Chem.* **278,** 41253–41258 CrossRef Medline
18. Malik, S., deRubio, R. G., Trembley, M., Irannejad, R., Wedegaertner, P. B., and Smrcka, A. V. (2015) G protein βγ subunits regulate cardiomyocyte hypertrophy through a perinuclear Golgi phosphatidylinositol 4-phosphate hydrolysis pathway. *Mol. Biol. Cell* **26,** 1188–1198 CrossRef Medline
19. Wu, D., Katz, A., and Simon, M. I. (1993) Activation of phospholipase Cβ2 by the α and βγ subunits of trimeric GTP-binding protein. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 5297–5301 CrossRef Medline
20. Crespo, P., Xu, N., Simonds, W. F., and Gutkind, J. S. (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein βγ subunits. *Nature* **369,** 418–420 CrossRef Medline
21. Bunney, T. D., Harris, R., Gandarillas N. L., Josephs, M. B., Roe, S. M., Sorli, S. C., Paterson, H. F., Rodrigues-Lima, F., Esposito, D., Ponting, C. P., Gierschik, P., Pearl, L. H., Driscoll, P. C., and Katan, M. (2006) Structural and mechanistic insights into Ras association domains of phospholipase Cε. *Mol. Cell* **21,** 495–507 CrossRef Medline
22. Gandarillas, N. L., Bunney, T. D., Josephs, M. B., Gierschik, P., and Katan, M. (2009) *In vitro* reconstitution of activation of PLCε by Ras and Rho GTPases. *Methods Mol. Biol.* **462,** 379–389 Medline
23. Zhang, L., Malik, S., Pang, J., Wang, H., Park, K., Yule, D., Blaxall, B. C., and Smrcka, A. V. (2013) Phospholipase Cε hydrolyzes perinuclear phosphatidylinositol 4-phosphate to regulate cardiac hypertrophy. *Cell* **153,** 216–227 CrossRef Medline
24. Wang, H., Oestreich, E. A., Maekawa, N., Bullard, T. A., Vikstrom, K. L., Dirksen, R. T., Kelley, G. G., Blaxall, B. C., and Smrcka, A. V. (2005) Phospholipase C ε modulates β-adrenergic receptor–dependent cardiac contraction and inhibits cardiac hypertrophy. *Circ. Res.* **97,** 1305–1313 CrossRef Medline
25. Kozasa, T., and Gilman, A. G. (1995) Purification of recombinant G proteins from Sf9 cells by hexahistidine tagging of associated subunits. Characterization of α12 and inhibition of adenylyl cyclase by αz. *J. Biol. Chem.* **270,** 1734–1741 CrossRef Medline
26. Davis, T. L., Bonacci, T. M., Sprang, S. R., and Smrcka, A. V. (2005) Structural definition of a preferred protein interaction site in the G protein $\beta_1\gamma_2$ heterodimer. *Biochemistry* **44,** 10593–10604 CrossRef Medline
27. Lehmann, D. M., Yuan, C., and Smrcka, A. V. (2007) Analysis and pharmacological targeting of phospholipase C β interactions with G proteins. In *Lipidomics and Bioactive Lipids: Lipids and Cell Signaling* (H. Alex Brown, ed.), Methods in Enzymology, Vol. 434, pp. 29–48, Academic Press, San Diego, CA
28. Bonacci, T. M., Ghosh, M., Malik, S., and Smrcka, A. V. (2005) Regulatory interactions between the amino terminus of G-protein βγ subunits and the catalytic domain of PLCβ2. *J. Biol. Chem.* **280,** 10174–10181 CrossRef Medline