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Differential Regulation of Phospholipase C-β_2 Activity and Membrane Interaction by Gα_q, G$\beta_1\gamma_2$, and Rac2*

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We combined fluorescence recovery after photobleaching (FRAP) beam-size analysis with biochemical assays to investigate the mechanisms of membrane recruitment and activation of phospholipase C-β_2 (PLCβ_2) by G protein α_q and $\beta\gamma$ dimers. We show that activation by α_q and $\beta\gamma$ differ from activation by Rac2 and from each other. Stimulation by α_q enhanced the plasma membrane association of PLCβ_2, but not of PLC$\beta_2\Delta$, which lacks the α_q-interacting region. Although α_q resembled Rac2 in increasing the contribution of exchange to the FRAP of PLCβ_2 and in enhancing its membrane association, the latter effect was weaker than with Rac2. Moreover, the membrane recruitment of PLCβ_2 by α_q occurred by enhancing PLCβ_2 association with fast-diffusing (lipid-like) membrane components, whereas stimulation by Rac2 led to interactions with slow diffusing membrane sites. On the other hand, activation by $\beta\gamma$ shifted the FRAP of PLCβ_2 and PLC$\beta_2\Delta$ to pure lateral diffusion 3- to 5-fold faster than lipids, suggesting surfing-like diffusion along the membrane. We propose that these different modes of PLCβ_2 membrane recruitment may accommodate contrasting functional needs to hydrolyze phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) in localized *versus* dispersed populations. PLCβ_2 activation by Rac2, which leads to slow lateral diffusion and much faster exchange, recruits PLCβ_2 to act locally on PtdInsP_2 at specific domains. Activation by α_q leads to lipid-like diffusion of PLCβ_2 accompanied by exchange, enabling the sampling of larger, yet limited, areas prior to dissociation. Finally, activation by $\beta\gamma$ recruits PLCβ_2 to the membrane by transient interactions, leading to fast "surfing" diffusion along the membrane, sampling large regions for dispersed PtdInsP_2 populations.

Phospholipase C-β (PLCβ)[4] isozymes hydrolyze phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) to produce inositol 1,4,5-trisphosphate and diacylglycerol (1–3). They are activated to different extents by heterotrimeric G protein α_q subunits (α_q) and $\beta\gamma$ dimers ($\beta\gamma$) (1–3). PLCβ_2 is also activated by the Rho GTPases Rac and Cdc42 (4–7). Activation by Rac has also been demonstrated for PLCγ_2 (8). PLCβ_2 has long been known to be expressed in hematopoietic cells (2, 3) but is also encountered in a variety of other cell types and tissues, including smooth muscle cells (9) and several brain regions (10, 11). Moreover, PLCβ_2 was shown to be essential for taste perception via certain G protein-coupled oral taste receptors (12).

Activation of PLCβ_2 by α_q and related α subunits requires the C-terminal region of the enzyme; mutants with deletions in this region (*e.g.* PLC$\beta_2\Delta$, that lacks the Phe^{819}–Glu^{1166} segment) are resistant to stimulation by α_q, but undergo activation by $\beta\gamma$ and Rac/Cdc42 (7, 13–15). Recent results show that $\beta\gamma$ and Rac/Cdc42 activate PLCβ_2 by interacting, at least in part, with different regions of the effector enzyme. Thus, although the pleckstrin homology (PH) domain of PLCβ_2 is dispensable for activation of the enzyme by $\beta\gamma$ dimers (6), this domain also interacts with $\beta\gamma$, suggesting that the latter binds to at least two sites on the enzyme (16). In contrast, direct interaction of the PH domain with activated Rho GTPases is both necessary and sufficient for their stimulatory function (6, 17, 18). Functional evidence for a connection between PLCβ_2 and Rho GTPases in cells is provided by the chemoattractant receptor system, which activates Rac/Cdc42 and PLCβ_2 (19–22).

The mechanisms by which heterotrimeric G proteins and Rho GTPases regulate PLCβ isozymes are only partially understood (16, 23, 24), especially in live cells. Because both PLCβ substrate(s) and stimulators are membrane-bound (16, 23, 24), recruitment of PLCβ from the cytoplasm to the membrane is clearly required for effective enzyme activation. This view is consistent with the loss of PLCβ activation following mutations that interfere with the membrane association of α_q or $\beta\gamma$ (25, 26). PtdInsP_2, the substrate of PLCβ enzymes, is located at the internal plasma membrane leaflet in both dispersed and localized populations (27–33) and diffuses in the cytoplasmic leaflet of cell membranes with a lateral diffusion coefficient (D) of 0.5–1 $\mu m^2/s$ (32, 34). The PH domains of several proteins (*e.g.* PLCδ_1 and the *Dictyostelium discoideum* CRAC protein) were found to undergo dynamic membrane-cytoplasm exchange along with lateral diffusion over short distances (35, 36). Interestingly, we found a similar behavior for PLCβ_2 (7), although this protein is likely to interact with the membrane through several distinct sites; these include the catalytic triosephosphate isomerase barrel, the C-terminal region, and possibly the EF hands motif and the C2 domain, as well as its PH domain,

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[4] The abbreviations used are: PLCβ, phospholipase C-β; D, lateral diffusion coefficient; GFP, green fluorescent protein; PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; r, travel range; R_f, mobile fraction; τ, apparent characteristic fluorescence recovery time; wt, wild type; FRAP, fluorescence recovery after photobleaching; PH, pleckstrin homology.

which, unlike that of PLCδ, appears to be unable to bind phosphoinositides (16, 18).

We have formerly studied the interactions of green fluorescent protein (GFP)-tagged PLCβ_2 (PLCβ_2-GFP) and PLC$\beta_2\Delta$-GFP with the plasma membrane in live cells (7). Using FRAP beam-size analysis (37), which discriminates between recovery by lateral diffusion and exchange, we demonstrated that activation by Rac2 enhances the association of PLCβ_2 and PLC$\beta_2\Delta$ with the plasma membrane via binding to slow diffusing membrane proteins. However, the effects of the G protein subunits α_q and $\beta\gamma$ on the membrane interactions of PLCβ_2 and their potential relevance to PLCβ_2 stimulation were not explored. Here, we show that α_q and $\beta\gamma$ recruit PLCβ_2 to the plasma membrane by distinct mechanisms, which differ from the mechanism employed by Rac2 and from each other. Each stimulator leads to a specific ratio between the rates of exchange and lateral diffusion characterizing the interaction of PLCβ_2 with the membrane, and this in turn allows the enzyme to act preferentially on localized or dispersed PtdInsP$_2$ populations.

EXPERIMENTAL PROCEDURES

Materials and Plasmids—Murine anti-GFP antibodies were from Roche Applied Science, and peroxidase-conjugated goat anti-mouse IgG was from Sigma. The cDNAs of wild-type (wt) and G12V mutant human Rac2 (Rac2(wt) and Rac2(G12V), respectively), mouse α_q(wt) and α_q(R183C), human β_1, bovine γ_2(wt) and γ_2(C68S), and human PLCβ_2 were ligated into pcDNA3.1(+) or pcDNA3.1(−) (Invitrogen). Bovine and human γ_2 have identical amino acid sequences, and mouse α_q differs from human α_q in but one residue (S141A). There are several indications that this residue has no role in α_q function: (i) In mouse α_q, Ser^{141} is in a long loop connecting helices αE and αF of the helical domain (PDB accession codes 2BCJ and 2RGN); it does not reside in any of the three switch domains or in regions known to interact with PLCβ isozymes and is not among the residues that contact the bound guanine nucleotide and G$\beta\gamma$ (38, 39). (ii) Secondary structure prediction algorithms do not predict a structural difference between mouse and human α_q αE-loop-αF region (40, results not shown). (iii) Mouse α_q has been successfully reconstituted with many signaling proteins from several other species, including human, for functional and structural analyses (*e.g.* 41–43). The α_q(wt) and α_q(R183C) cDNAs were a gift from B. R. Conklin and H. R. Bourne (University of California, San Francisco, CA). The cDNAs of PLCβ_2 and the deletion mutant PLC$\beta_2\Delta$, which lacks a C-terminal region necessary for stimulation by α_q (Phe^{819}–Glu^{1166}), were inserted in-frame with the cDNA of GFP into the EcoRI/SalI site of pEGFP-N1 (Clontech) to generate the plasmids encoding PLCβ_2-GFP and PLC$\beta_2\Delta$-GFP (7). Plasmids (pcDNA3.1) encoding the N-terminal half (residues 1–155) of the venus fluorescent protein fused to the N terminus of human γ_2 (venus 1–155-γ_2) or venus residues 156–239 fused to the N terminus of human β_1 (venus 156–239-β_1) were described earlier (44) and donated by N. A. Lambert (Medical College of Georgia, Augusta, GA). A vector encoding the *Salmonella typhimurium* SigD protein cloned in pEGFP-N1 lacking the EGFP-encoding sequences (45), with SigD starting at residue 28 to increase stability, was a gift from B. Brett Finlay (University of British Columbia, Vancouver, Canada).

Cell Culture and Transfection—Cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum (7). For FRAP experiments, COS-7 cells grown on glass coverslips in 35-mm dishes for 24 h were transfected using DEAE-dextran (46) with 150 ng of plasmid DNA encoding one of the PLCβ_2-GFP derivatives together with 850 ng of empty vector or expression vectors encoding the various activating proteins: human β_1 along with bovine γ_2(wt) ($\beta_1\gamma_2$), β_1 plus the isoprenylation-defective γ_2(C68S) ($\beta_1\gamma_2$(C68S)), murine α_q(wt), constitutively active α_q(R183C), and human Rac2(wt) or Rac2(G12V). After 24 h, the cells were taken for the FRAP studies.

For studies of inositol phosphate formation, COS-7 cells (1.5×10^5/well) were seeded into 12-well plates. After 24 h, the cells were incubated with fresh medium (1 ml, 1 h), and co-transfected with vector encoding a GFP-tagged PLCβ_2 derivative (250 ng) together with 750 ng of vectors encoding the various activating proteins. Transfection was done with Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's instructions. At 48 h post-transfection, the cells were taken for experiments on inositol phosphate formation. For experiments on subcellular fractionation of PLCβ_2, COS-7 cells (2.5×10^6/10-cm dish) were co-transfected as above with PLCβ_2-GFP or PLC$\beta_2\Delta$-GFP (7 μg of DNA) together with 7 μg of vectors encoding one of the PLCβ_2 stimulators or empty vector.

FRAP—FRAP studies (47, 48) were conducted as described (7). The experiments were performed 24–26 h post-transfection on COS-7 cells transfected with PLCβ_2-GFP derivatives as described above. All experiments were conducted at 22 °C, in Hanks' balanced salt solution supplemented with 20 mM HEPES, pH 7.2. The monitoring argon ion laser beam (488 nm and 1.2 microwatts) was focused through the microscope (Zeiss Universal, Carl Zeiss MicroImaging) to a Gaussian spot with a radius $\omega = 0.85 \pm 0.02$ μm (63×/1.4 numerical aperture (NA) oil-immersion objective) or 1.36 ± 0.04 μm (40×/0.75 NA objective). Experiments were conducted with each beam size (beam-size analysis; described previously (7, 37)). The ratio between the illuminated areas ($\omega^2(40\times)/\omega^2(63\times)$) was 2.56 ($n = 39$). After a brief measurement at the monitoring intensity, a 5-milliwatt pulse (4–6 ms or 10–20 ms for the 63× and 40× objectives, respectively) bleached 50–70% of the fluorescence in the spot. Fluorescence recovery was followed by the monitoring beam. The apparent characteristic fluorescence recovery time (τ) and the mobile fraction (R_f) were derived from the FRAP curves by nonlinear regression analysis, fitting to a lateral diffusion process with a single τ value (49).

Statistical Analysis of FRAP Data—The significance of differences between τ values measured with the same laser beam size was evaluated by Student's t test. To compare ratio measurements ($\tau(40\times)/\tau(63\times)$ and $\omega^2(40\times)/\omega^2(63\times)$), we employed bootstrap analysis, which is preferable for comparison between ratio values (50). The $\tau(40\times)$ and $\tau(63\times)$ values were resampled with replacement using Excel, and average values from each group of resampled data ($\tau(40\times)^{Boot}$ and $\tau(63\times)^{Boot}$) were derived. For each beam size, 1000 averaged samples were generated, followed by calculation of the bootstrap ratio dividing

$\tau(40\times)^{Boot}$ by $\tau(63\times)^{Boot}$. To evaluate whether the τ ratios thus obtained differ significantly from the beam-size ratio calculated by the same method ($\omega^2(40\times)^{Boot}/\omega^2(63\times)^{Boot}$), the set of the τ bootstrap ratios was divided by the set of beam area bootstrap ratios, and the *p* value was derived from the spread of the resulting histogram at ~1.

Radiolabeling of Inositol Phospholipids and Analysis of Inositol Phosphate Formation—Twenty-four hours after transfection, COS-7 cells were washed once with 0.5 ml/well of buffer A (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.4), followed by addition of 0.4 ml/well of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 10 μCi/ml *myo*-[2-^{3}H]inositol (Amersham Biosciences) and 10 mM LiCl. After incubation for 20 h, the cells were washed once with 0.4 ml/well of buffer A and lysed in 0.2 ml/well 10 mM ice-cold formic acid (51). Samples were incubated on ice for 30 min, neutralized with 0.3 ml/well of 10 mM NH_4OH, and centrifuged for 5 min at 15,000 × *g*. The total inositol phosphates in the supernatants were separated on columns of Dowex® 1 × 8–200 ion exchange resin (Sigma), as described (51, 52), and the radioactivity was quantified by liquid scintillation counting.

Subcellular Fractionation—COS-7 cells grown in 10-cm dishes were transiently transfected as described above by vectors encoding wt or mutant GFP-PLCβ_2 and various activators. After 20 h, the cells were serum-starved (24 h) to avoid serum-induced activation, scraped into 8 ml of buffer A, and centrifuged at 300 × *g* for 5 min. The cells were lysed in 70 μl of ice-cold buffer B (20 mM Tris/HCl, pH 7.5, 2 mM EDTA, 2 μg/ml soybean trypsin inhibitor, 3 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 1 μM leupeptin, and 1 μg/ml aprotinin) by freezing in liquid nitrogen and thawing followed by homogenization, forcing the suspension ten times through a 0.40- × 20-mm syringe needle. After removal of unbroken cells and nuclei (300 × *g*, 10 min, 4 °C), particulate (*P*) and soluble (*S*) fractions were separated by centrifugation at 100,000 × *g* for 60 min at 4 °C. The *P* fraction was washed once with 100 μl of buffer B and resuspended in 25 μl of buffer B. To enable direct comparison of the relative distribution of PLCβ_2-GFP (wt or mutant) between the two fractions, equal proportions (identical percentages by volume, v/v) of pairs of the *P* and *S* fractions were analyzed by SDS-PAGE and immunoblotting using anti-GFP antibodies followed by peroxidase goat anti-mouse and ECL. The bands were quantified by densitometry using EZQuant-Gel 2.2 (EZQuant Ltd.).

Miscellaneous—Protein concentrations were determined according to Bradford (53) using bovine IgG as standard. SDS-PAGE and immunoblotting were as described before (7); immunoreactive proteins were visualized using the ECL™ Western blotting Detection System (Amersham Biosciences).

RESULTS

PLCβ_2 Activation by Different Stimulators Can Occur at Varying Extents of Membrane Recruitment—PLCβ_2 can be activated by G protein subunits (α_q, $\beta\gamma$) and by Rac with different orders of efficacy and potency (1, 2, 6). Because the different stimulators interact with distinct regions of the enzyme, they may differ in their ability to recruit PLCβ_2 to the membrane, as well as in the resulting mode of membrane interactions. We

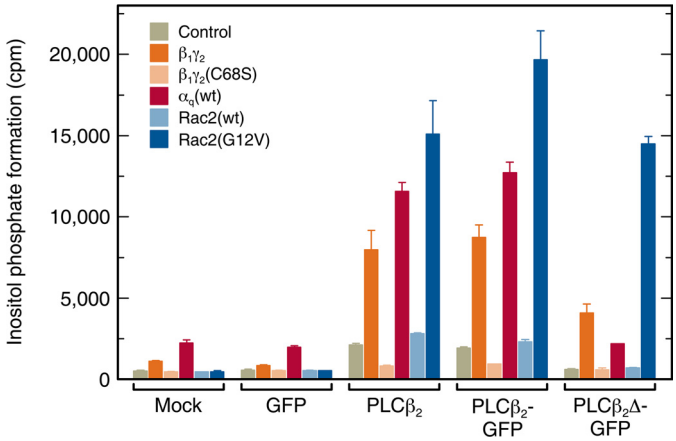

FIGURE 1. **Regulation of wild-type PLCβ_2, PLCβ_2-GFP, and PLC$\beta_2\Delta$-GFP by heterotrimeric G protein subunits and Rac2 in intact cells.** COS-7 cells were co-transfected as indicated at the *abscissa* with 250 ng per well of either empty vector (*Mock*) or vector encoding GFP, PLCβ_2, PLCβ_2-GFP, or PLC$\beta_2\Delta$-GFP together with empty vector (*Control*) or vectors encoding the activating proteins as shown in the *inset* (750 ng in all cases, composed of 375 ng each for β_1 and γ_2; Rac2-expressing vectors were added at 50 ng, completed to 750 ng of DNA by empty vector). At 24-h post-transfection, the cells were incubated for 20 h in the presence of *myo*-[2-^{3}H]inositol (10 μCi/ml) and 10 mM LiCl, and the levels of inositol phosphates were determined as described under "Experimental Procedures." The values shown correspond to the means ± S.D. of triplicate determinations. Co-transfection with α_q(R183C) (data not shown) yielded saturating levels of inositol phosphate formation in all cases (25,000–34,000 cpm), including the mock- and GFP-transfected controls, because high levels of activated α_q stimulate the activity of PLCβ isozymes endogenously present in COS-7 cells.

therefore compared, in cells and under identical conditions, the activation of PLCβ_2 and the PLC$\beta_2\Delta$ mutant by the different stimulators (Fig. 1). Because a major part of the studies involves PLCβ_2-GFP chimeric proteins, we validated that the GFP tagging does not interfere with the activation of the enzyme by all the stimulators. To this end, we compared side-by-side (Fig. 1) the ability of $\beta_1\gamma_2$, α_q, and Rac2 (wt or mutated) to stimulate inositol phosphate formation by PLCβ_2, PLCβ_2-GFP, and PLC$\beta_2\Delta$-GFP. Inositol phosphate formation was measured in COS-7 cells co-transfected with vectors encoding one of the above PLCβ_2 constructs (replaced by empty or GFP-encoding vectors as controls) together with: (i) $\beta_1\gamma_2$ (β_1 along with γ_2(wt)); (ii) $\beta_1\gamma_2$(C68S) (β_1 along with the isoprenylation mutant γ_2(C68S)); (iii) α_q(wt); (iv) Rac2(wt); or (v) Rac2(G12V). Comparison between the inositol phosphate formation levels in cells transfected with PLCβ_2 *versus* PLCβ_2-GFP (*third* and *fourth bar groups* in Fig. 1) clearly demonstrates that PLCβ_2-GFP was activated by all stimulators to an extent similar to that of untagged PLCβ_2. These results confirm the earlier demonstration that PLCβ_2-GFP is effectively stimulated by activated Rac2 (7), and extend them to show that attachment of GFP to the C terminus of PLCβ_2 does not affect the regulation of the enzyme by the heterotrimeric G protein subunits. For both PLCβ_2 and PLCβ_2-GFP, the degree of stimulation was Rac2(G12V) > α_q(wt) > $\beta_1\gamma_2$. Considering that only a portion of α_q(wt) is activated under the conditions used here, this is consistent with the rank order of potencies (which cannot be directly determined in cells) of the three stimulators in *in vitro* assays: activated α_q > activated Rac2 > $\beta_1\gamma_2$ (6, 14, 54). As shown in Fig. 1, stimulation by $\beta_1\gamma_2$ required membrane anchorage of γ_2, as suggested by the lack of PLCβ_2 activation

upon replacement of γ_2 by the isoprenylation-resistant mutant γ_2(C68S). Rac2(wt) had only a weak effect, in accord with the notion that it should undergo activation to stimulate PLCβ_2 (7). It should be noted that α_q(wt) induced a marked stimulatory response in cells expressing PLCβ_2 or PLCβ_2-GFP, but not in cells expressing PLC$\beta_2\Delta$-GFP, in line with the inability of the latter to bind α_q. However, PLC$\beta_2\Delta$-GFP remained responsive to Rac2(G12V) or $\beta_1\gamma_2$, in accord with earlier *in vitro* studies on the untagged form of this mutant (6). Constitutively active α_q(R183C) gave a very high stimulation of inositol phosphate formation (up to ~60-fold; not shown in Fig. 1 due to the out-of-range value); this robust activation was obtained already in mock- or GFP-transfected cells, suggesting that it reflects activation of endogenous PLCβ isozymes other than PLCβ_2, which are present in COS-7 cells (13, 55). Constitutively active Rac2(G12V) did not stimulate inositol phosphate formation in the absence of a co-transfected PLCβ_2 construct (Fig. 1), because it specifically activates PLCβ_2, whose endogenous expression level in these cells is very low.

PLCβ_2 must be recruited to the membrane to interact with its substrate, PtdInsP_2, which is membrane-associated. We therefore investigated the ability of the different stimulators to translocate PLCβ_2-GFP and PLC$\beta_2\Delta$-GFP to the membrane fraction (Fig. 2). To this end, COS-7 cells were co-transfected with a vector encoding PLCβ_2-GFP or PLC$\beta_2\Delta$-GFP together with either empty vector or with vectors encoding various PLCβ_2 activators, and the relative distribution of PLCβ_2-GFP or PLC$\beta_2\Delta$-GFP between the cytosolic (*S*) and membrane (*P*) fractions was determined by cell fractionation as described under "Experimental Procedures." Fig. 2*A* shows that singly expressed PLCβ_2-GFP was mostly in the cytosol (*S* fraction). Co-expression with the different PLCβ_2 activators increased the proportion (%) of PLCβ_2-GFP in the particulate fraction to varying degrees (Fig. 2*A*), to extents that correlated with their abilities to stimulate the enzymatic activity of PLCβ_2 (*cf.* Fig. 1): Rac2(G12V) > α_q(wt) > $\beta_1\gamma_2$. Because the fractionation experiment specifically detects the localization of PLCβ_2-GFP, it is not masked by activation of endogenous PLCβ enzymes (unlike the stimulation of PLCβ activity), enabling us to measure the effects of constitutively active α_q(R183C) on PLCβ_2-GFP membrane association. This mutant had a slightly higher effect than α_q(wt), which was hard to detect due to the limited sensitivity of the fractionation assay, but is supported by the more sensitive biophysical FRAP studies of PLCβ_2 membrane interactions (see Fig. 4). Interestingly, even though both Rac2(G12V) and α_q(R183C) are both constitutively active, Rac2(G12V) recruited PLCβ_2-GFP to the particulate fraction to a higher extent than α_q(R183C). Together with the lower membrane recruitment and lower activation of PLCβ_2 by $\beta_1\gamma_2$ (Figs. 1 and 2*A*), this raises the possibility that the extent and/or mode of membrane recruitment mediated by the distinct stimulators are different. This view gains strong support from the FRAP beam-size analysis experiments (Fig. 4), which detect with high sensitivity the membrane association dynamics of PLCβ_2-GFP in live cells; it is corroborated by fractionation studies on cells expressing PLC$\beta_2\Delta$-GFP (Fig. 2*B*). Here, $\beta_1\gamma_2$ did not induce a measurable increase in the percentage of PLC$\beta_2\Delta$-GFP in the membrane pellet, contrasting with its ability to induce mild, but

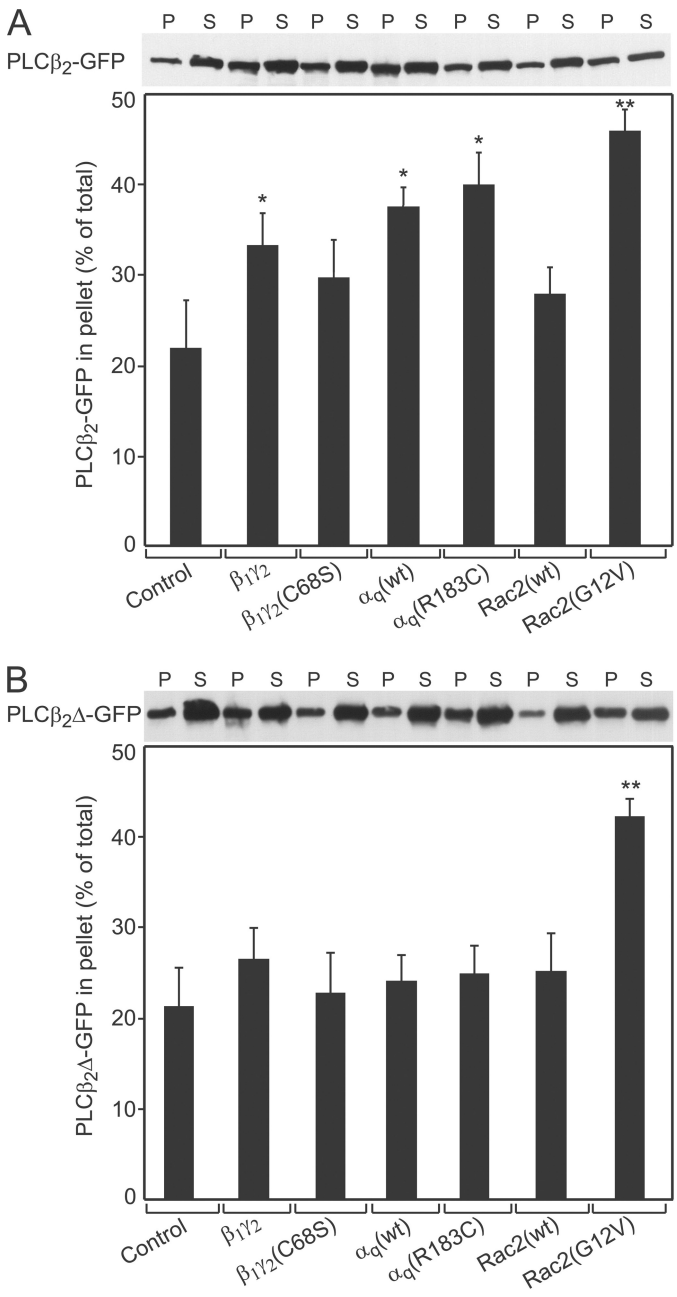

FIGURE 2. **Effect of G protein subunits and Rac2 on the subcellular distribution of PLCβ_2-GFPand PLC$\beta_2\Delta$-GFP.** COS-7 cells grown in 10-cm dishes were co-transfected with 7 μg per dish of vector encoding PLCβ_2-GFP (*A*) or PLC$\beta_2\Delta$-GFP (*B*) together with either empty vector (*Control*) or vectors encoding the activating proteins shown in the abscissa (7 μg of DNA, composed of 3.5 μg each for β_1 and γ_2). After 20 h, the cells were serum-starved for another 24 h, homogenized, and fractionated into postnuclear particulate (*P*) and soluble (*S*) fractions as described under "Experimental Procedures." The two fractions were well separated, as controlled by immunoblotting for RhoGDIα (cytosolic) and Gβ_{1-5} (membrane-bound, not shown). Equal proportions (v/v) of the *P* and *S* fractions of each sample were subjected to SDS-PAGE and quantified by immunoblotting and densitometry using anti-GFP antibodies. The regions shown are those around 160 kDa (*A*) and 125 kDa (*B*); no other immunoreactive bands were detected. The immunoblots shown (*upper panels*) are of a representative experiment, whereas the *bar graphs* (*lower panels*) depict the means ± S.E. ($n = 3$) of multiple experiments quantified by densitometry. *Asterisks* indicate a significant increase in the percentage of PLCβ_2-GFP (*A*) or PLC$\beta_2\Delta$-GFP (*B*) in the membrane fraction (*P*) relative to the control (**, $p < 0.01$; *, $p < 0.05$; Student's *t* test).

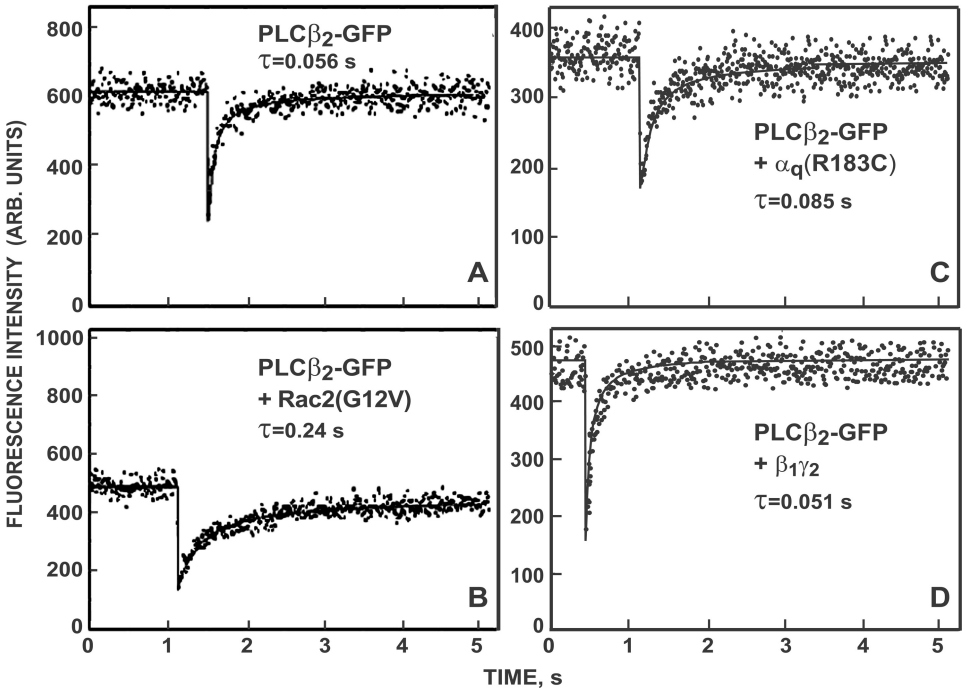

FIGURE 3. **Typical FRAP curves demonstrating that the FRAP rate of PLCβ_2-GFP is modulated differently by the various activators.** COS-7 cells were transfected with a plasmid encoding PLCβ_2-GFP and an excess of empty vector (*A*) or vectors encoding Rac2(G12V) (*B*), α_q(R183C) (*C*), or β_1 and γ_2 (*D*). FRAP experiments were conducted at 22 °C using a 63× objective (see "Experimental Procedures"). The *solid lines* show the best fit of a non-linear regression analysis (49). The τ values derived for the specific curves are depicted in each *panel*; the mobile fractions (R_f) were above 0.93 in all cases, and are therefore not shown.

clear stimulation of PLC$\beta_2\Delta$-GFP enzymatic activity (Fig. 1). The ability of $\beta_1\gamma_2$ to activate PLCβ_2 or PLC$\beta_2\Delta$ despite its weak effect on their recruitment to the particulate fraction is likely to reflect transient (as opposed to stable) membrane recruitment, enabling significant dissociation of the enzyme from the membrane during fractionation. The notion that $\beta_1\gamma_2$ elicits transient association of the enzyme with the membrane was validated by the biophysical studies described later (see Figs. 4 and 5). In line with the inability of PLC$\beta_2\Delta$ to bind α_q, neither α_q(wt) nor α_q(R183C) affected its membrane association. On the other hand, Rac2(G12V) was highly effective in recruiting PLC$\beta_2\Delta$ to the particulate fraction, in accord with the concept that activated Rac/Cdc42 GTPases interact with the N-terminal PH domain also present in the mutant (6, 7, 17, 18).

FRAP Studies Demonstrate Distinct Modes of PLCβ_2 Membrane Recruitment by the Different Stimulators—The subcellular fractionation experiments (Fig. 2) provide a measure for the population of PLCβ_2 molecules that exhibit relatively stable association with the total membrane fraction. To investigate the effects of the various PLCβ_2 activators on the mode and dynamics of PLCβ_2 interactions with the plasma membrane of live cells, we expressed PLCβ_2-GFP in COS-7 cells and employed FRAP to measure its lateral diffusion and membrane association dynamics in the presence or absence of the various stimulators. Typical FRAP experiments are depicted in Fig. 3; quantitative results on multiple cells using two different sizes of a Gaussian laser beam (FRAP beam-size analysis) are shown in Fig. 4. The beam-size analysis (7, 37) explores the membrane interaction mode of proteins capable of both lateral diffusion in the membrane and of exchange between membrane-associated and cytoplasmic pools. If FRAP occurs exclusively by diffusion, the characteristic fluorescence recovery time τ is identical to the characteristic diffusion time τ_D, which is proportional to the bleached area ($\tau = \tau_D = \omega^2/4D$, where ω is the Gaussian radius of the beam, and D is the lateral diffusion coefficient) (49). In the current studies, the ratio between the recovery times obtained with the two beam sizes generated using the 40× and 63× objectives, $\tau(40\times)/\tau(63\times)$, should be 2.56 (the measured ratio between the illuminated areas). When FRAP occurs by exchange, τ reflects the chemical relaxation time, which is independent of the bleached area; *i.e.* $\tau(40\times)/\tau(63\times)$ should equal 1. Intermediate τ ratios suggest mixed recovery, where the faster process has a higher contribution (7, 37).

Because PLCβ_2-GFP (and PLC$\beta_2\Delta$-GFP) have a significant cytoplasmic fraction (*cf.* Fig. 2), we focused the laser beam on flat cell regions near the cell periphery, resulting in low contribution of cytoplasmic fluorescence due to the thin cell volume in such regions. Moreover, the FRAP rate of free PLCβ_2-GFP in the cytoplasm is very fast, showing immediate recovery on the time scale of the current experiments, ensuring no contribution of cytoplasmic diffusion to the FRAP curves (7). The results shown in Figs. 3 and 4 demonstrate that the different stimulators each induce a distinct effect on the membrane interactions mode of PLCβ_2. Prior to stimulation, FRAP beam-size analysis of PLCβ_2-GFP yielded a $\tau(40\times)/\tau(63\times)$ ratio of 2.0, intermediate between the ratios characterizing FRAP by lateral diffusion (2.56 with the current beam sizes used) and by exchange (τ ratio = 1), suggesting a mixed contribution of the two mechanisms (7, 37). The contribution of exchange does not allow an accurate calculation of the lateral diffusion coefficient; yet, an estimate of D can be calculated from $\tau(63\times)$ and the beam size with the 63× objective, because the recovery at this smaller beam size contains a higher contribution of lateral diffusion (56). This yields $D = \omega^2/4\tau_D = (3.2 \pm 0.2)\ \mu m^2/s$, faster than D of the lipid probe $DiIC_{16}$ (1 $\mu m^2/s$) in the same cells (7) or the 0.5–1 $\mu m^2/s$ value reported for $PtdInsP_2$ (32, 34), in accord with the lack of stable binding of the enzyme to the plasma membrane and the significant contribution of exchange to the FRAP measurements.

Stimulation of PLCβ_2-GFP by co-expression with Rac2-(G12V) led to a large and highly significant increase in the FRAP times (τ; slower recovery rates) of PLCβ_2-GFP (Fig. 4*A*), in line with the marked increase in its membrane-associated fraction (Fig. 2*A*). Importantly, this was accompanied by a shift of the FRAP mechanism to recovery by nearly pure exchange

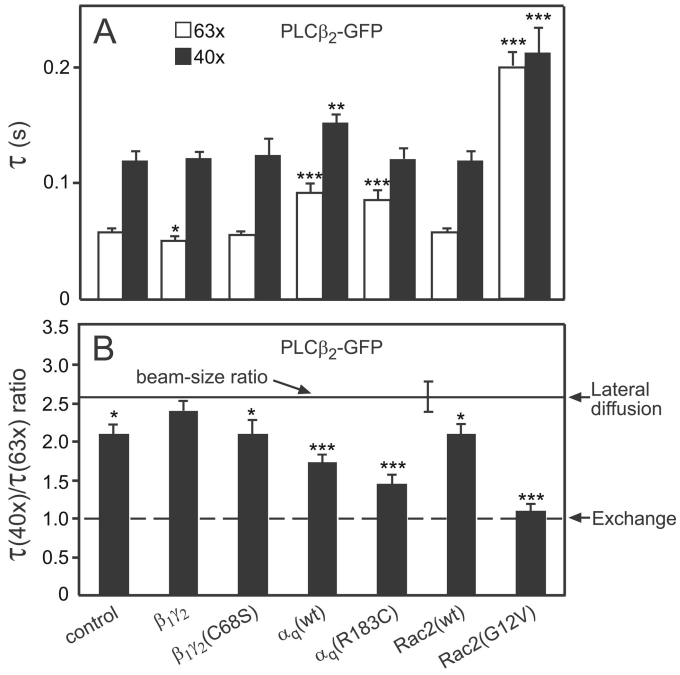

FIGURE 4. **FRAP beam-size analysis suggests activator-dependent distinct modes of PLCβ_2-GFP interactions with the plasma membrane.** FRAP experiments were conducted at 22 °C as in Fig. 3, on COS-7 cells transfected with PLCβ_2-GFP, and an excess of empty vector (*Control*) or vectors encoding the indicated proteins as described under "Experimental Procedures." Two beam sizes were generated using a 63× and 40× objectives (see "Experimental Procedures"), and the τ values were determined with each. The ratio between the areas illuminated by the two beams, $\omega^2(40\times)/\omega^2(63\times)$, was 2.56 ($n = 39$). This ratio is expected for FRAP by lateral diffusion, whereas a ratio of 1 is expected for recovery by exchange (37). The R_f values were high in all cases (≥0.93). *A*, τ values. *Bars* are means ± S.E. of 40–60 measurements, each conducted on a different cell. Comparing τ values measured with the same beam size, Rac2(G12V) and α_q induced significant increases in τ of PLCβ_2-GFP relative to the control (***, $p < 10^{-6}$; **, $p < 0.005$; Student's *t* test). $\beta_1\gamma_2$ had no significant effect on $\tau(40\times)$, but reduced $\tau(63\times)$ (*, $p < 0.02$). *B*, $\tau(40\times)/\tau(63\times)$ ratios. The ratio values (τ ratios and the beam-size ratio) and their S.E. were calculated from the experimentally measured values ($\tau(40\times)$ and $\tau(63\times)$ for τ ratio, $\omega^2(40\times)$ and $\omega^2(63\times)$ for the beam-size ratio) using bootstrap analysis. The bootstrap analysis (see "Experimental Procedures") showed that the τ ratios of PLCβ_2 differ significantly from the 2.56 beam-size ratio predicted for FRAP by lateral diffusion in all cases (***, $p < 10^{-6}$; *, $p < 0.02$), except for co-expression with $\beta_1\gamma_2$ ($p > 0.3$). Comparison of the τ ratios to 1 (the value expected for FRAP by exchange) using bootstrap analysis shows that in the presence of Rac2(G12V) the τ ratio of PLCβ_2-GFP is not significantly different from 1 ($p > 0.4$).

($\tau(40\times)/\tau(63\times) \approx 1$; Fig. 4*B*), suggesting that the characteristic diffusion time (τ_D) must be at least an order of magnitude slower than τ for exchange (τ_{ex}), resulting in a negligible contribution of the lateral diffusion to the fluorescence recovery. Thus, in the presence of Rac2(G12V), τ_D of PLCβ_2-GFP is at least 2 s, 10-fold slower than the measured $\tau(63\times)$ (0.2 s, reflecting exchange), providing an upper limit of $D = 0.09\ \mu m^2/s$. This D value is much lower than D of lipid probes in the plasma membrane, indicating that the enhanced association of PLCβ_2 with the plasma membrane following activation by Rac2(G12V) is due to interactions with membrane proteins and/or localized protein/lipid clusters. In addition, because the dissociation rate governs fluorescence recovery due to exchange (57, 58), τ measured in the FRAP experiment under conditions where the recovery is due to exchange (τ_{ex}) reflects the time constant for dissociation from the plasma membrane. This allows the calculation of the distance that a given fraction of the membrane-associated protein will diffuse laterally prior to dissociation into the cytoplasm (travel range); for a fraction comprising 63% of the protein population, this range (r) is given by $r = (2 \times D \times \tau)^{1/2}$ (59). For Rac2(G12V)-activated PLCβ_2-GFP, this calculation yields $r = (2 \times 0.09 \times 0.2)^{1/2} = 0.19\ \mu m$, suggesting that recruitment of PLCβ_2 to the membrane by Rac2(G12V) targets the activated PLCβ_2 mainly to laterally restricted small ranges, where it acts until it dissociates back to the cytoplasm.

To examine whether $PtdInsP_2$ hydrolysis following PLCβ_2 activation is involved in altering the membrane interaction dynamics of PLCβ_2-GFP upon activation, we co-transfected COS-7 cells with PLCβ_2-GFP (150 ng of DNA) together with a 7-fold excess of a plasmid encoding SigD, a bacterial inositol phosphatase shown to hydrolyze $PtdInsP_2$ and reduce its cellular level (60, 61). FRAP studies conducted 24 h post-transfection showed no change in PLCβ_2-GFP FRAP parameters, suggesting that $PtdInsP_2$ hydrolysis *per se* does not significantly affect the dynamics of PLCβ_2 membrane interactions. This notion is further supported by the finding that each PLCβ_2 activator has a distinct effect on the FRAP kinetics of the stimulated PLCβ_2 (Figs. 4 and 5; see below), although $PtdInsP_2$ hydrolysis by PLCβ_2 is elicited in all cases (Fig. 1).

Unlike the robust effect of Rac2(G12V) on the FRAP dynamics of PLCβ_2-GFP, co-expression with α_q (wt or constitutively active) induced modest, albeit significant, effects on the FRAP parameters of the enzyme. This correlated with the biochemical fractionation experiments (Fig. 2*A*), where Rac2(G12V) induced a higher increase in the percentage of PLCβ_2-GFP associated with the membrane fraction. Accordingly, α_q(R183C) and α_q(wt) modulated the FRAP parameters of PLCβ_2 toward the same direction as Rac2(G12V), but to a lower extent (Fig. 4). They mildly increased the τ values of PLCβ_2-GFP and shifted its $\tau(40\times)/\tau(63\times)$ ratio to lower values, albeit still higher than 1. Note that the sensitivity of the FRAP beam-size analysis demonstrates that α_q(R183C) has a stronger effect than α_q(wt) on the τ ratio (Fig. 4*B*), a difference that was too mild to detect by the fractionation studies. Because the exchange rates of PLCβ_2-GFP stimulated by α_q (wt or mutant) were distinctively faster than after stimulation by active Rac2, the increase in the τ values was very mild. This is especially valid for $\tau(40\times)$, which contains a higher contribution of exchange (due to the larger beam size) and was only weakly affected by α_q(wt) and even less by α_q(R183C). Therefore, the τ ratio can detect the stronger effect of α_q(R183C) with higher sensitivity. The results depicted in Fig. 4 suggest that, although stimulation by α_q(R183C) or α_q(wt) enhances the mobility-retarding interactions of PLCβ_2-GFP with the plasma membrane, the interactions are weaker than those induced by Rac2(G12V) (7; see Fig. 4), and may involve association with different targets in the membrane. Estimation of D for PLCβ_2-GFP co-expressed with α_q(R183C) or α_q(wt) from the $\tau(63\times)$ values (which are very similar for the two α_q proteins) yields $D = (2.1 \pm 0.2)\ \mu m^2/s$, somewhat higher than lipid probe diffusion, most likely due to the residual contribution of exchange. Because the $\tau(40\times)/\tau(63\times)$ ratio of PLCβ_2-GFP upon co-expression with an α_q protein is intermediate between the values expected for recovery by diffusion and exchange, the characteristic exchange time τ_{ex} should be in the same range as τ_D and can be

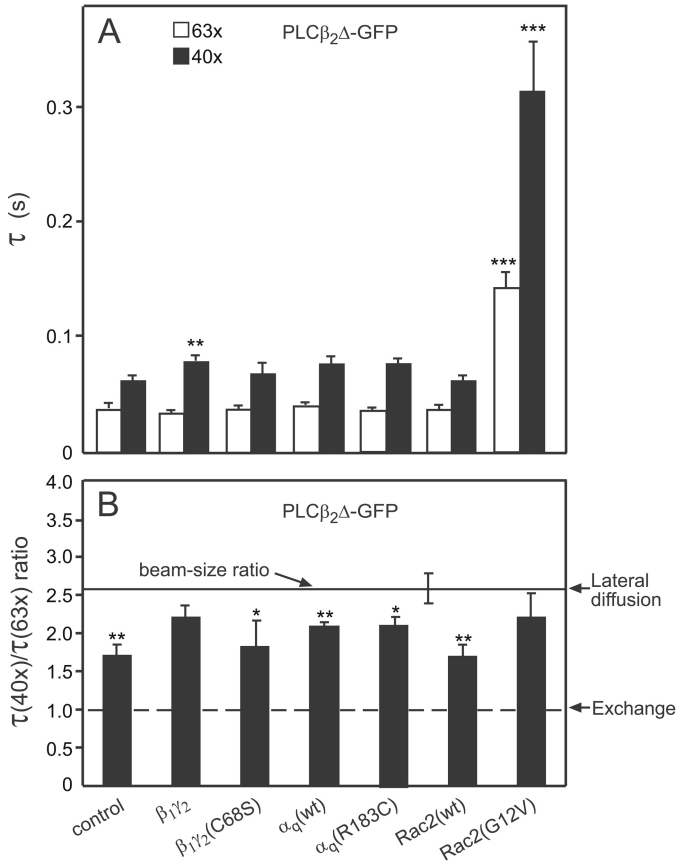

FIGURE 5. **FRAP beam-size analysis of the effects of various activators on the mode of the membrane interactions of PLC$\beta_2\Delta$-GFP.** FRAP experiments and beam-size analyses were conducted as in Fig. 4, except that PLC$\beta_2\Delta$-GFP replaced PLCβ_2-GFP. The R_f values were high in all cases (≥95%; not shown). *A*, τ values. *Bars* are means ± S.E. of 40–60 measurements. Comparison of the values measured for cells co-transfected with the various activators relative to control (cells singly transfected with PLC$\beta_2\Delta$-GFP) showed highly significant increase in both $\tau(63\times)$ and $\tau(40\times)$ for PLC$\beta_2\Delta$-GFP co-expressed with Rac2(G12V) (***, $p < 10^{-6}$; Student's *t* test). $\beta_1\gamma_2$ had a weaker but significant effect on $\tau(40\times)$ of PLC$\beta_2\Delta$-GFP (**, $p < 0.005$). In all other cases, the differences from the τ values measured with the same beam size in the control cells were not significant ($p > 0.05$). *B*, $\tau(40\times)/\tau(63\times)$ ratios. All the ratio values and their S.E. were calculated using bootstrap analysis. This analysis (see "Experimental Procedures") showed that co-expression with $\beta_1\gamma_2$ or with Rac2(G12V) shifts the τ ratio of PLC$\beta_2\Delta$-GFP to values essentially similar to the 2.56 ratio expected for FRAP by lateral diffusion ($p > 0.15$ for co-expression with $\beta_1\gamma_2$, and $p > 0.3$ for co-expression with constitutively active Rac2). In all other cases, $\tau(40\times)/\tau(63\times)$ of PLC$\beta_2\Delta$ differed significantly from the 2.56 beam-size ratio (**, $p < 0.005$; *, $p < 0.02$), except for co-expression with $\beta_1\gamma_2$ ($p > 0.3$), indicative of a significant contribution of exchange to the FRAP mechanism.

estimated from $\tau(40\times)$ (τ measured with the larger beam size, where the relative contribution of exchange is higher). The $\tau(40\times)$ values for α_q(R183C) and α_q(wt) are 0.12 and 0.15 s, respectively, suggesting that the travel range of PLCβ_2 following activation by α_q is roughly $r = (2 \times D \times \tau_{ex})^{1/2} = (2 \times 2.1 \times 0.135)^{1/2} = 0.75$ μm, 4-fold larger than after stimulation with Rac2(G12V). Thus, activation by α_q leads to a less confined recruitment of PLCβ_2 to the plasma membrane, because the active enzyme can diffuse laterally a longer distance prior to dissociation to the cytoplasm.

Unexpectedly, the effects of $\beta_1\gamma_2$ on the FRAP parameters of PLCβ_2-GFP were highly different from those of Rac2(G12V) and α_q. Unlike the marked increase in τ of PLCβ_2-GFP induced by the latter two stimulators, activation by $\beta_1\gamma_2$ had only very subtle effects on the τ values (Fig. 4*A*). Concomitantly, $\beta_1\gamma_2$ shifted the $\tau(40\times)/\tau(63\times)$ ratio of PLCβ_2 in a direction opposite to that mediated by Rac2(G12V) and α_q, resulting in a τ ratio indistinguishable from lateral diffusion (Fig. 4*B*). This suggests that, following stimulation by $\beta_1\gamma_2$, the exchange rate of PLCβ_2-GFP between the plasma membrane and the cytoplasm becomes much slower than its lateral diffusion rate, indicating enhanced membrane interactions. Yet, these interactions are highly transient. This notion is supported by the biochemical fractionation studies, which showed a markedly weaker recruitment of PLCβ_2 to the membrane fraction by $\beta_1\gamma_2$ relative to Rac2(G12V) or α_q (Fig. 2*A*). The *D* value of PLCβ_2-GFP co-expressed with $\beta_1\gamma_2$ can be accurately calculated from the FRAP experiments, because in the presence of $\beta_1\gamma_2$ the τ ratio between the two beam sizes is as expected for lateral diffusion. Interestingly, the *D* value thus obtained, (3.6 ± 0.2) $\mu m^2/s$, is markedly higher than *D* of lipid probes such as $DiIC_{16}$ (1 $\mu m^2/s$) or $PtdInsP_2$ (0.5–1 $\mu m^2/s$) (7, 32, 34). It is much higher than the lateral diffusion of $\beta_1\gamma_2$ at the plasma membrane of COS-7 cells, which we measured by FRAP on cells co-transfected with 850 ng DNA (1:1 ratio) of venus 1–155-γ_2 and venus 155–239-β_1. The two halves of the venus protein are not fluorescent separately and form a stable fluorescent complex due to bifunctional fluorescence complementation upon association (44, 62). These measurements yielded $D = (0.21 \pm 0.2)$ $\mu m^2/s$ ($n = 31$). This value, which is similar to the 0.23 $\mu m^2/s$ value reported for $\beta_1\gamma_2$ in HEK293 cells (62), was not altered by co-expressing the venus-tagged $\beta_1\gamma_2$ constructs with PLCβ_2 (150 ng of plasmid DNA). This suggests that $\beta_1\gamma_2$-stimulated PLCβ_2 exhibits a surfing-like diffusion along the plasma membrane, spending some of the time transiently bound to membrane lipids and/or fatty acid-anchored proteins, including the $\beta_1\gamma_2$ complex itself. The latter notion is supported by the failure of $\beta_1\gamma_2$(C68S), where γ_2 cannot undergo isoprenylation, to modulate the FRAP parameters of PLCβ_2 (Fig. 4). These findings have important implications for the travel range of PLCβ_2 following stimulation by $\beta_1\gamma_2$. A lower limit for this travel range can be derived based on the assumption that the exchange time (τ_{ex}) of PLCβ_2-GFP co-expressed with $\beta_1\gamma_2$ is at least 10-fold slower than the diffusion time τ_D, which is essentially equal to the measured τ value (*e.g.* $\tau(63\times) = \tau_D(63\times) = 0.045$ s, and thus τ_{ex} is at least 0.45 s). Therefore, the lower limit of the travel range is $r = (2 \times D \times \tau_{ex})^{1/2} = (2 \times 3.6 \times 0.45)^{1/2} = 1.8$ μm, ~10-fold larger than following stimulation with Rac2(G12V). We conclude that activation of PLCβ_2 by $\beta\gamma$ involves a mechanism that recruits the enzyme to the plasma membrane by inducing interactions that enable it to roam relatively large membrane regions prior to detachment to the cytoplasm, as required for hydrolysis of dispersed $PtdInsP_2$ populations.

To validate the specificity of the effects of the various stimulators on the FRAP parameters of PLCβ_2, we conducted analogous FRAP studies to measure their effects on PLC$\beta_2\Delta$-GFP (Fig. 5), which does not respond to α_q due to the F819-E1166 C-terminal deletion. In line with the loss of the response of this mutant to α_q as measured by both activation and recruitment to the membrane (Figs. 1 and 2), neither α_q(R183C) nor α_q(wt) modulated the τ values or the $\tau(40\times)/\tau(63\times)$ ratios of PLC$\beta_2\Delta$-GFP (Fig. 5). On the other hand, the effects of $\beta_1\gamma_2$ on the FRAP

dynamics of PLC$\beta_2\Delta$ closely resembled their effects on PLCβ_2, inducing only a very minor effect on the τ values, while increasing the $\tau(40\times)/\tau(63\times)$ ratio very close to the ratio expected for FRAP by pure lateral diffusion. These findings support the notion that $\beta_1\gamma_2$ expression induces highly transient interactions of PLCβ_2 and PLC$\beta_2\Delta$ with the plasma membrane, in line with its inability to measurably enhance the fraction of PLC$\beta_2\Delta$-GFP in the membrane pellet (Fig. 2*B*). Accordingly, the very fast lateral diffusion of $\beta_1\gamma_2$-stimulated PLC$\beta_2\Delta$ ($D = (5.2 \pm 0.3)\ \mu m^2/s$, calculated from the $\tau(63\times)$ in Fig. 5*A*)) suggests surfing-like diffusion (even faster than that of $\beta_1\gamma_2$-stimulated PLCβ_2), resulting in a high travel range prior to dissociation to the cytoplasm; assuming that τ_{ex} for PLC$\beta_2\Delta$-GFP is at least 10-fold slower than $\tau(63\times)$, the lower limit of $\beta_1\gamma_2$-stimulated PLC$\beta_2\Delta$ travel range is $r = (2 \times D \times \tau_{ex})^{1/2} = (2 \times 5.2 \times 0.33)^{1/2} = 1.9\ \mu m$, similar to $\beta_1\gamma_2$-stimulated PLCβ_2. Finally, activation of PLC$\beta_2\Delta$-GFP by Rac2(G12V) markedly increased the τ values (Fig. 5*A*), as in the case of PLCβ_2-GFP, supporting the notion that activation by Rac2 enhances the membrane association of PLC$\beta_2\Delta$, in accord with the fractionation studies (Fig. 2). However, the effect of Rac2(G12V) on the $\tau(40\times)/\tau(63\times)$ ratio differed markedly between PLCβ_2 and PLC$\beta_2\Delta$ (*cf.* Figs. 5*B* and 4*B*), shifting the τ ratio of PLC$\beta_2\Delta$ toward recovery by pure lateral diffusion. As shown and discussed by us earlier (37), when recovery occurs by lateral diffusion and exchange, their contribution to the measured FRAP is determined by the *relative* rates of the two processes, with the faster process prevailing. Thus, the shift of Rac2-stimulated PLC$\beta_2\Delta$ to FRAP by lateral diffusion directly demonstrates that its exchange rate is at least 10-fold slower than its lateral diffusion rate. This situation differs from that observed for Rac2-stimulated PLCβ_2, where exchange becomes the dominant mechanism, reflecting a much slower lateral diffusion rate for the full-length, Rac2-stimulated PLCβ_2 (compare Figs. 5*B* and 4*B*). This difference, discussed by us extensively earlier (7), is in line with the suggestion (7) that the C-terminal region missing in PLC$\beta_2\Delta$ has a role in the membrane interactions of full-length PLCβ_2, mainly with membrane proteins that diffuse slower than lipid probes. In its absence (PLC$\beta_2\Delta$), the interactions with slow diffusing membrane proteins become weaker, resulting in a loss of the diffusion-restricting interactions with the above proteins and in faster, lipid-like diffusion of Rac2-stimulated PLC$\beta_2\Delta$ ($D = (1.3 \pm 0.2)\ \mu m^2/s$, calculated from $\tau(63\times)$ of Rac2(G12V)-stimulated PLC$\beta_2\Delta$ in Fig. 5*A*). Under these conditions, the diffusion of PLCβ2Δ becomes fast relative to its exchange rate, as indicated by the diffusion-dominated FRAP mechanism (Fig. 5*B*). Thus, τ_{ex} of Rac2-stimulated PLCβ2Δ should be higher than the measured $\tau(63\times)$ by at least 10-fold (*i.e.* $\tau_{ex} \geq 1.4$ s). This would increase the travel range of Rac2-stimulated PLC$\beta_2\Delta$, with a lower limit estimate of $r = (2 \times D \times \tau_{ex})^{1/2} = (2 \times 1.3 \times 1.4)^{1/2} = 1.9\ \mu m$. This in turn indicates that, unlike the limited travel range of Rac2(G12V)-activated PLCβ_2, the travel range on the plasma membrane of the Rac2-activated PLC$\beta_2\Delta$ mutant is 10-fold higher, strongly compromising the localized nature of the membrane recruitment.

DISCUSSION

The mechanisms regulating the membrane recruitment and activation of PLCβ isozymes by their activators are not fully understood. Here, we investigated these issues in live cells for PLCβ_2 activated by several stimulators (Rac2, α_q, and $\beta_1\gamma_2$). Our findings demonstrate that each activator causes a distinct mode of PLCβ_2 membrane association, ranging between recruitment to confined regions (activation by Rac2, and to a lesser degree by α_q) and fast, surfing-like diffusion of the enzyme along the cytoplasmic leaflet of the plasma membrane (activation by $\beta_1\gamma_2$). The diversity of these mechanisms has important implications for the $PtdInsP_2$ populations targeted by PLCβ_2, because the first mechanism targets the activated enzyme to act on discrete $PtdInsP_2$ populations localized at or near the recruitment sites, while the second directs the enzyme to act on dispersed $PtdInsP_2$ populations.

In the current study, we combined FRAP beam-size analysis with biochemical and signaling assays to investigate the mechanisms by which Rac2, α_q, and $\beta_1\gamma_2$ mediate membrane recruitment and activation of PLCβ_2. Because the FRAP studies and some of the biochemical studies employed GFP-tagged PLCβ_2 or PLC$\beta_2\Delta$, we first validated that the GFP-tagged enzymes are as responsive as their untagged counterparts to the various PLCβ_2 activators (Fig. 1). Moreover, the specificity of the activation was kept in the GFP-tagged constructs, as shown by the loss of their response to $\beta_1\gamma_2$ upon mutational removal of the γ_2 membrane anchor site (C68S) and the loss of α_q activation in the PLC$\beta_2\Delta$ mutant (Fig. 1).

The fractionation studies demonstrate that PLCβ_2-GFP and PLC$\beta_2\Delta$-GFP are mainly cytosolic prior to activation (Fig. 2). However, transient association can be overlooked in such studies, which require relatively stable association with the membrane. Indeed, the FRAP beam-size analysis shows that unstimulated PLCβ_2 and PLC$\beta_2\Delta$ do interact transiently with the plasma membrane, as indicated by the mixed contribution of lateral diffusion and exchange to their FRAP kinetics, which are much slower than that of free cytoplasmic GFP (Figs. 3–5; see also Ref. 7). Thus, although mainly cytoplasmic, unstimulated PLCβ_2 (and PLC$\beta_2\Delta$) experiences some mobility-retarding interactions with plasma membrane constituents, which are insufficient for stable association. The *D* values estimated from $\tau(63\times)$ for PLCβ_2 and PLC$\beta_2\Delta$ prior to activation (3.2 and 4.8 $\mu m^2/s$, respectively) are ~4-fold higher than those of lipids and of $PtdInsP_2$ (7, 32, 34), most likely due to contributions of exchange and possibly of surfing-like diffusion along the cytoplasmic face of the membrane, a diffusion mode discussed later in the context of $\beta_1\gamma_2$-activated PLCβ_2. It should be noted that the lack of significant enzymatic activity in the unstimulated enzymes (Fig. 1), despite their transient interactions with the plasma membrane, suggests that such interactions *per se* are not sufficient to activate PLCβ_2, and that transient recruitment to the membrane (as observed following $\beta_1\gamma_2$ stimulation) should be accompanied by an additional event (*e.g.* a conformational change) to induce activation.

A striking finding of the current studies is that, although all the stimulators enhance the membrane interactions of PLCβ_2, they induce these effects by different mechanisms. Expression

of constitutively active Rac2(G12V) induced a robust recruitment of PLCβ_2-GFP to the membrane fraction (Fig. 2*A*), accompanied by a major modulation of its membrane interaction dynamics (significantly longer τ values and a drastic shift of the $\tau(40\times)/\tau(63\times)$ ratio to ~1, indicative of exchange-dominated FRAP (Fig. 4)). The significantly slower τ values at both laser beam sizes indicate that both the lateral diffusion and exchange of PLCβ_2-GFP are retarded following activation by Rac2(G12V), while the simultaneous shift to recovery dominated by exchange suggests that the lateral diffusion of PLCβ_2 is inhibited at least 10-fold more than its exchange, resulting in a negligible contribution of lateral diffusion to the FRAP. Such slow diffusion (the upper limit estimate of D is 0.09 $\mu m^2/s$; see "Results") is well below the typical D values of lipid probes, which are in the 1 $\mu m^2/s$ range (7, 32, 34), indicating that Rac2(G12V) recruits PLCβ_2 to the plasma membrane by enhancing its interactions with slow diffusing entities such as transmembrane proteins or protein-lipid clusters. This has important implications for the $PtdInsP_2$ populations targeted by Rac2-stimulated PLCβ_2; calculation of the resulting travel range prior to dissociation of the majority (63%) of the Rac-stimulated PLCβ_2 molecules from the membrane (59) yields 0.19 μm (see "Results"), indicating that the enzyme is recruited to act on substrate populations localized in distinct limited regions or clusters. Interestingly, the localized nature of PLCβ_2 recruitment and activation by Rac2(G12V) is disrupted in the PLC$\beta_2\Delta$ mutant, which is effectively recruited by activated Rac2 to the membrane fraction (Fig. 2*B*), but shifts to FRAP by lateral diffusion (τ ratio very close to the ratio expected for pure lateral diffusion (Fig. 5)). The D value obtained for Rac2-stimulated PLC$\beta_2\Delta$ is 1.3 $\mu m^2/s$, similar to the typical values for lipid probe diffusion. These results indicate that the modulation of PLCβ_2 membrane interactions by activated Rac2 involve not only the enzyme's PH domain, which was shown to interact with activated Rho GTPases (6, 17, 18), but also the C-terminal region, a major portion of which is missing in PLC$\beta_2\Delta$. The C-terminal region appears to contribute to the interactions of full-length PLCβ_2 with the slow diffusing membrane constituents, and its deletion in PLC$\beta_2\Delta$ interferes with these interactions, leading to faster lateral diffusion relative to exchange. Due to the higher D (1.3 $\mu m^2/s$) and slower exchange time ($\tau_{ex} \geq 1.4$ s), the travel range of Rac2-stimulated PLC$\beta_2\Delta$ increases by at least an order of magnitude (lower estimate, 1.9 μm).

The C-terminal region of PLCβ_2 is essential for activation by α_q (7, 13–15), in line with the failure of α_q or α_q(R183C) to activate PLC$\beta_2\Delta$ and to modulate its membrane interactions (Figs. 1, 2*B*, and 5). This contrasts with the ability of activated Rac2 and $\beta_1\gamma_2$ to activate PLC$\beta_2\Delta$, suggesting that the recruitment and activation mechanisms of PLCβ_2 by α_q may be different. Indeed, although α_q(R183C) and α_q enhanced the recruitment of PLCβ_2-GFP to the membrane fraction (Fig. 2*A*), this effect was weaker than that mediated by activated Rac2, and the ability of the α_q proteins to modulate the membrane interaction dynamics of PLCβ_2 was much milder (Fig. 4). Thus, the effect of α_q on prolonging the τ values was evident but much weaker, and the shift toward a τ ratio of 1 (recovery by exchange) was partial, indicating that, although stimulation by α_q increases the contribution of exchange relative to diffusion, the latter still has a contribution to the fluorescence recovery. This suggests that the retardation of the lateral diffusion of PLCβ_2 following stimulation by α_q is less than that induced by Rac2(G12V); indeed, the D value estimated for α_q-stimulated PLCβ_2 (which may contain some contribution of exchange) is 2.1 $\mu m^2/s$, close to but somewhat higher than D of lipid probes. This indicates that α_q-stimulated PLCβ_2 molecules interact with membrane constituents different from those targeted by Rac2 activation, possibly including lipids (*e.g.* via the PH and/or C2 domains) and/or lipid-anchored proteins. Direct association with α_q, which can interact with the membrane via its single fatty-acyl residue, may also contribute to these interactions. Nevertheless, the association of α_q-stimulated PLCβ_2 with membrane constituents (including α_q itself) must be dynamic, as indicated by the contribution of exchange to the FRAP kinetics (Fig. 4) and by the lower D value (0.47 $\mu m^2/s$) measured for a Gα subunit (α_{oA}) (63). Based on the D and τ_{ex} values, the travel range of α_q-stimulated PLCβ_2 is estimated to be 0.75 μm, suggesting that PLCβ_2 recruited by α_q stimulation is less constrained than the Rac2-stimulated enzyme, roaming larger (albeit still limited) membrane regions.

$\beta_1\gamma_2$ dimers can interact with the PH domain of PLCβ_2 (16) but also appear to interact with other portions of the enzyme (6). In line with the latter, distinct interactions, the modulation of the membrane interactions of PLCβ_2 by $\beta_1\gamma_2$ are very different from those mediated by Rac2 or α_q. Unlike activation by the latter two, activation by $\beta_1\gamma_2$ induced highly transient association with the membrane fraction, as evidenced by the fractionation experiments (Fig. 2). Yet, despite this highly transient nature, the FRAP of $\beta_1\gamma_2$-activated PLCβ_2 (or PLC$\beta_2\Delta$) is strongly dominated by fast lateral diffusion (Figs. 4 and 5), characterized by D values 3- to 5-fold faster than lipids or lipid-anchored proteins such as G protein α or $\beta\gamma$ subunits (7, 32, 34, 62, 63). Combining the highly transient interactions and the very fast diffusion along the plasma membrane, we propose that stimulation by $\beta_1\gamma_2$ induces surfing-like diffusion of PLCβ_2 enzymes along the cytoplasmic face of the plasma membrane. Thus, when a $\beta_1\gamma_2$-stimulated PLCβ_2 molecule dissociates from the membrane, it diffuses a short distance and quickly re-associates with another membrane component, remaining in the juxtamembrane vicinity and diffusing along the membrane. Juxtamembrane diffusion was recently found for paxillin and vinculin above focal adhesions (58). This unique mechanism enables diffusion of $\beta_1\gamma_2$-stimulated PLCβ_2 along the membrane at rates much faster than those of lipids or lipidated proteins, including the $\beta_1\gamma_2$ dimers, which diffuse with $D = 0.2$ $\mu m^2/s$ (62). The diffusion of $\beta_1\gamma_2$-stimulated PLCβ_2 (or PLC$\beta_2\Delta$) is also much faster than that of $PtdInsP_2$, which has D values of 0.5–1 $\mu m^2/s$ (32, 34), enabling very fast spatial dispersal of the activated enzyme, not limited by the lateral diffusion of either lipidated protein targets or the substrate. This mechanism is likely relevant under physiological conditions, because PLCβs are typically much less abundant (at least 100-fold) in cells than $\beta\gamma$ dimers (64, 65), enabling sequential interaction of a single PLCβ molecule with multiple $\beta\gamma$ dimers by dissociation and fast re-association. An important consequence of this fast diffusion is a dramatic increase in the travel range of $\beta_1\gamma_2$-activated PLCβ_2 (or PLC$\beta_2\Delta$) to at least 1.9 μm. This implies

that stimulation by $\beta_1\gamma_2$ recruits PLCβ_2 to act on dispersed $PtdInsP_2$ populations.

The divergent mechanisms described above may have evolved for stimulation of PLCβ_2 (and possibly other PLCβ isozymes) to accomplish different tasks of cellular regulation. $PtdInsP_2$ has long been known to occur in cells not only in dispersed populations, but also to form gradients and locally enriched regions (27–33). We propose that activation by Rac/Cdc42 recruits PLCβ_2 to hydrolyze $PtdInsP_2$ in discrete, spatially restricted zones. On the other hand, activation by $\beta_1\gamma_2$ dimers results in signals that rapidly propagate along the plasma membrane, because the activated PLCβ_2 can roam large areas along the membrane, hydrolyzing $PtdInsP_2$ with low spatial resolution. Activation by α_q yields an intermediate situation, which may fit conditions where relatively shallow gradients of $PtdInsP_2$ hydrolysis are beneficial.

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