Phospholipase Cβ2 Binds to and Inhibits Phospholipase Cδ1

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Phospholipase Cβ (PLCβ) isoforms, which are under the control of Goα and Gβγ subunits, generate Ca2+ signals induced by a broad array of extracellular agonists, whereas PLCδ isoforms depend on a rise in cytosolic Ca2+ for their activation. Here we find that PLCβ2 binds strongly to PLCδ1 and inhibits its catalytic activity in vitro and in living cells. In vitro, this PLC complex can be disrupted by increasing concentrations of free Gβγ subunits. Such competition has consequences for signaling, because in HEK293 cells PLCβ2 suppresses elevated basal [Ca2+]i and inositol phosphates levels and the sustained agonist-induced elevation of Ca2+ levels caused by PLCδ1. Also, expression of both PLCs results in a synergistic release of [Ca2+]i upon stimulation in A10 cells. These results support a model in which PLCβ2 suppresses the basal catalytic activity of PLCδ1, which is relieved by binding of Gβγ subunits to PLCβ2 allowing for amplified calcium signals.

The binding of an agonist to its target G protein-coupled receptor stimulates heterotrimeric G proteins, which in turn can result in an increase in intracellular Ca2+ through the activation of phospholipase Cβ (PLCβ)1 (1, 2). PLCs catalyze the hydrolysis of a minor lipid component, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), to release the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (Ins(1,4,5)P3). These messengers in turn activate protein kinase C and stimulate signals. At maximum [Ca2+]i concentrations, the specific catalytic activity of purified PLCδ1 is typically 50–100-fold greater than that of unstimulated PLCβ or PLCγ. When reconstituted into permeabilized PC12 and HL60 cells, PLCδ1, but not PLCβ1 or PLCγ1, shows substantial activation by physiologic calcium levels (4). Overexpression of PLCδ1 in PC12 and Chinese hamster ovary cells enhances the increase in intracellular Ca2+ and soluble inositol phosphate levels produced by bradykinin (5) and thrombin (6). The Ca2+-amplification role of PLCδ1 has been clearly defined in keratinocytes derived from PLCδ1-null mice in which the sustained elevation of cytoplasmic Ca2+ that follows a PLCγ1-stimulated rapid rise does not occur but can be reconstituted when PLCδ1 is introduced (7).

Several studies suggest that activation of PLCδ1 is under more complex control than the simple rise in cytoplasmic Ca2+. In frog oocytes expressing thrombin and platelet-derived growth factor receptors, microinjection of PLCδ1 antibody specifically inhibits thrombin but not platelet-derived growth factor-induced calcium mobilization (8). In Chinese hamster ovary cells, overexpression of PLCδ1 enhances the amount of inositol phosphates generated by ionomycin, but this increment is much smaller than the increase observed during thrombin stimulation (6). Similar results are obtained in bradykinin-stimulated PC12 cells expressing high levels of PLCδ1 (5).

Here, raising calcium with high extracellular potassium, thapsigargin, or ionomycin induces a measurable increase in inositol trisphosphate, yet this increment is substantially less than that observed with a maximum dose of bradykinin. Thus, although these observations support a generalized amplification hypothesis, they suggest that receptor-generated signals other than calcium also contribute to PLCδ1-dependent inositol phosphate generation.

Whereas protein regulators of most mammalian PLCs have been identified, those for PLCδ1 have not been well established. A novel form of RhoGAP associates strongly with PLCδ1 in cell lysates (9), stimulating its catalytic activity at low levels of calcium (0.1–1 μM). There is compelling evidence that PLCδ1 is also regulated by an atypical GTP-binding protein, Gz, or transglutaminase (10). Gz is controlled by α1-adrenergic receptor α1A and α1D in heart and liver (11–13), as well as oxytocin receptors in myometrium (14). PLCδ1 stimulates GDP/GTP exchange on TGI/Gz (11, 15), and reciprocally, Gz allows PLCδ1 to be stimulated at lower Ca2+ concentrations. Although these studies show that TGI/Gz couples heptahelical receptors to PLCδ1, the fraction of the total cellular response that this represents is unclear.

Other regulators of PLCδ1 have been suggested. Association of PLCδ1 to GAP43 results in a rise in cytoplasmic Ca2+, although it is uncertain whether this is because of increased membrane

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‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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The abbreviations used are: PLC, phospholipase C; PH, pleckstrin homology; PtdIns, phosphatidylinositol; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; YFP, yellow fluorescent protein; BiFC, bimolecular fluorescence complex; HBSS, Hanks’ balanced salt solution; DEPC, diethyl pyrocarbonate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PTX, pertussis toxin; HEK, human embryonic kidney; Ins(1,4,5)P3, inositol 1,4,5-trisphosphate; DABCYL, 4-(dimethylamino)phenylazo-phenyl-4-sulfonyl chloride succinyl ester.

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association of PLCδ1 (16). The activity of PLCβ3 is suppressed by a inactive PLCδ4-Alt3 variant (17), but it is unclear whether comparable regulators of other PLCδ isoforms exist.

Most PLCβ and PLCδ isoforms are widely expressed with varying tissue distribution. PLCβ2, which is the focus of this study, is expressed at high levels in cells of hematopoietic origin. Mice lacking this isoform have abnormal chemokine signaling, possibly through Gβγ released from Gαo-coupled receptors (18). PLCδ1 is the most widely distributed PLCδ isotype and is most strongly expressed in skeletal muscle, some layers of the skin, the spleen, testis, and lung (1, 3, 19). In the adult brain, PLCδ1 is found primarily in glial cells (20, 21); however, like PLCβ2, its presence is low in most neurons.

Previous work in our laboratories has focused on characterizing the regulation of PLCδ6 and PLCβ2. Here we report that PLCδ inhibits PLCδ and that this inhibition is relieved upon binding of Gβγ to PLCβ2. Our results suggest a novel mechanism in which G protein stimulation has the ability to amplify PLCβ-mediated Ca2+ signals through simultaneous activation of PLCδ1 under some cellular conditions.

Materials and Methods

In Vitro Fluorescence Studies—Gβγ2 subunits and PLCβ2 were expressed in Sf9 cells by using a baculovirus system (22). Gβγ2 subunits were reconstituted into preformed lipid bilayers by simple addition (23). PLCδ2 was bacterially expressed and purified (24). Association measurements between membrane-bound proteins were carried out in large unilamellar vesicles composed of 67% anionic 1-palmitoyl-2-oleoyl-sn-phosphatidylcholine and 33% 1-palmitoyl-2-oleoyl-sn-phosphatidyl ethanolamine, which both PLc enzymes are membrane-bound above 250 μM total lipid (data not shown) (25, 26). Protein-protein association was assessed using the fluorescence methods described previously (27). Briefly, proteins were labeled with either cyantrycyl- or cyantrycyl-sulfonate-modified photoreactive reagent (26, 28). The labeled proteins were excited at 340 nm and scanned from 350 to 500 nm. The emission intensity was taken from the integrated area of the spectrum. We found that the emission intensity of labeled PLCδ2 showed a substantial and reproducible increase upon the addition of unlabeled PLCδ3, and gave a titration curve that showed the appropriate shift in midpoint when the initial concentration of acrylodan-PLCδ was changed, thereby reflecting protein-protein association. Protein-protein associations were also assessed by fluorescence resonance energy transfer using coumarin- and DABCYL-labeled proteins and were carried out similarly by using the methods and analysis described previously (28).

PLC Activity Measurements—PLC activity measurements were conducted by using purified proteins and detergent-mixed micelles subsequent previously (26, 28).

Construction of BiFC Plasmids—The vectors used to construct YFP fusion proteins were generously provided by Dr. Thomas Kerppola (University of Michigan). These vectors contained YFP fragments (FLAG/Y-Nmd3 or FLAG/YC-Smad4) that can associate to form a bimolecular fluorescent complex (BiFC). The coding sequence of human PLCβ2 was amplified by using PCR with PLCβ2-pVL1392 plasmid as template and was subcloned through EcoRI/XbaI sites into the FLAG/Y-Nmd3 plasmid. The plasmid set was 5′-CGGGGCGGGGCTGGGATCCGCAAAAGGAGAAGGCAGGGAATTTCTGCAAAGAGAAGACG-3′ and 3′-CCCAACGGTCCAGCTAAGGGTGCGGTCCTTCTTTTCTGCGGGG-5′, which contained restriction enzyme sites EcoRI and XbaI (underlined), respectively. PLCβ2 was subcloned through an EcoRI/XbaI site into FLAG/Y/Nmd3 vector. Similarly, human PLCδ2, was amplified by using PCR with PLCδ2-pet3a plasmid as template (the primer set was 5′-GTTTAACTGGTACCAGGAGATAAGGTTTACTCCGTC-3′ and 3′-CTCACCGAGGGGCCCTCCCTAGATCTTTCAGCCTAGTCC-5′, which contained restriction enzyme sites KpnI and XbaI (underlined), respectively) and was subcloned through KpnI/XbaI sites into FLAG/Y-Cmd3 vector. The PLCβ2-FLAG/Y-Nmd3 and PLCδ2-FLAG/YC-Smad4 plasmids were sequenced by checking their reading frames.

Cell Culture and Overexpression of PlCαs—HEK293 cells and A10 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. For A10 cells, the medium was supplemented with 1 mM sodium pyruvate at 37 °C in 5% CO2. For fluorescence studies, HEK293 cells were cotransfected with BiFC-PLCβ2/PLCδ1 vectors (15 μg/106 cells in 60-mm dish) by calcium phosphate coprecipitation. HEK293 cells transfected with either BiFC-PLCβ2/PLCδ1 vectors or vectors lacking the BiFC tags were used in the Ca2+ and inositol phosphate studies as both produced identical results. A Western blot showing the levels of expression is given in Fig. 3. In the pertussis toxin (PTX)-treated cells, HEK293 cells were incubated with 100 ng/ml for 16–20 h.

Imaging of Fluorescence in Living Cells—24 h after transfection, cells from 60-mm dishes were plated into Labtek chambered glass coverslips (Bio-Rad). Fluorescence emission from living cells was measured 48–72 h after transfection on a Zeiss Axiovert microscope using 63× 1.4 numerical aperture objective.

Emission spectra of BiFC complexes from a 100-mm dish of living cells were taken on an ISS spectrofluorometer (Champaign, IL). Cells expressing BiFC-PLCβ2/PLCδ1 were placed in a 1-cm cuvette with stirring at an adjusted concentration of 1 × 106 cells/ml. Spectra were taken at λexc = 500 nm and scanning from 535 to 600 nm. Cut-off filters (525 nm, Corion Optical) were placed before the monochromators to remove the amount of scattered light. Background spectra were obtained from cultures transfected with only one of the BiFC constructs. Background spectra, which contributed 12–25% of the signal, were subtracted from the corresponding sample spectra.

For immunostaining, cells were grown on Labtek chambers, fixed in 4% formaldehyde solution in PBS, and permeabilized in 0.1% Triton X-100. Cells were blocked in PBS containing 5% goat serum, 1% BSA, and 50 mM glycine overnight. The monoclonal antibody anti-PLCδ1 (Upstate Biotechnology, Inc.) was used as the primary antibody. Polyclonal antibody against PLCβ2 (Santa Cruz Biotechnology) was also used as the primary antibody. Primary antibodies were diluted 1:200 in PBS containing 0.5% BSA. Cells were incubated in primary antibody at 37 °C for 1 h. This was followed by three washes of 7 min each in PBS. Secondary antibodies were diluted at 1:2000 in PBS, 0.5% BSA. Fluorescence was excited with cyantrycyl- or cyantrycyl-sulfonate-modified photoreactive reagent. Secondary antibodies were added to detect PLCδ1 antibody and DsRed-conjugated anti-mouse secondary antibody was used to detect antibody against PLCβ2. After 1 h of incubation at 37 °C, the cells were washed by PBS (three times at 7 min each). Finally, PBS was added to the cells, and the specimens were viewed under the Zeiss Axiovert fluorescence microscope.

Measurement of Cellular [Ca2+].—Cellular [Ca2+]i was determined with the fluorescent calcium indicator dye fura-2/AM in an ISS spectrofluorometer. Briefly, cell monolayers were labeled with the fluorescent calcium indicator dye fura-2/AM in an ISS spectrofluorometer. Briefly, cell monolayers were washed with Hanks’ balanced salt solution (HBSS) (118 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 1 mM Heps, 1% BSA, pH 7.4), and the cells were detached by a buffer stream. Suspended cells were labeled with 1 μM fura-2/AM for 45 min at 37 °C in HBSS with rotation. Thereafter, cells were washed twice and incubated in HBSS for another 20 min. After centrifugation, the cells were resuspended at a density of 1 × 106 cells/ml and were measured in a continuously stirred cell suspension at room temperature. The ratio of fluorescence emitted at 340 and 380 nm (340:380 ratio) of cells was converted to Ca2+ concentration by the relation: [Ca2+]i = (R−Rmin)/Rmax−R×(F380/F380)×Kd (nm). The Fura-2/AM Kd value is ~225 nM. Fmax is the fluorescence at that wavelength in EDTA buffer, and F380 is the fluorescence in the presence of detergent and excess calcium. R is the measured ratio, and Rmin and Rmax are the ratios corresponding to the EDTA and detergent/excess calcium conditions, respectively. In some experiments, [Ca2+]i was determined in the absence of extracellular Ca2+ by treatment cell with 1 mM EDTA.

Measurements of Inositol Phosphate Formation and Phosphoinositide Analysis in intact Cells—Cells were prelabeled with myo-[3H]inositol (1 μCi; Amersham) 2 days in medium. The cells were harvested, kept in suspension, and incubated for 10 min at 37 °C with HBSS/LiCl (118 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM t-glucose, and 15 mM Hepes, pH 7.4, supplemented with 10 mM LiCl), before challenging with agonist. For agonist stimulation, the cells were incubated with agonist (5 μM carbachol) in the presence of LiCl for 30 min at 37 °C. All reactions were stopped by removing the incubation medium and lysing the cells in 1 ml of ice-cold methanol. After the addition of 1 ml of
chloroform and 0.5 ml of H2O, phase separation was performed by centrifugation at 2000 g for 10 min at 4 °C. The aqueous upper phase was applied to AG 1-X8 anion exchange columns to isolate myo-[3H]-inositol phosphate formation. For determination of phosphoinositide levels, the lower phase was collected and evaporated by vacuum drying, and the lipids were resuspended in 50 l of chloroform and spotted onto LK5D linear-k silica gel TLC plates (Whatman). The plates were developed in chloroform, methanol, 2.5M ammonium hydroxide (9:7:2, v/v). The areas corresponding to authentic PtdIns (Rf 0.64), PtdIns(4)P (Rf 0.45), and PtdIns(4,5)P2 (Rf 0.25) were scraped into vials, and the radioactivity was measured by liquid scintillation counting. The amount of disintegrations/min was normalized for protein content.

RESULTS

PLCβ2 and PLCδ1 Strongly Interact on Model Membrane Surfaces—This study began with the unexpected finding that PLCβ2 and PLCδ1 form heteromeric complexes in solution and on membrane surfaces. We directly measured the formation of PLCβ2-PLCδ1 complexes by using fluorescence methods to quantify the association energy between proteins. Protein-protein association was monitored by the change in the fluorescence emission of the probe acrylodan covalently linked to PLCβ2 (see “Materials and Methods”). This probe undergoes an increase in emission intensity and energy upon the association of Gβγ subunits with PLCβ2 and yields a dissociation constant identical to that obtained by using fluorescence resonance energy transfer (27). In Fig. 1A, we show the normalized increase in acrylodan-PLCβ2 fluorescence when PLCδ1 is added in solution. This increase was not observed when buffer or unlabeled PLCβ2 was substituted for PLCδ1. These data can be fit to a bimolecular association curve to give an apparent Kd value (23). Control studies substituted buffer or unlabeled PLCβ2 for PLCδ1. B, identical data were obtained at 0 and 12 μM free Ca2+ (data not shown). C, decrease in the fluorescence of the energy transfer donor, coumarin-PLCδ1, upon the addition of a nonfluorescent energy transfer acceptor, DABCYL-PLCβ2, due to transfer. Fluorescence energy transfer, measured by the decrease in intensity, is inhibited in the presence of 10 nM Gβγ. All data are an average of 3–5 measurements, and S.E. is shown.

FIG. 1. PLCβ2 binds to PLCδ1 in vitro at a site that is mutually exclusive with Gβγ subunits. Association of PLCδ1 to 50 nM acrylodan-PLCβ2 in solution (A) or on 300 μM phosphatidylcholine/phosphatidylserine (1:2) lipid bilayers (B) as determined by the increase in acrylodan fluorescence that occurs upon protein association. Experimental data were corrected for dilution and background and fit to a bimolecular association curve to give an apparent Kd value (23). Control studies substituted buffer or unlabeled PLCβ2 for PLCδ1. B, identical data were obtained at 0 and 12 μM free Ca2+ (data not shown). C, decrease in the fluorescence of the energy transfer donor, coumarin-PLCδ1, upon the addition of a nonfluorescent energy transfer acceptor, DABCYL-PLCβ2, due to transfer. Fluorescence energy transfer, measured by the decrease in intensity, is inhibited in the presence of 10 nM Gβγ. All data are an average of 3–5 measurements, and S.E. is shown.
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PLCβ₂/PLCδ₁ complex, we chemically treated PLCδ₁ with diethyl pyrocarbonate (DEPC). This agent forms a covalent adduct with one or both of the catalytic His residues, thereby inactivating the histidine-dependent enzyme (34). Treatment of PLCδ₁ resulted in complete loss of PLCδ₁ activity but did not affect its ability to keep PLCδ₁ membrane-bound as long as substrate is present in the membrane and the amount of product in the aqueous phase is low. In contrast, the corresponding pleckstrin homology of PLCβ₂ binds to membranes with little specificity (33). Addition of Ins(1,4,5)P₃ to PLCδ₁ should greatly reduce its activity because it will inhibit the binding of the PH domain to substrate-containing membranes. Additionally, the presence of product will compete for substrate in the catalytic site, although very high levels of product are needed for inhibition by this latter process. Conversely, product inhibition of PLCβ₂ will only occur at high levels of product. We measured the ability of Ins(1,4,5)P₃ to inhibit the PLCβ₂/PLCδ₁ mixture, and we found that the activity of the mixtures was not significantly changed. This result suggests that the activity of PLCδ₁ may be inhibited when bound to PLCβ₂.

To better isolate which enzyme is inhibited in the PLCβ₂/PLCδ₁ complex, we chemically treated PLCδ₁ with diethyl pyrocarbonate (DEPC). This agent forms a covalent adduct with one or both of the catalytic His residues, thereby inactivating the histidine-dependent enzyme (34). Treatment of PLCδ₁ resulted in complete loss of PLCδ₁ activity but did not affect its ability to...
bind substrate membrane, and the apparent affinity with PLCβ2 on membrane surfaces fell within the error of its unmodified enzyme (K<sub>app</sub> = 10 nM on 200 µl lipid). Most importantly, addition of DEPC-treated PLCδ1 to wild type PLCβ2 up to 20 nM did not change its rate of PI(4,5)P<sub>2</sub> hydrolysis (Fig. 2B).

We further tested whether PLCβ2 inhibits PLCδ1 activity by using a point mutant of PLCβ2, H327N. This residue is necessary to stabilize the transition state charge of the phosphate group, and mutation of the corresponding residue on PLCδ2 profoundly reduces activity (35, 36). We find that the enzymatic activity is reduced 50–100-fold, and as expected, its binding to membranes decreases 4-fold with increasing amounts of H327N-PLCβ2, with an EC<sub>50</sub> similar to the K<sub>d</sub> for PLCδ1 association (Fig. 2B). These results demonstrate that PLCβ2 suppresses the catalytic activity of PLCδ1 at activating levels of Ca<sup>2+</sup>.

**Gβγ Subunits Disrupt PLCβ-PLCδ Association in Vitro—** PLCβ<sub>2</sub> and PLCδ<sub>1</sub> both bind to Gβγ subunits, although binding to the former enzyme is of higher affinity and results in enzyme activation (33). We tested whether Gβγ subunits could interfere with PLCβ-PLCδ association by measuring complex formation on membranes in the absence or presence of Gβγ subunits by fluorescence resonance energy transfer using the coumarin-DABCYL donor-acceptor pair. Addition of DABC-SYL-PLCβ<sub>2</sub> to coumarin-PLCδ<sub>1</sub> resulted in a decrease in donor fluorescence as the two proteins associate (Fig. 1C), but this decrease was largely prevented by the addition of 10 nM Gβγ.

**Association of PLCβ2 and PLCδ1 in Living Cells—** To determine whether the two PLC subtypes associate in living cells, we initially used indirect immunofluorescence to view endogenous PLC in fixed rat aorta smooth muscle cells (A10) because these cells express both PLCs (see Ref. 37 and Supplemental Material). Images of these cells showed several pools of colocalized PLCβ2 and -δ1 that were significantly reduced when the cells were stimulated with acetylcholine before fixing and staining, although the relatively low signal limited interpretation of these images (data not shown). As an alternative approach, we used the technique of bimolecular fluorescence complementation (BiFC) (38). In this method, the N-terminal portion of YFP is linked to a target protein, whereas the C-terminal region encompassing the four missing β strands is linked to a potential binding partner (39). Association between the proteins reconstitutes the fluorescent YFP fluorophore. BiFC-PLCδ1/PLCβ2 plasmids were transfected in HEK293 cells, which exhibit relatively low levels of endogenous PLCδ1 and -β2. We find that although a small amount of fluorescence is cytosolic, most of the reconstituted YFP fluorescence is confined to the plasma membrane (Fig. 3).

Epifluorescence images of HEK293 cells expressing BiFC-PLCδ1/PLCβ2 before and after stimulation with acetylcholine are shown in Fig. 4. An intense yellow was observed, which rapidly decreased following stimulation. In contrast, no fluo-

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**Behavior of BiFC-PLCβ2 / PLCδ1 in Transfected HEK Cells**

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![Epifluorescence images of HEK293 cells expressing BiFC-PLCδ1/PLCβ2 before and after stimulation with acetylcholine are shown in Fig. 4. An intense yellow was observed, which rapidly decreased following stimulation. In contrast, no fluorescence was detected in the cytosol.](image-url)
rescence was observed in cells transfected with only one of the BiFC-PLC plasmids or with the BiFC-PLC/HP2/Fos pair. Positive controls using BiFC-Fos/Jun showed bright fluorescence restricted to the nucleus (Fig. 4). Also, no fluorescence could be detected for the BiFC-PLC/HP2/Fos under conditions where a significant population of Fos was cytosolic (40), suggesting that the BiFC tags were not strongly promoting association between nonphysiologic protein partners.

In Fig. 4 we also show a representative epifluorescence image of a BiFC-PLC/HP2/PLC/HP2-transfected cell that was stimulated with the G protein-coupled receptor agonist acetylcholine. We find a significant decrease in total BiFC-PLC/HP2/PLC/HP2 fluorescence after correcting for photobleaching in the first few minutes following agonist addition (Fig. 4, graph). At longer times (5–30 min), the intensities of all samples monitored were recovered to near the initial values. These studies demonstrate a dissociation of the BiFC PLC/HP2/PLC/HP2 upon G protein stimulation.

To determine whether the loss in BiFC PLC/HP2/PLC/HP2 fluorescence resulted from specific pools of BiFC PLC/HP2/PLC/HP2, we monitored changes of the cellular distribution of the proteins in HEK293 cells by confocal microscopy (e.g., Fig. 3). We found also that stimulation of the cells using 1 μM carbachol diminished the overall fluorescence. Digitizing the images and comparing the changes in intensity from the plasma membrane population versus the cytosolic compartment suggested that the loss of YFP fluorescence was from the plasma membrane pool.

To sample the effect of G protein stimulation on a larger population of cells, we repeated the experiments with a suspension of cells in a spectrofluorometer. In Fig. 5A (closed circles), we show that addition of acetylcholine, but not the unrelated agonist insulin, causes a significant drop in the BiFC-PLC/HP2/PLC/HP2 fluorescence within the first 5 min. This response, which is similar to that seen for the attached cells, indicates a disruption of the BiFC-PLC/HP2/PLC/HP2 complex in response to G protein activation. The extent of this drop varied from 18 to 47% of the initial fluorescence, which appeared to be related to differences in the expression level of the proteins (Fig. 5A). All samples recovered to near initial values at longer times.

Acetylcholine will release activated Gαq and Gαi subunits as well as Gβγ subunits. To test which was involved in the changes of BiFC-PLC/HP2/PLC/HP2 fluorescence, we pre-treated the cells with PTX to inhibit activation of Gαi, because previous studies have shown HEK293 cells to be PTX-sensitive (41, 42). PTX treatment, which prevents Gβγ release from Gαi, was
found to eliminate completely the decrease in fluorescence (Fig. 5, open squares). These results imply that Gβ subunits released from Goi are required for the decrease in BiFC-PLCδ2/PLCβ2 fluorescence.

To assess directly the ability of Gβγ subunits to disrupt BiFC-PLCβγ/PLCδ1 complexes, membranes were prepared from HEK293 cells transfected with the BiFC-PLCβγ/PLCδ1 plasmids. We then measured the reduction in YFP fluorescence...
as Gβγ subunits or unlabeled PLCβ2 is added in vitro. As shown in Fig. 5B, we find that we can completely eliminate BiFC-PLCβ2/PLCδ1 fluorescence by the addition of unlabeled PLCβ2 or Gβγ subunits.

The Effect of Increased PLCβ2 and/or PLCδ1 on Intracellular Ca2+ and Inositol Phosphate Levels—The physical association between the two proteins described above suggested that the activity of PLCδ1 would be suppressed by cotransfection with PLCβ2. This prediction was first tested by monitoring cellular Ca2+ levels in living HEK293 cells transfected with PLCβ2 or PLCδ1 expression plasmids or both by using the calcium indicator dye Fura-2. The results are presented in Fig. 6. Transfection of these cells with PLCβ2 does not significantly perturb either the basal or stimulated levels of Ca2+. In contrast, transfection with PLCδ1 results in a significant increase in both the basal and stimulated levels of Ca2+. However, cotransfecting PLCδ1 with PLCβ2 reduces these Ca2+ levels to values much closer to those of control untransfected cells. These data support the idea that PLCβ2 binds to PLCδ1 and inhibits its PI(4,5)P2 hydrolyzing activity.

In Fig. 6 we also present traces showing the recovery of elevated Ca2+ after stimulation from a single series of studies. Pooling 9–12 sets performed in triplicate allowed us to analyze the time dependence of the decrease in elevated cellular Ca2+ following stimulation. We find that the recoveries of all but PLCδ1-transfected cells could be fit to a single exponential decay to give 0.0192 ± 0.003 nM [Ca2+]/s (control cells), 0.0168 ± 0.007 [Ca2+]/s (PLCβ2-transfected cells), and 0.0177 ± 0.004 [Ca2+]/s (PLCβ2/PLCδ1-transfected cells). However, PLCδ1-transfected cells either did not recover (5 of 12 studies) or recovered too slowly to be fit to an exponential (7 of 12 studies). This persistence in Ca2+ levels due to PLCδ1 most likely results from enhancement of PI(4,5)P2 levels in the cells. It is interesting that coexpression of PLCβ2 with PLCδ1 reduces this persistent period to control levels. Recovery occurs during the time that the two enzymes are separated (see Fig. 5), suggesting that their coexpression entails feedback mechanisms in addition to or other than PLCβ2 regulation.

To verify that changes in cellular Ca2+ shown in Fig. 6 reflected inositol phosphate generation, we measured changes in the total inositol phosphate production in the basal and stimulated states in cells transfected with PLCβ2, PLCδ1, or both. The results, presented in Fig. 7A, show that cells transfected with PLCβ2 display a small increase in inositol phosphate levels, whereas transfection with PLCδ1 alone resulted in much higher inositol phosphate production. Transfection with both enzymes returns the inositol phosphate levels to those seen for PLCβ2 alone. This same suppression of PLCδ1-
induced increase in inositol phosphate levels could be seen upon stimulation with 5 μM carbachol. Thin layer chromatography of the inositol lipids showed that the basal levels of [3H]PI(4,5)P$_2$ are maintained in cells transfected with PLC$_{\beta 2}$, whereas the levels of this lipid are elevated when the cells are transfected with PLC$_{\delta 1}$ alone (Fig. 7B).

The in vitro results predict a synergistic release of Ca$^{2+}$ upon agonist stimulation that was not clearly seen in HEK293 cells. However, signaling in these cells may be limited at the level of receptor. To test this idea, we cotransfected Gβ$_1$γ$_2$, with BiFC-PLC$_{\beta 2}$/PLC$_{\delta 1}$ in HEK293 cells. We found that overexpression of Gβ$_1$γ$_2$ reduced BiFC from PLC$_{\beta 2}$/PLC$_{\delta 1}$ fluorescence from an initial normalized value of 1.00 ± 0.35 to 0.30 ± 0.01 (n = 3) relative to control cells that were transfected with empty vector. Thus, the presence of excess Gβγ results in a 70% reduction in BiFC.

We further tested whether enhanced Ca$^{2+}$ release by dissociation of PLC$_{\beta 2}$/PLC$_{\delta 1}$ complexes was limited at the receptor/G protein level in another series of studies utilizing rat vascular smooth muscle A10 cells. Western blot analysis has suggested that these cells have high endogenous levels of PLC$_{\beta 2}$ and only trace amounts of PLC$_{\delta 1}$ (37). We transfected these cells with small amounts of PLC$_{\delta 1}$ and observed a 30% increase in Ca$^{2+}$ release (r = 0.004) over mock-transfected cells with stimulation using 10 μM carbachol. However, transfection with higher amounts of PLC$_{\delta 1}$ resulted in increased basal Ca$^{2+}$ and a corresponding rise in stimulated levels indicating that there is not enough available PLC$_{\beta 2}$ to control the robust PLC$_{\delta 1}$ activity. Therefore, it is possible to observe a synergistic release of Ca$^{2+}$ with PLC$_{\delta 1}$ over a narrow range of expression.

**DISCUSSION**

The studies presented here demonstrate that PLC$_{\beta 2}$ controls PLC$_{\delta 1}$ activity. This control involves inhibition of PLC$_{\delta 1}$ through its physical association with PLC$_{\beta 2}$ at physiological concentrations of Ca$^{2+}$. The association between these PLCs can be recapitulated in living cells as can the inhibition of increased cellular Ca$^{2+}$ and inositol phosphate levels due to overexpression of PLC$_{\beta 2}$. These findings connect the regulation of PLC$_{\delta 1}$ activity with G protein-coupled receptor signaling.

By using purified proteins on model membranes, we find that PLC$_{\beta 2}$ and PLC$_{\delta 1}$ associate and that this association is not simply because of nonspecific aggregation of the proteins. These proteins associate with moderate affinity in solution and a 20-fold more strongly when bound to lipid bilayers. Binding of proteins to a membrane surface confines them to a more restricted area and increases their effective concentration. We have previously treated the difference in dissociation constants between proteins in solution and bound to membranes, and we found the decrease in affinity between PLC$_{\beta 2}$ and PLC$_{\delta 1}$ to be appropriate (23). PLC$_{\beta 2}$ binds strongly and fairly nonspecifically to membranes (26) in contrast to PLC$_{\delta 1}$, which only binds strongly to membranes that are highly negatively charged or if PI(4,5)P$_2$ is present (25). The strong affinity for the two proteins on membrane surfaces allows PLC$_{\beta 2}$ to laterally associate with PLC$_{\delta 1}$ to suppress its activity under unstimulated conditions.

It is noteworthy that the strength of PLC$_{\beta 2}$/PLC$_{\delta 1}$ association on membrane surfaces is only ~5-fold weaker than that of PLC$_{\beta 2}$/Gβγ (28) and may thus be competitive under many physiological conditions. The mechanism of inhibition, however, is not clear. Although PLC$_{\delta 1}$ binds strongly and specifically to membranes containing PI(4,5)P$_2$, no such specificity is observed for PLC$_{\beta 2}$, and so inhibition is not because of competition of the enzyme for substrate or membrane binding. Because the PH domains of both enzymes play a role in catalytic activity, and because the PH domains of both enzymes bind Gβγ subunits, this region is a reasonable candidate for inhibition.

We note that we have preliminary evidence suggesting that the PH domain of PLC$_{\delta 1}$ binds to PLC$_{\beta 2}$ with an affinity in range of the whole enzyme. We speculate that the PLC$_{\beta 2}$/PLC$_{\delta 1}$ molecular association may be similar to the inhibitory association between PLC$_{\delta 1}$ and the inactive PLC$_{\delta 2}$/Alf3, which also appears to involve the PH domain. Molecular models that dock the crystal structures of the PH and catalytic domains of PLC$_{\delta 1}$ suggest a very close proximity of the PH domain to the catalytic site (27), leading to the idea that altered domain interactions could inhibit the conformational changes needed for effective catalysis. We note that while this study focused on PLC$_{\beta 2}$ which binds strongly to Gβγ subunits (28), it is likely that similar results would be obtained with the widely distributed PLC$_{\beta 2}$/Gβγ isozymes, which are also regulated by Gβγ subunits (43), and we find the PLC$_{\delta 1}$ association for PLC$_{\beta 2}$ to be similar to PLC$_{\beta 2}$. Cellular association between the two former proteins is currently under investigation.

PLC$_{\delta 1}$ is activated by increases in cellular Ca$^{2+}$ and inhibited by a high local concentration of Ins(1,4,5)P$_3$ that prevents substrate binding (29). Regulation of PLC$_{\delta 1}$ by RhoGAP and transglutaminase (9, 44) is through lowering the Ca$^{2+}$ concentration that induces PLC$_{\delta 1}$ activation. We find here that PLC$_{\beta 2}$ inhibition of PLC$_{\delta 1}$ occurs even at high physiological levels of Ca$^{2+}$, suggesting the mechanism of regulation is quite different from RhoGAP and transglutaminase. By maintaining PLC$_{\delta 1}$ in an inactive state, PLC$_{\beta 2}$ confers agonist-specific control on this Ca$^{2+}$-stimulated enzyme. The inability of Ca$^{2+}$ to disrupt the PLC$_{\beta 2}$/PLC$_{\delta 1}$ complex suggests that only PLC$_{\delta 1}$ populations not associated with PLC$_{\beta 2}$ will be activated upon an increased in Ca$^{2+}$ pathways other than those involving heterotrimeric G proteins. Of course, this regulation mechanism of PLC$_{\delta 1}$ will only occur in cell lines that express both proteins. Although there are many reports identifying coexpression of these proteins by Western blot analysis in cultured cells lines, such as A10 and PC12 (37, 45, 46), we note that almost all of these studies employed the same commercial polyclonal PLC$_{\beta 2}$ antibody, which may not have high specificity. Weak anti-PLC$_{\delta 1}$/PLC$_{\delta 1}$ interaction precludes identifying these complexes by communoprecipitation, and so we verified expression of mRNA of both PLC$_{\beta 2}$ and PLC$_{\delta 1}$ in heart and brain tissue and in A10 and PC12 cells by reverse transcription-PCR (Supplemental Material). These results suggest that some cell lines express both proteins. It is also noteworthy that preliminary studies show that more widely expressed PLC$_{\beta 2}$, which is also stimulated by Gβγ subunits, similarly inhibits PLC$_{\delta 1}$ in vitro.

Most interestingly, we find that in living cells the PLC$_{\beta 2}$/PLC$_{\delta 1}$ complex is mainly localized on the plasma membrane, with a significant population diffusely distributed throughout the cytosol. Our studies suggest that G protein stimulation disrupts the plasma membrane-localized complexes as compared with the cytosolic. Whereas association between internal PLC$_{\beta 2}$/PLC$_{\delta 1}$ is expected to suppress phosphatidylinositol lipid hydrolysis at these internal sites, it is unclear what causes dissociation of the cytoplasmic PLC$_{\beta 2}$/PLC$_{\delta 1}$ complex, although it is unlikely to be the rise in cytoplasmic free calcium concentration, because complex stability seems unperturbed by this dvalent cation. The simplest mechanism could involve the generation of excess free Gβγ subunits at the plasma membrane that would shift the dynamic equilibrium in favor of PLC$_{\beta 2}$/PLC$_{\delta 1}$ plasma membrane association, which is supported by recent work (47). From this latter work, it is also possible that cytosolic PLC$_{\beta 2}$ is regulated by RhoGTPases, which may also inhibit its association with cytoplasmic PLC$_{\delta 1}$.

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2 S. Scarlata and M. Rebecchi, unpublished results.

3 Y. Guo and S. Scarlata, unpublished results.
PLCβ2-PLCδ1 association in living cells was monitored by BiFC. This technique has recently been established to detect in vivo protein-protein associations (38). Protein-protein association, which is measured as increased fluorescence, is technically easier to assess than fluorescence resonance energy transfer. Because the formation of the BiFC-YFP chromophore may typically easier to assess than fluorescence resonance energy transfer, which is measured as increased fluorescence, is technically easier to assess than fluorescence resonance energy transfer.

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