Specificity and Structural Requirements of Phospholipase C-β Stimulation by Rho GTPases Versus G Protein βγ Dimers

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Phospholipase C-β2 (PLCβ2) is activated both by heterotrimeric G protein α- and βγ-subunits and by Rho GTPases. In this study, activated Rho GTPases are shown to stimulate PLCβ isoforms with the rank order of PLCβ2 > PLCβ2 ≥ PLCβ1. The sensitivity of PLCβ isoforms to Rho GTPases was clearly different from that observed for G protein βγ dimers, which decreased in the following order: PLCβ2 > PLCβ2 > PLCβ1 for βγ12 and PLCβ2 > PLCβ2 ≥ PLCβ2 for βγ2 γ2. Rac1 and Rac2 were found to be more potent and efficacious activators of PLCβ2 than was Cdc42Hs. The stimulation of PLCβ2 by Rho GTPases and G protein βγ dimers was additive, suggesting that PLCβ2 activation can be augmented by independent regulation of the enzyme by the two stimuli. Using chimeric PLCβ1-PLCβ2 enzymes, βγ dimers, and Rho GTPases are shown to require different regions of PLCβ2 to mediate efficient stimulation of the enzyme. Although the catalytic subdomains X and Y of PLCβ2 were sufficient for efficient stimulation by βγ, the presence of the putative pleckstrin homology domain of PLCβ2 was absolutely required for the stimulation of the enzyme by Rho GTPases. Taken together, these results identify Rho GTPases as novel PLCβ regulators, which mediate PLCβ isozyme-specific stimulation and are potentially involved in coordinating the activation of PLCβ2 by extracellular mediators in intact cells.

Many extracellular signaling molecules elicit intracellular responses by activating inositol phospholipid-specific phospholipases C (PLCs),1 which hydrolyze phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) to produce the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. These two second messengers modulate intracellular events through the regulation of intracellular free Ca²⁺ and protein kinase C isozymes, respectively. The mammalian PLC isoforms can be divided into four major families: PLCα, PLCγ, PLCδ, and PLCε (1). The PLCβ and PLCγ subclasses have been shown to be regulated through G protein-coupled and protein-tyrosine kinase-linked receptors, respectively. The mechanisms by which PLCβ isoforms and PLCε are coupled to membrane receptors are less well understood (for recent reviews, see Refs. 1–4). Stimulation of PLCβ, of which four isozymes (PLCβ1-PLCβ4) are known, is mediated by members of the αq subfamily of G protein α subunits and, excepting PLCβ1, by G protein βγ dimers (1–4). Activated αq subunits stimulate PLCβ in the rank order of efficacy of PLCβ1 > PLCβ2 > PLCβ2. PLCβ2 is also activated by αq subunits. The sensitivity of PLCβ isoforms to βγ dimers decreases in the order: PLCβ1 > PLCβ2 > PLCβ2 (3, 4).

Mammalian PLCβ isoforms are differentially expressed in various tissues and cell types (5, 6). At the protein level, PLCβ1 is highly expressed in the central nervous system but is also present in several other tissues, e.g. adrenal gland, parotid gland, lung, and kidney (7–9). The PLCβ2 polypeptide is present at high levels in neutrophils and cultured myeloid cells but has also been detected in other cell types and tissues, including platelets (10), T lymphocytes (11), osteoblasts (12), vascular and tracheal smooth muscle cells (13, 14), cerebellum (12), spleen, and thymus (15). Myeloid cells have been found to contain both PLCβ2 and PLCβ2 (9, 16). However, PLCβ2 appears to be predominantly important in these cells, because inactivation of the PLCβ2 gene caused an almost complete loss of formyl peptide receptor-stimulated inositol phosphate formation in mouse neutrophils (15). Furthermore, PLCβ2, but not PLCβ1, was activated by complement C5a and formyl peptide receptors in transfected cells (17). The latter findings are intriguing in light of the fact that PLCβ3 has been shown to be stimulated to a remarkable extent by G protein βγ dimers in cell-free assays (9). In addition to myeloid cells, various other cell types and tissues contain the PLCβ1 isoform (8, 9). In contrast, the PLCβ2 protein shows a more limited tissue distribution and is primarily found in the retina and in specific regions of the brain (18, 19).

We have previously reported the identification of a PLCβ2-stimulating GTP-binding protein present in cytosolic fractions of bovine neutrophils (20). This G protein was shown to be a member of the Rho subfamily of GTPases, Cdc42Hs and/or Rac, associated with the Rho GDP dissociation inhibitor LyGDI (21). Rho GTPases form a subgroup of the Ras superfamily of GTP-binding proteins that have been shown to regulate a wide spectrum of cellular functions, including gene expression, cell cycle progression, and reorganization of the actin cytoskeleton (22–25). The activity of the Rho GTPases is determined by the ratio of their GTP/GDP-bound forms, which is regulated by at least three regulatory proteins: guanine nucleotide dissociation inhibitors, guanine nucleotide exchange factors, and GTPase-activating proteins (25).

Using purified proteins, we have previously demonstrated that PLCβ2 is activated by GTPγS-ligated Cdc42Hs and Rac1, but not by RacA, through direct protein–protein interaction (21). This stimulation has been shown to be independent of LyGDI but to require both C-terminal processing of the Rho

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1 The abbreviations used are: PLC, phospholipase C; LyGDI, a Rho guanine nucleotide dissociation inhibitor originally identified in lymphocytes, but subsequently also found in other cells; GTP-γ-S, guanosine 5′-O-(3-thiotriphosphate); PH, pleckstrin homology; PI-3-P, phosphatidylinositol 3-phosphate; PI-4,5-P₂, phosphatidylinositol 4,5-bisphosphate; PI-3,4,5-P₃, phosphatidylinositol 3,4,5-trisphosphate.
GTAPs and the integrity of their effector regulating domain (21). Like G protein βγ dimers, activated Rho GTAPs stimulated a deletion mutant of PLCβ2, PLCβ2Δ, lacking a C-terminal region necessary for stimulation by G protein α subunits (21). The specificity of PLCβ stimulation by Rho GTAPs and the mechanisms of enzyme activation by Rho GTAPs remained unknown. The goal of the study was to elucidate the sensitivity of PLCβ isozymes to stimulation by Rho GTAPs versus G protein βγ dimers, known activators of these enzymes, and to identify and to compare the structural requirements of PLCβ stimulation by these two regulatory proteins. The results show that the three PLCβ isozymes tested, PLCβ1, PLCβ2, and PLCβ3, are differentially sensitive to stimulation by activated Rho GTAPs and βγ dimers. The specificity of Rho GTAPase-mediated PLCβ stimulation also differs from that reported for αm-mediated PLCβ stimulation, with PLCβ2 being considerably less sensitive than PLCβ1 and PLCβ2 (1–4). This study identifies PLCβ2 as the PLCβ isozyme most sensitive to stimulation by Rho GTAPs, especially Rac1 and Rac2. Using PLCβ1 and PLCβ2 chimeras constructed on the basis of structural domains predicted by the crystal structure of PLCβ, we demonstrate that the presence of the catalytic subdomains X and Y of PLCβ2 is sufficient for βγ-dimer stimulation. In contrast, the presence of the putative pleckstrin homology (PH) domain of PLCβ2 is absolutely required for stimulation of the enzyme by activated Rho GTAPs. Taken together, the results demonstrate, for the first time, the unique regulation of the activity of PLCβ2 by monomeric GTAPs and G protein βγ dimers requiring different structural elements of this enzyme.

EXPERIMENTAL PROCEDURES

Recombinant PLCβ Isoforms, C-terminally Deleted PLCβ Mutants, and PLCβ1–PLCβ3 Chimeras—Construction of recombinant baculoviruses for expression of the bovine PLCβ and human PLCβ have been described (26, 20). The cDNA of human PLCβ2 (27) was cloned into the EcoRI site of the baculovirus transfer vector pVL1393 (Invitrogen, Carlsbad, CA).

The cDNAs of C-terminally deleted PLCβ constructs were generated such that the proteins retained their C-terminal-most portions (GEN-PLGKEDTPL, PLCβ2; QDPLIAKADAESRL, PLCβ2; and GAD-SEQQENTQ, PLCβ3) which had been used as synthetic peptides to generate PLCβ subtype-specific antisera in rabbits (28). PLCβ2Δ, a deletion mutant of human PLCβ2, lacking a C-terminal region (Phe139–Glu146) necessary for stimulation by αm, has been shown previously to be indistinguishable from wild-type PLCβ2 in terms of its interaction with PI-4,5-P2, Ca2+, and βγ dimers (29). The deletion mutants PLCβ2Δ and PLCβ3Δ, lacking the corresponding C-terminal regions, Val147–Leu203 and Ala227–Ser236, respectively, were generated by the PCR overlap extension method (30). Two PCR amplifications were performed using bovine PLCβ3 cDNA (GenBank accession number J03137) cloned into pVL1392 as template and the following two pairs of oligonucleotides as primers: 5′-CTGGGATTCTACTAATATGCG-3′ (upstream, sense), 5′-GGTTCTCCCTACTGATGTTGAC-GAAGC-3′ (internal, antisense), 5′-TCACATCGATGAGGACAA-AACCCGGAAAGACG-3′ (internal, sense), and 5′-CAGGATTTAACCAAAGATATG-3′ (downstream, antisense). The two amplified fragments, flanking the sequence to be deleted, were re-amplified using the upstream and downstream primers. The final PCR product and the vector pVL1392-PLCβ1 were digested with Nhel and BamHI, and the wild-type fragment was replaced by the corresponding mutant fragment. In the case of PLCβ3Δ, the two fragments flanking the upstream and downstream regions of the deletion were amplified using pVL1390-PLCβ3, as template and the following two pairs of oligonucleotides as primers: 5′-TGAGCCGAGGTCGCCGG-3′ (upstream, sense), 5′-ACAGGTCTCGGCTTGATGTTGACAGGC-3′ (internal, antisense), 5′-GATGGACGAGGAGGCGGACCAGGGCCG-3′ (internal, sense), and 5′-GGTTCTCCCTACTGATGTTGAC-GAAGC-3′ (downstream, antisense). The final PCR product and the vector pVL1392-PLCβ3 were digested with Nhel and the wild-type fragment was replaced by the corresponding mutant fragment. The PLCβ1–PLCβ3 chimeras were generated by the PCR overlap extension method. In chimera A, the N-terminal amino acids of PLCβ3 (residues 1–138) were replaced by the corresponding residues of PLCβ1 (residues 1–142), so that the putative PH domain was substituted. Substitution of the PH domain and the putative four EF-hand motives of PLCβ3 (residues 1–303) for the corresponding residues of PLCβ1 (residues 1–307) resulted in chimera B. Chimera A was constructed from the vectors pVL1392-PLCβ1 and pVL1393-PLCβ3 by PCR amplification using the following two pairs of primers: 5′-GAACTATGGGC-GGGGCCACAGGC-3′ (upstream, sense), 5′-GGAGGCCCTGGCTGCTGCGC-TCTCCTGCGGCGC-3′ (internal, antisense), 5′-ACACCTGCTGACGGCCACCGCCCTCCC-3′ (internal, sense), 5′-GGATCCAGGGCCCTGCTTCC-3′ (downstream, antisense). The two amplified fragments were re-annealed and re-amplified using the upstream and the downstream primers, which introduced an EcoRI and a BamHI recognition site, respectively. Chimera B was constructed using PCR upstream and downstream primers and the following two inner primers: 5′-GAAAAATGAGTTGCTGCGCCGAGACAGGACGAC-GCTG3′ (internal, antisense) and 5′-CAGCTTTGCTCGGGCGACGACGACGTC-3′ (internal, sense). The final fragments were then ligated at their EcoRI and BamHI sites, and the resulting constructs were inserted into the baculovirus transfer vector pVL1392. The entire PCR-amplified regions were sequenced and found to be identical to the expected sequences. Production of recombinant baculoviruses, expression, and isolation of PLCβ isoforms were carried out according to published protocols (26).

Recombinant Rho GTAPs—The production of recombinant baculoviruses using BaculoGold DNA (BD Pharmingen, San Diego, CA) and the cDNAs of the bovine PLCβ isozymes cloned into pVL1392 as template and the following two pairs of oligonucleotides as primers for expression of the bovine PLCβ and human PLCβ have been described (26, 20). The production of recombinant baculoviruses required solubilization of Rho GTAPs and/or 5 μl of purified βγ were supplemented with 10 μl of soluble fraction of PLCβ-baculovirus-infected insect cells and incubated at 30 °C in an incubation mixture (40 μl) containing 25 mM Hepes-NaOH, pH 8.0, 1 mM EDTA, 1 mM dithiorthiol, 20 mM MgCl2, 0.1% (v/v) GENAPOL X-100 (Calbiochem, La Jolla, CA), and 100 mM FRET-GTP-S (296 GBq/mmol). The incubation was terminated after 6 h as described previously (31). We also used [3H]GTP-S binding to estimate the concentrations of activated GTPγS-bound Rho GTAPs under conditions of the PLC assay (see below) in the presence of 100 μM [3H]GTPγS (115 GBq/mmol).

Phospholipase C Assay—Phospholipase C activity was determined as described (21) with minor modifications. In brief, 5 μl of detergent-solubilized Rho GTAPs and/or 5 μl of purified βγ were supplemented with 10 μl of soluble fraction of PLCβ-baculovirus-infected insect cells and incubated at 25 °C for time periods as indicated in the figure legend. In the absence of 50 μl of coupling medium, 50 mM Hepes-NaOH, pH 7.2, 75 mM KCl, 3 mM EDTA, 2 mM dithiorthiol, 33 μM [3H]H-Pip4-5P2, 185 GBq/mmol, 536 μM phosphatidyl ethanolamine, and 150 mm free Ca2+ in all experiments comparing the effects of Rac2 and βγ, the final concentration of sodium cholate was 2 mM. Only βγ-mediated stimulation of wild-type versus C-terminally deleted PLCβ isozymes (Fig. 6) was measured in the absence of sodium cholate. Results obtained in control experiments using purified PLCβ3Δ (31) were indistinguishable from that obtained with soluble fractions of PLCβ2Δ–baculovirus-infected insect cells.

Miscellaneous—Purification of βγ γ isolated from bovine retinal rod outer segment membranes has been described elsewhere (32). The membrane-associated used to purify membrane-associated recombinant βγ dimers is described in a previous study (33). SDS-PAGE, immunoblotting, and determination of protein concentrations were performed as described previously (21). Polyclonal antibodies against Rho GTAPs were from Santa Cruz Biotechnology (Santa Cruz, CA). Specific antigens against PLCβ1, PLCβ2, and PLCβ3 were a kind gift from Dr. P. J. Parker (28). The data are presented as means ± S.D. of triplicate determinations.

RESULTS

Stimulation of Recombinant PLCβ Isozymes by βγ Dimers—To determine the specificity of PLCβ stimulation by G protein βγ dimers, the PLCβ isozymes PLCβ1, PLCβ2, and PLCβ3 were produced as recombinant proteins in Sf9 insect cells. Fig. 1A shows that all three isozymes were expressed as soluble proteins in infected insect cells and migrated at molecular masses of ~150, 140, and 160 kDa, respectively, corre-
Fig. 1. Expression of PLCβ1, PLCβ2, and PLCβ3 in baculovirus-infected insect cells. A, soluble fractions of S9 insect cells that had been infected with baculovirus-encoding bovine PLCβ1 (10 μg of protein) (lane 1), human PLCβ2 (30 μg of protein) (lane 2), and human PLCβ3 (60 μg of protein) (lane 3) were subjected to SDS-PAGE and immunoblotting using PLCβ subtype-specific antisera. The apparent molecular weights of the marker proteins are indicated. The PLCβ1, PLCβ2, and PLCβ3, migrated at ~150, 140, and 160 kDa, respectively. Additional immunoreactive proteins of lower molecular masses were detected in all three preparations. Because soluble fractions of non-infected insect cells did not contain proteins reactive with the antisera used in this experiment (not shown), these proteins most likely correspond to proteolytic fragments of the full-length PLC isozymes. B, aliquots of the three soluble fractions were incubated in the presence of 150 nM free Ca2+ for the times indicated at the abscissa with phospholipid vesicles containing PI-4,5-P2. The reaction was terminated by the addition of chloroform/methanol/concentrated HCl, and the mixture was analyzed for inositol phosphates. See “Experimental Procedures” for details. Prior to this experiment, the amounts of the samples containing soluble PLCβ1, PLCβ2, and PLCβ3 were adjusted to give equal maximal PLC activities in the presence of 1 mM free Ca2+ and 3.3 mM sodium cholate (42) (not shown) (PLCβ1, 0.4 μg of protein/sample; PLCβ2, 1.5 μg of protein/sample; PLCβ3, 5.6 μg of protein/sample).

Stimulation of Recombinant PLCβ Isozymes by Rho GTPases—To investigate the specificity of PLCβ2 stimulation by Rho family members, the recombinant Rho GTPases Rac1, Rac2, and Cdc42Hs were produced in baculovirus-infected insect cells, extracted from the membranes of infected cells with detergent-containing buffer, and reconstituted with a recombinant C-terminal deletion mutant of PLCβ2, PLCβ2Δ, in the presence of 100 μM GTPγS. Fig. 3 shows that both Rac1 and Rac2 caused a marked (~13-fold) stimulation of PLCβ2Δ. Rac2 was slightly more potent than Rac1. Thus, high-maximal stimulation was observed at ~40 nM Rac2 and 100 nM Rac1. Cdc42Hs was a less potent (EC50: 400 nM) and less efficacious (~8-fold) stimulator of PLCβ2Δ than Rac1 and Rac2. In additional experiments, we observed a similar rank order of potency of Rho GTPases (Rac2 > Rac1 > Cdc42Hs) to stimulate PLCβ2Δ when full-length enzyme rather than PLCβ2Δ was used (not shown). RhoA had no effect on full-length PLCβ2 or PLCβ2Δ when tested under the same conditions (not shown).

Next, the most potent Rho GTPase, Rac2, was incubated at increasing concentrations in the presence of either GDP or GTPγS with soluble preparations of recombinant wild-type PLCβ1, PLCβ2, and PLCβ3. Fig. 4 shows that PLCβ2 was clearly the PLCβ isoform most sensitive to stimulation by GTPγS-activated Rac2. Thus, half-maximal and maximal (~4-fold) stimulation was observed at approximately 80 and 500 nM Rac2, respectively. Rac2 also appeared to stimulate PLCβ2Δ in the presence of GDP, albeit to a much lesser (~1.6-fold) extent. At high concentrations of Rac2, a reduction of PLCβ2 stimulation was observed both in the presence of GDP and GTPγS, suggesting an inhibitory effect of the membrane extracts on PLC activity. PLCβ1 and PLCβ3 were also activated by Rac2, but to a much lower extent and only at much higher concentrations of GTPγS-activated Rac2 (2 μM). Additional measurements (not shown) of PLCβ stimulation by C-terminally modified Cdc42Hs and Rac1 revealed an at least 10-fold higher
FIG. 3. Stimulation of PLCβΔ by the human Rho GTPases Cdc42Hs, Rac1, and Rac2. The recombinant Rho GTPases were extracted with buffer containing sodium cholate from membranes of baculovirus-infected insect cells and incubated at increasing concentrations with soluble proteins of insect cells expressing PLCβΔ (0.2 μg of protein/sample) and phospholipid vesicles containing PI-4,5-P2. PLC activity was measured for 1 h in the presence of 100 μM GTPγS. The concentrations of Rac1 (filled triangles), Rac2 (filled circles), and Cdc42Hs (filled squares) were estimated by determining the binding of [35S]GTPγS under the same buffer conditions. The Rho GTPases did not affect the activity of PLCβΔ in the presence of 100 μM GDP (not shown).

potency of these GTPases toward PLCβ2 than toward PLCβ1 or PLCβ3. RhoA, a Rho GTPase incapable of stimulating PLCβ2 (21), affected neither PLCβ1 nor PLCβ2 activity under the same conditions. These data show that Rho GTPases stimulate PLCβ2 with a rank order of potency of Rac2 > Rac1 > Cdc42Hs and that, among the PLCβ isozymes tested, PLCβ2 is most sensitive to stimulation by activated Rac2.

Comparison of Full-length and C-terminally Deleted PLCβ Isozymes—A truncated PLCβ isoyme related to PLCβ3 has previously been reported to be remarkably sensitive to activation by βγ dimers (36). Similarly, C-terminal truncation of PLCβ3, from human platelets, has been shown to result in a marked augmentation of βγ stimulation (37). PLCβ1 has been shown to be cleaved by calpain between residues 880 and 881, generating two fragments of 100 and 45 kDa, respectively (38). The presence of 45- to 50-kDa proteins in the preparations of recombinant PLCβ isoymes, which are likely to represent C-terminal proteolytic fragments of the enzymes (cf. Fig. 1), raised the possibility that the observed order of PLCβ stimulation by Rac2 was a consequence of C-terminal proteolysis of the PLCβ isoymes. To challenge this hypothesis, we examined and compared the ability of Rac2 to stimulate the activity of C-terminal deletion mutants of PLCβ1, PLCβ2, and PLCβ3. A schematic representation of the wild-type and mutant PLCβ isoymes is shown in Fig. 5A. As shown in Fig. 5B, all three mutants were expressed in baculovirus-infected insect cells as soluble proteins and migrated on SDS-polyacrylamide gels at the expected molecular weights. The deletion mutants displayed Ca2+ sensitivities indistinguishable from those of their wild-type counterparts (not shown). Reconstitution of the wild-type and mutant PLCβ isoymes with GTPγS-activated Rac2 (500 nM) or β1γ1 (300 nM) showed that, surprisingly, the deletion of the C-terminal regions of each PLCβ isoyme did not change the efficiency of β1γ1 to cause PLCβ stimulation (Fig. 6). In agreement with earlier findings (20), the removal of the C-terminal part of PLCβ3 enhanced the extent of Rac2-mediated stimulation of the enzyme. Importantly, however, no enhancement of Rac2-mediated stimulation was observed for the C-terminally deleted variants of PLCβ1 and PLCβ3. Therefore, removal of the C-terminal regions of PLCβ isoymes does not change the rank order of specificity of PLCβ stimulation by both Rac2 and G protein βγ subunits.

Simultaneous Stimulation of PLCβ2 by Rac2 and βγ Subunits—The next experiments were designed to examine whether the different specificities of PLCβ isoyme stimulation by Rho GTPases and βγ dimers may reflect independent regulation of PLCβ2 by both stimulators. We have previously shown that β2γ2H3 is a more potent stimulator of PLCβ2Δ than is β1γ1H3, and that the latter, but not the former βγ dimer, activates PLCβ3 (33). A comparison of the effects of β2γ2H3 and β1γ1H3 on the activity of wild-type PLCβ1, PLCβ2, and PLCβ3 is shown in Fig. 7. Among the PLCβ isoymes tested, PLCβ3...
was the isoform most sensitive to stimulation by \( \beta_1\gamma_1 \) in Rac2, followed by PLC\( \beta_2 \) and PLC\( \beta_3 \), which was hardly activated by this \( \beta_\gamma \) preparation. In marked contrast, PLC\( \beta_2 \) and PLC\( \beta_3 \) proved to be a potent and efficacious activator of full-length PLC\( \beta_2 \), but it did not affect full-length PLC\( \beta_1 \) or PLC\( \beta_3 \). Because activation of PLC\( \beta_2 \) by \( \beta_\gamma_2 \) occurred at low concentrations (EC\(_{50}\) ~10 nM) and reached saturation within the range of \( \beta_\gamma \) dimer concentrations tested, PLC\( \beta_2 \) was chosen as the \( \beta_\gamma \) dimer to compare the effects of Rac2 and \( \beta_\gamma \) dimers on PLC\( \beta_2 \) activity when added alone or in combination at maximally effective concentrations (Fig. 8). Because stimulation of PLC\( \beta_2 \) by \( \beta_\gamma \) dimers is sensitive to high concentrations of detergent (32), detergent-free Rac2-LyGDI heterodimers were used as a source of Rac2. In our hands, Rac2-LyGDI heterodimers and monomeric Rac2 stimulated PLC\( \beta_2 \) with equal potency and efficacy in the presence of phospholipid vesicles containing PI-4,5-P\(_2\). The results are given as the percentage of the basal activities, which were set to 100%. For any given pair of wild-type and mutant PLC\( \beta_2 \) isoforms, the basal activities varied by less than a factor of two (not shown).
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obtained by β5γ2,3Hus, suggesting independent stimulation of PLCβ2 by Rho GTPases and βγ subunits. Interestingly, partial inhibition of β5γ2-Hus-mediated PLCβ2 stimulation by Rac2-LyGDI was measured in the presence of GDP. Because there was no effect of Rac2-LyGDI on basal PLCβ2 activity, this result may indicate that inactive Rac2-LyGDI may interact with β5γ2-Hus and/or noncompetitively interfere with β5γ2-Hus-mediated PLCβ2 activation.

Structural Requirements of PLCβ Stimulation by Rho GTPases versus βγ Dimers—The independent stimulation of PLCβ2 by βγ subunits and Rho GTPases prompted us to delineate the structural elements of PLCβ2 required for enzyme activation. The site of interaction of PLCβ2 with βγ was localized by others to the region between the catalytic subdomain Y residues Glu574 and Lys583 (39, 40). However, βγ dimers have also been reported to bind to the isolated PH domains of PLCβ1 and PLCβ2 (41). To identify the sites on PLCβ2 relevant for activation by Rac2 and βγ, we took advantage of the fact that both stimulators elicited only a slight effect on PLCβ1Δ, but markedly activated PLCβ2Δ. The effects of the two stimulators were examined on the activity of PLCβ2Δ—PLCβ2 chimera in which N-terminal portions of PLCβ2Δ had been replaced by the corresponding regions of PLCβ1Δ (Fig. 9). Two chimera, designated A and B, carrying the putative PH domain and the PH domain together with the four EF-hand motifs of PLCβ1Δ, respectively, were analyzed. A third chimera, comprising the PH domain, the EF-hand motifs, the catalytic subdomain X of PLCβ1Δ, and the catalytic subdomain Y of PLCβ2Δ, was catalytically inactive and hence not used for further analysis. Although chimera A was expressed in baculovirus-infected insect cells at levels considerable lower than were PLCβ1Δ and chimeras B, the two chimeras and PLCβ2Δ were indistinguishable in terms of the dependence of their catalytic activity on Ca2+ (not shown). Fig. 10 shows that substitution of the N-terminal portions of PLCβ2Δ for the corresponding regions of PLCβ1Δ led to reduction of the degree of β1γ-mediated stimulation. Specifically, at the highest concentration of β1γ tested (4 μM), the degrees of stimulation were 129%, 88%, 66%, and 8-fold for PLCβ1Δ, chimera A, chimera B, and PLCβ2Δ (Fig. 10A). Because the effects of β1γ did not reach saturation within the range of concentrations tested, it is currently unclear whether the reduced stimulation is due to a decrease in the potency or efficacy of β1γ. Very interestingly, substitution of the putative PH domain of PLCβ2Δ for its counterpart of PLCβ1Δ caused an almost complete (>95%) loss of stimulation by GTPγS-activated Rac2 (Fig. 10B). In additional experiments (not shown), we found that chimeras A and B were also resistant to stimulation by GTPγS-activated Cdc42Hs. Taken together, these results not only show that the structural requirements of PLCβ2 stimulation by βγ dimers and by the Rho GTPases Rac2 and Cdc42Hs are distinct but also suggest that the putative PH domain of PLCβ2 is critically involved in mediating its activation by Rho GTPases.

**DISCUSSION**

Specificity of PLCβ Stimulation by Rho GTPases—We have previously shown that the Rho GTPases Rac1 and Cdc42Hs, but not RhoA, stimulate the activity of PLCβ2 (21, 31). In this study, we demonstrate that the PLCβ isozymes PLCβ1, PLCβ2, and PLCβ3 are differentially sensitive to stimulation by Rho GTPases and G protein βγ dimers. Activated Rac2 is shown to stimulate PLCβ isozymes with the rank order of potency and efficacy of PLCβ2Δ > PLCβ3Δ ≥ PLCβ1Δ. This rank order is clearly different from the order observed for G protein βγ dimers, which is PLCβ3Δ > PLCβ2Δ > PLCβ1Δ for most βγ dimers, e.g. β1γ1 or β1γ-Hus (cf. Figs. 2 and 7 and Refs. 9, 42, and 43) and PLCβ2Δ > PLCβ1Δ for β5γ2-Hus (cf. Fig. 7). Furthermore, the results reported here show that both Rac1 and Rac2 are more
potent activators of PLCβ2 than Cdc42Hs. All three PLCβ-stimulating Rho GTPases, Rac2, Rac1, and Cdc42Hs, preferentially activate PLCβ2 (PLCβ2 \( > \) PLCβ1 \( \geq \) PLCβ3) (not shown). RhoA did not stimulate any of the three PLCβ isozymes tested under the same conditions (not shown). Notably, the specificity of PLCβ activation by Rho GTPases described here did not depend on the presence of detergents, which could conceivably differentially affect the activities of the PLCβ isozymes, because purified detergent-free Rac2-LyGDI heterodimers (31) stimulated the PLCβ isozymes studied here with the same rank order as did detergent-solubilized monomeric, C-terminally processed Rac2 (not shown). Interestingly, the rank order of PLCβ stimulation by Rho GTPases also differs from that reported for activation of these enzymes by members of the \( \alpha_q \) subfamily of G protein \( \alpha \) subunits: PLCβ1 \( \approx \) PLCβ2 > PLCβ3 (3, 4). Together with the observation that the C-terminal region of PLCβ2 isoforms is required for the stimulation by \( \alpha_q \) subunits (43, 44), but not for stimulation by Rho GTPases (20 and Fig. 6), this finding suggests that the stimulation of PLCβ by \( \alpha_q \) subunits of heterotrimeric G proteins differs mechanistically from stimulation of this enzyme by G protein GTPases. Thus, PLCβ isozymes can be activated, in the nanomolar concentration range, by both subunits of G proteins and by Rho GTPases but with distinct PLCβ isozyme specificities.

The Role of the C-terminal Regions of PLCβ isozymes in Their Regulation by Rho GTPases and \( \beta\gamma \)—Proteolytic cleavage of PLCβ2 by calpain at a site upstream of the C2 domain has been suggested to enhance \( \beta\gamma \)-mediated stimulation (36, 37). Our data show, however, that truncation of recombinant PLCβ1, PLCβ2, and PLCβ3, at a site corresponding to the calpain cleavage site in PLCβ1, had no effect on their sensitivity to \( \beta\gamma \) stimulation. The finding that the deletion of the C-terminal region of PLCβ2 did not enhance \( \beta\gamma \) stimulation suggests that the increased PLCβ2 stimulation following calpain cleavage reported by Banno and coworkers (37) may not simply result from the removal of an inhibitory constraint built up by the C-terminal amino acids of PLCβ2. Instead, it is more likely that the C-terminal region generated by treatment of PLCβ2 with calpain still interacts with the remaining part of the enzyme, to inhibit its activity, whereas it is absent from the deletion mutant studied here. Consistent with earlier results (20), Rac2 stimulation of PLCβ2 was enhanced in the absence of the C-terminal region of the enzyme. However, because this effect was not observed in the case of PLCβ1 and PLCβ3, the PLCβ2 specificity described here is clearly not a consequence of C-terminal proteolysis of the enzymes.

Simultaneous Stimulation of PLCβ Isozymes by Rho GTPases and G Proteins—The fact that the stimulatory effects of \( \beta_2\gamma_3 \) and GTPγS-activated Rac2 on PLCβ2 were strictly additive at saturating concentrations of the two activators suggests that there are separate sites on PLCβ2, for the interaction with \( \beta\gamma \) and Rac2. Similar observations have been made previously for the activation of PLCβ2 and PLCβ3 by \( \alpha_i \) and \( \beta\gamma \) (9, 45). In additional experiments, we have found that neither \( \beta\gamma \)-mediated activations nor \( \alpha_i \)-mediated activations of PLCβ2 or PLCβ3 were influenced by GTPγS-activated Rac2. Collectively, these results suggest that PLCβ isozymes can be isoyme-specifically activated by three different stimulators, G protein \( \alpha \) subunits, G protein \( \beta\gamma \) dimers, and Rho GTPases, via independent regulatory sites.

Structural Requirements of PLCβ Stimulation by Rho GTPases versus \( \beta\gamma \) Dimers—The generation of chimeric PLCβ enzymes made up of portions from an isozyme stimulated only poorly by \( \beta\gamma \) dimers and Rho GTPases, PLCβ2Δ, together with the remaining portions of PLCβ2Δ, an isoyme markedly sensitive to both activators, allowed to delineate the structural elements of PLCβ2 required for the regulation by \( \beta\gamma \) and Rac2. The fact that chimeras A and B were still markedly activated by \( \beta\gamma \) dimers suggests that the catalytic subdomains of PLCβ2 are both necessary and sufficient for \( \beta\gamma \) stimulation. This is
consistent with a previous report showing that a region within the catalytic Y domain of PLCβ2 contains the stimulatory βγ interaction site (40). The lower extent of stimulation of chimeric A and B by βγ relative to PLCβ3 is in line with the suggestion that an additional binding site for βγ stimulation may exist in the putative PH domain of PLCβ2 (41), which, albeit not absolutely required for βγ stimulation, may increase the affinity of the βγ-PLCβ2 interaction. In addition, our results suggest that the region corresponding to the putative PH domain of PLCβ1, an isozyme barely activated by βγ, is capable of substitute for the corresponding region of PLCβ2. This is consistent with the recent report describing interaction of an isolated PH domain of PLCβ1 with βγ dimers (41). Interestingly, construction of a chimera consisting of the putative PH domain of PLCβ2 and the catalytic subdomains X and Y of PLCα, an enzyme that is not regulated by βγ dimers, resulted in a βγ-regulated enzyme (46). This suggests that βγ may interact with multiple sites in phospholipase C isoforms and that these sites can be provided even by isoforms that are poorly (e.g. PLCβ1) or not at all (e.g. PLCβ3) sensitive to βγ stimulation. An important outcome of our experiments on chimeric PLCβ1-PLCβ2 enzymes is the observation that the substitution of the putative PH domain of PLCβ1 by the corresponding region of PLCβ1 abolished Rac2-mediated stimulation of the enzyme. This result not only demonstrates different structural requirements of PLCβ2 stimulation by βγ and Rac2, but also shows, for the first time, that the putative PH domain of PLCβ2 is specifically and critically involved in mediating the regulation of the activity of the PLCβ isozyme. It is currently unknown whether Rac2 directly binds to the PH domain of PLCβ2 or whether Rac2 induces a conformational change of the enzyme involving the PH domain. Interestingly, the PH domain of PLCβ2 was found to be required for both membrane targeting and catalytic activity of recombinant PLCβ2 in transfected COS-7 cells (44). The functional role of the region corresponding to the putative PH domain in PLCβ isozymes is poorly understood. The isolated PH domain of PLCβ1 has been reported to bind inositol phospholipids (PI-3-P > PI-4,5-P2, PI-3,4,5-P3) (47). A cooperative mechanism involving phosphatidylinositol 3-phosphate and βγ subunits has been proposed to regulate plasma membrane localization and activation of PLCβ through the putative PH domain of this enzyme. A similar scenario involving Rac2 and βγ could be depicted in the case of PLCβ2. Thus, PLCβ isozymes seem to act as a point of convergence of transmembrane signaling: PLCβ1 integrating signals emanating from inositol phospholipid 3-kinase and G protein αi subunits, PLCβ2 those from G protein αi and βγ subunits, and PLCβ2 those from G protein βγ subunits and Rac/Cdc42Hs.

The finding that the activity of PLCβ isozymes can be specifically regulated by three different stimulators, G protein αi subunits, G protein βγ dimers, and the Rho GTPases Rac and Cdc42Hs also enhances the cellular repertoire to coordinate, both spatially and temporally, responses to extracellular signals acting through stimulation of PLCβ isozymes and thereby to enhance the degree of signal specificity. An intriguing possibility is that the Rho GTPases Rac and Cdc42Hs act as organizers to mediate recruitment of PLCβ2 to allow activation by G protein-coupled receptors only at specific sites within the cell and/or only within a specific time frame during or after receptor activation. The mechanisms involved in the recruitment of PLCβ isozymes to their phospholipid are still not known. It seems clear, however, that the known activators, αi and βγ, are not involved in this process (48, 49). Interestingly, although PLCβ2 is strongly activated by βγ dimers in cell-free systems, this isoform is, in marked contrast to PLCβ2, not stimulated by chemoattractant receptor activation or exogenous βγ dimers in transiently transfected COS-7 cells (17). These results support the notion that each PLCβ isoform may require distinct additional components either for recruitment or stimulation of the catalytic activity. For example, activated members of the αi subfamily of G protein α subunits have been shown to permit PLCβ stimulation by receptors acting through βγ subunits of pertussis toxin-sensitive G proteins (50). The results presented herein suggest that Rac1, Rac2, and Cdc42Hs may contribute to the specificity and/or efficacy of PLCβ signaling. Interestingly, both PLCβ1 and Rac2 are the major representatives of the PLCβ and Rac GTPase subfamilies, respectively, in myeloid cells (16, 51). Both proteins are activated in response to activation of chemoattractant receptors in a pertussis-toxin sensitive manner (17, 52, 53, 54). The recent finding, that expression of a dominant-interfering form of Cdc42Hs in myeloid-differentiated HL-60 cells drastically reduced the formyl peptide receptor mediation of (α) formation of inositol 1,4,5-trisphosphate, (β) increase in the concentration of intracellular Ca2+, and (γ) rapid activation of Rac2 (55), supports our hypothesis that Rac2 and possibly Rac1 and Cdc42Hs are critically involved in receptor-mediated regulation of PLCβ activity in intact cells. Although the mechanisms by which chemoattractant receptors stimulate Rac/Cdc42 are not defined and the functional relevance of phosphatidylinositol 3-kinase to this process is a subject of debate, the recently identified βγ- and PI-3,4,5-P3-dependent Rac exchanger P-Rex 1 appears to fill the gap between chemoattractant receptors and Rac GTPases in leukocytes (56).

In conclusion, our results demonstrate that the specificity of PLCβ stimulation by the Rho GTPases Rac and Cdc42Hs differs from the specificity observed for αγi subunits and βγ dimers of heterotrimeric G proteins and that PLCβ2 represents the PLCβ isozyme most sensitive to stimulation by the Rho GTPases Rac and Cdc42 among the PLCβ isozymes investigated in this study. Moreover, there are separate sites on PLCβ isozymes for the regulation by Rho GTPases, G protein α subunits, and βγ subunits allowing independent activation by these stimulators.

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