The regulation of the two isoforms of phospholipase C-γ, PLCγ1 and PLCγ2, by cell surface receptors involves protein tyrosine phosphorylation as well as interaction with adapter proteins and phosphatidylinositol 3,4,5-trisphosphate (PtdInsP3) generated by inositool phospholipid 3-kinases (PI3Ks). All three processes may lead to recruitment of the PLCγ isoforms to the plasma membrane and/or stimulation of their catalytic activity. Recent evidence suggests that PLCγ may also be regulated by Rho GTPases. In this study, PLCγ1 and PLCγ2 were reconstituted in intact cells and in a cell-free system with Rho GTPases to examine their influence on PLCγ activity. PLCγ2 but not PLCγ1 was markedly activated in intact cells by constitutively active Rac1G12V, Rac2G12V, and Rac3G12V but not by Cdc42G12V and RhoA G14V. The mechanism of PLCγ2 activation was apparently independent of phosphorylation of tyrosine residues known to be modified by PLCγ-activating protein-tyrosine kinases. Activation of PLCγ2, by Rac2G12V in intact cells coincided with a translocation of PLCγ2 from the soluble to the particulate fraction. PLCγ isoform-specific activation of PLCγ2 by Rac GTPases (Rac1 > Rac2 > Rac3), but not by Cdc42 or RhoA, was also observed in a cell-free system. Herein, activation of wild-type Rac GTPases with guanosine 5′-(3-O-thio)triphosphate caused a marked stimulation of PLCγ2 but had no effect on the activity of PLCγ1. PLCγ1 and PLCγ2 have previously been shown to be indiscriminately activated by PtdInsP3 in vitro. Thus, the results suggest a novel mechanism of PLCγ2 activation by Rac GTPases involving neither protein tyrosine phosphorylation nor PI3K-mediated generation of PtdInsP3.

Inositol phospholipid-specific phospholipases C (PLCs) catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP2) to produce inositol 1,4,5-trisphosphate and diacylglycerol. While the products of this reaction have long been known to act as intracellular second messengers (reviewed in Refs. 1–4), it has only recently become clear that the phospholipid substrate itself may also be considered an intracellular signaling molecule in that its local concentration in the plasma membrane and possibly in other cellular membranes regulates the activities and/or subcellular distribution of a number of regulatory or structural proteins. These include enzymes, ion channels and transporters, transcription factors, scaffolding proteins, cytoskeletal proteins, as well as proteins involved in the regulation of exocytosis, endocytosis, and membrane trafficking (reviewed in Refs. 5–8).

The mammalian PLCs are divided into six subfamilies, designated β, γ, δ, ε, ζ, and η (reviewed in Refs. 9 and 10). The four members of the PLCβ subfamily are activated by βy dimers of heterotrimeric G proteins and/or members of the αs subfamily of G protein α subunits and by certain Rho GTPases (11–14). The two members of the PLCγ family are activated by receptor and nonreceptor protein-tyrosine kinases (reviewed in Refs. 9, 10, 15, and 16). In addition, alternative mechanisms exist to regulate the activity of the two PLCγ isoforms (reviewed in Ref. 17). The mechanisms of PLCζ regulation are less well understood. Members of this subfamily have been reported to be under stimulatory and/or inhibitory control of a number of low molecular weight substances and proteins, including p122-RhoGAP, RhoA, the α subunit of the heterodimeric GTP-binding protein Gs, tissue transglutaminase (2 reviewed in Refs. 9, 10, and 18) as well as GAP43, PLCβ2, RalA, and RalB (19–21). PLCε is subject to regulation by βy dimers, α12 and α13 subunits of heterotrimeric G proteins, and several members of the Ras superfamily of small GTPases, including H-Ras, Rap1A, Rap2B, RhoA, RhoB, and RhoC (reviewed in Ref. 22). PLCζ is specifically expressed in mammalian sperm and is capable of inducing oscillations of intracellular Ca2+ and subsequent early embryonic development when introduced into mammalian eggs upon sperm-egg fusion (23). Very recently, two members of a novel subfamily, PLCζ1 and PLCζ2, have been identified and characterized (24, 25). While it seems clear that PLCζ2 and PLCζ1 isozymes are activated by Ca2+ (24–26), it is currently unknown whether their activity is controlled by regulatory proteins.

We (13) and others (14) have previously reported that constitutively active Rac2 activates PLCβ2 in intact cDNA-transfected COS-7 and HEK293 cells and causes translocation of the enzyme from the cytosol to the plasma membrane. Both effects are mediated by the putative amino-terminal pleckstrin homology domain of PLCβ2 (12–14). Rac2 is specifically expressed in cells of hematopoietic origin (27, 28), and mice genetically deficient in Rac2 are characterized by defects in cellular functions of hematopoietic stem cells (29, 30), neutrophils (31), mast cells (32), T cells (33), and B lymphocytes (34, 35). In the latter cells, the absence of Rac2 caused a reduction in the B cell antigen receptor (BCR)-mediated increase in cytosolic Ca2+ (34, 35). The fact that PLCγ2, rather than PLCβ2, mediates this response in B cells (reviewed in Refs. 15 and 36) prompted us to examine whether PLCγ2 is regulated by Rac2. The results presented herein demonstrate that PLCγ2, but not PLCγ1, is activated by Rac GTPases in both cellular and cell-free systems by a mechanism that involves translocation of the cytosolic PLCγ2 isozyme to cellular membranes but is apparently independent of protein tyrosine phosphorylation and inositol phospholipid 3-kinase-mediated generation of PtdInsP3.
Stimulation of Phospholipase C-γ2 by Rac GTPases

EXPERIMENTAL PROCEDURES

Materials—Polyclonal antisera reactive against human PLCγ2 (catalog numbers sc-9015 and sc-407), rat PLCβ1 (catalog number sc-7520), human Rac2 (catalog number sc-96), Gβ1-4 (catalog number sc-378), and RhoGDIA and -β (catalog number sc-359) were from Santa Cruz Biotechnology. Polyclonal antisera reactive against bovine PLCβ1, human PLCβ2, human PLCβ3, and bovine PLCγ2 (37) were gifts of Dr. Peter J. Parker. The cDNA of human Rac3 inserted into pcDNA3.1 (+) was obtained from the University of Missouri-Rolla cDNA Resource Center (Rolla, MO).

Plasmids—The cDNAs of human Cdc42, human RhoA (GenBank™ accession number X05026), human Rac1, human Rac2, human Rac3, bovine PLCβ1, human PLCβ2, and human PLCβ3 were ligated into pcDNA3.1 (+) or pcDNA3.1 (−) (Invitrogen). The cDNAs of rat PLCβ1, bovine PLCγ1, and human PLCγ2 were obtained in pMT2 (38) from Dr. Matilda Katan. For production of recombinant baculoviruses, the cDNAs of bovine PLCγ1 and human PLCγ2 were ligated into the XbaI site of pVL1392. The cDNA of human Rac3 was ligated into the Smal/ XbaI site of pVL1393.

Cell Culture and Transfection—COS-7 and HEK293 cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen, catalog number 41965-039) supplemented with 10% (v/v) fetal calf serum (Invitrogen, catalog number 10270-106) and 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 1 mM sodium bicarbonate, and 25 mM HEPES buffer solution (all from PAA Laboratories, Co¨lbe, Germany). Prior to transfection, COS-7 or HEK293 cells were seeded into 12-well plates at medium containing fetal calf serum and supplements as specified above, at 12,000 cells per well, respectively, and grown without changing the medium. For transfection of HEK293 cells, the cells were scraped into 0.15 ml of ice-cold buffer B containing 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. The cells were homogenized by forcing the suspension ten times through a 0.25 × 25-mm needle attached to a disposable syringe. After removal of unbroken cells and nuclei by centrifugation at 300 × g for 10 min at 4 °C, particulate (P) and soluble (S) fractions were separated by centrifugation at 12,000 × g for 15 min at 4 °C.

Production of Recombinant Rho GTPases and Recombinant PLCγ Isozymes in Baculovirus-infected Insect Cells—For production of recombinant Rho GTPases, Sf9 cells (Invitrogen) were grown at 27 °C in suspension culture in TTNM-FH medium containing 10% (v/v) fetal calf serum (catalog number P04–83500, PAN Biotech, Aidenbach, Germany) supplemented with 0.2% (w/v) Pluronic® F-68 (Invitrogen), 50 μg/ml gentamicin (PAA Laboratories), and 2.5 μg/ml amphotericin B (Fungizone®, Invitrogen) in a 1800-ml Fernbach culture flask. Cells (1.2 × 10⁸) were incubated at 27 °C with recombinant baculovirus in 400 ml of medium at 80 rpm on a rotary shaker with an amplitude of 25 mm. Three days after infection, the cells were harvested at room temperature by centrifugation at 300 × g for 5 min followed by two washes with 100 ml each of buffer A. To obtain detergent-solubilized Rho GTPases, the cells were resuspended in 15 ml per 10⁷ intact cells at the time of cell harvesting of ice-cold buffer C containing 20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 3.75 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 3 μM GDP and homogenized using a precooled 5-ml Teflon-glass homogenizer. Nuclei and unbroken cells were removed by centrifugation at 300 × g for 10 min at 4 °C. The membrane fraction was collected from the resulting supernatant by centrifugation at 12,000 × g for 15 min at 4 °C. Rho GTPases were solubilized by resuspending the membranes in 2 ml per 10⁶ intact cells at the time of cell harvesting of ice-cold buffer C containing 23 mM sodium cholate and incubating this mixture for 90 min at 4 °C with vigorous vortexing every 10 min. Insoluble material was removed from this suspension by centrifugation at 12,000 × g for 15 min at 4 °C. The resulting detergent extract was aliquoted, snap-frozen in liquid N₂, and stored at −80 °C. For production of recombinant PLCγ isozymes, Sf9 cells were grown at 27 °C in adherent culture in TNM-FH medium (catalog number T3285, Sigma) supplemented with 10% (v/v) fetal calf serum (catalog number P04–83500, PAN Biotech, Aidenbach, Germany) supplemented with 0.2% (w/v) Pluronic® F-68 (Invitrogen), 50 μg/ml gentamicin (PAA Laboratories), and 2.5 μg/ml amphotericin B (Fungizone®, Invitrogen) in a 1800-ml Fernbach culture flask. Cells (1.2 × 10⁸) were incubated at 27 °C with recombinant baculovirus in 400 ml of medium at 80 rpm on a rotary shaker with an amplitude of 25 mm. Three days after infection, the cells were harvested at room temperature by centrifugation at 300 × g for 5 min followed by two washes with 100 ml each of buffer A. To obtain detergent-solubilized Rac GTPases, the cells were resuspended in 15 ml per 10⁷ intact cells at the time of cell harvesting of ice-cold buffer C containing 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.

Subcellular Fractionation—HEK293 cells (2.7 × 10⁶) were grown on 100-mm dishes and transiently transfected as described above using the CalPhos™ mammalian transfection kit. Twenty-four hours after transfection, the cells were scraped into 0.15 ml of ice-cold buffer B containing 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. The cells were homogenized by forcing the suspension ten times through a 0.25 × 25-mm needle attached to a disposable syringe. After removal of unbroken cells and nuclei by centrifugation at 300 × g for 10 min at 4 °C, particulate (P) and soluble (S) fractions were separated by centrifugation at 12,000 × g for 15 min at 4 °C.
Twenty-four hours after transfection, the cells were incubated for 20 h in the presence of GDP containing 23 mM sodium cholate and then incubated at 30 °C. 24 h after transfection, the cells were incubated for 18 h in the presence of 100 mM sodium deoxycholate and then assayed as described previously (11) with minor modifications. Briefly, detergent extracts prepared from membranes of Rho-GTPase-negative baculovirus-infected insect cells were diluted 15-fold in buffer C with 0.1 M 2-mercaptoethanol, 53.6 mM phosphatidylethanolamine, 33.4 μM [3H]PtdInsP2 (185 GBq/mol), and the concentrations of sodium deoxycholate and free Ca²⁺ specified in the legends to Figs. 5–8. The concentration of CaCl₂ required to adjust the concentration of free Ca²⁺ to the desired value was calculated using the program EqCal for Windows (Biosoft, Ferguson, MO). The reaction was terminated, and the samples were analyzed for inositol phosphates as described (41).

Miscellaneous—Recombinant baculoviruses were produced as described (43). A baculovirus-encoding Escherichia coli β-galactosidase (44) was a gift from Dr. Michael Ruffing. Protein concentrations were determined according to Bradford (45) using bovine IgG as standard. SDS-PAGE and immunoblotting were performed according to standard protocols (46), except that immunoreactive proteins were visualized using the ECL Western blotting detection system (Amersham Biosciences). The sources of all other reagents are specified in Refs. 11–13. All experiments were performed at least three times. Similar results and identical trends were obtained each time. Data from representative experiments are shown as means ± standard deviation of triplicate determinations.

RESULTS

We have previously shown that a constitutively active mutant of Rac2, Rac2G12V, causes a marked stimulation of a fusion protein of PLCβ2 and enhanced green fluorescent protein, PLCβ2-enhanced green fluorescent protein in intact cDNA-transfected COS-7 and HEK293 cells (13). To examine the PLC isozyme specificity of this effect, the formation of inositol phosphates was measured in COS-7 cells that had been cotransfected with vector encoding wild-type Rac2 or Rac2G12V together with vector encoding PLCβ1, PLCβ2, PLCβ3, PLCγ1, PLCγ2, or PLCδ1. Fig. 1 shows that expression of the PLC isozymes caused a variable increase in inositol phosphate formation in the absence of Rac2 and Rac2G12V, which was maximal (~3.9-fold) for PLCβ2, followed by PLCγ1, PLCγ2, PLCδ1, and PLCδ2. Expression of wild-type Rac2 had no effect on inositol phosphate formation both in the absence and in the presence of exogenous PLC isozymes. Expression of Rac2G12V did not affect inositol phosphate formation in the absence of exogenous PLC isozymes and in the presence of PLCβ1, PLCβ2, PLCγ1, or PLCδ1 but caused a marked stimulation of inositol phosphate formation in cells

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expressing PLCβ2. Interestingly, expression of Rac2G12V also caused a marked stimulation of inositol phosphate formation in cells expressing PLCγ2. Taking into account that only about 74 and 29% of basal inositol phosphate formation in cells expressing PLCβ2 and PLCγ2, respectively, was due to the activity of the exogenous PLC isozyme and that Rac2G12V caused no change in inositol phosphate formation in the absence of exogenous PLC isozymes, Rac2G12V caused an about 4.9- and 24.0-fold stimulation of PLCβ2 and PLCγ2, respectively, in this experiment. In additional experiments, we found that PLCγ2 was stimulated by Rac2G12V to a similar extent in cotransfected HEK293 cells (results not shown). The inset of Fig. 1 shows that PLCγ2 was in fact expressed in COS-7 cells transfected with the corresponding cDNA and that the levels of expression of PLCβ2, PLCγ1, and PLCγ2 were similar in the absence or presence of wild-type or constitutively active Rac2. Similar results were obtained for the expression of PLCβ2, PLCβ2, and PLCβ2 in cotransfected COS-7 cells (data not shown). Thus, the stimulation of inositol phosphate formation by Rac2G12V in cells expressing PLCβ2 or PLCγ2 was not explained by an increase in PLC expression, and the inability of Rac2G12V to stimulate inositol phosphate formation in cells that had been cotransfected with the cDNAs of PLCβ2, PLCβ2, and PLCβ2 was not due to the lack of expression of these PLC isoforms. We occasionally observed increased expression of exogenous proteins in COS-7 cells coexpressing these proteins with high levels of Rac2G12V (data not shown). The reason(s) for these changes are currently unknown.

Fig. 2 shows that the ability to stimulate the activity of PLCβ2 and PLCγ2 in cotransfected COS-7 cells was not restricted to Rac2G12V but was also observed for the constitutively active mutants of Rac1 and Rac3, Rac1G14V and Rac3G14V, but not for the corresponding mutant of RhoA, RhoAG14V. The stimulation of both PLCβ2 and PLCγ2 was maximal in cells that coexpressed Rac3G12V, followed by cells coexpressing Rac2G12V and cells coexpressing Rac1G12V. Note that this rank order does not necessarily reflect the rank order of sensitivity of the two PLC isoforms to the three Rac GTases, since the relative abundance of the latter proteins in the cotransfected cells analyzed in Fig. 2 remained unknown. Consistent with earlier in vitro findings (11, 12), expression of Cdc42G12V caused an about 2.3-fold increase of inositol phosphate formation in cells expressing PLCβ2. In contrast, Cdc42G12V did not affect this activity in cells expressing PLCγ2. Thus, although the sensitivity of PLCβ2 and PLCγ2 to stimulation by activated Rac1, Rac2, and Rac3, respectively, appears to be similar, the sensitivity to stimulation by activated Cdc42 distinguishes PLCβ2 from PLCγ2. Only marginal, if any, changes of inositol phosphate formation were observed in cells coexpressing the constitutively active Rho GTPases with PLCγ1. In additional experiments (results not shown), we found that coexpression of RhoAG14V, but not of wild-type RhoA, with human PLCγ1 in COS-7 cells caused an about 13.5-fold stimulation of this PLC isozyme, indicating that RhoAG14V was in fact constitutively active in this system.

Activation of PLCγ2 by cell surface receptors has previously been shown to involve protein phosphorylation at one or several of four tyrosine residues present at positions 753, 759, 1197, and 1217 of PLCγ2 (47–50). To examine whether phosphorylation of any one of these residues was involved in stimulation of inositol phosphate formation by activated Rac GTases in COS-7 cells expressing PLCγ2, mutants of PLCγ2 carrying substitutions of one, two, or all four tyrosine residues by phenylalanine residues were coexpressed with either wild-type Rac2 or Rac2G12V. Fig. 3A shows that expression of Rac2G12V, but not of wild-type Rac2, caused a marked increase in inositol phosphate formation in cells expressing PLCγ2, regardless whether Rac2G12V was coexpressed with wild-type or with mutant PLCγ2. Although the formation of inositol phosphates was lower in cells expressing Rac2G12V together with the mutant PLCγ2 polypeptides, basal inositol phosphate formation was enhanced ~4.1-fold by Rac2G12V even in cells expressing the PLCγ2 mutant lacking all four tyrosine residues concurrently, PLCγ2FFF. Fig. 3B shows that the expression of wild-type and mutant PLCγ2 was slightly variable in this experiment, which may contribute, at least to some extent, to the differences in Rac2G12V-stimulated inositol phosphate formation observed in Fig. 3A. In additional experiments (results not shown), we found that PLCγ2FFF was consistently expressed at lower levels than wild-type PLCγ2 in cotransfected COS-7 cells. These caveats notwithstanding, the results shown in Fig. 3 clearly demonstrate that phosphorylation of the tyrosine residues present in positions 753, 759, 1197, and 1217 is not required for Rac2G12V to cause stimulation of inositol phosphate formation by PLCγ2.

To investigate the mechanisms by which Rac2G12V mediates stimulation of inositol phosphate formation in COS-7 cells and HEK293 cells expressing PLCγ2, the influence of wild-type Rac2 and constitutively active Rac2G12V on the subcellular distribution of PLCγ2 was examined in HEK293 cotransfected with vector encoding PLCγ2 and either empty vector or vector encoding wild-type Rac2 or Rac2G12V. Homogenates of transfected cells were fractionated, and aliquots of the postnuclear particulate fraction containing plasma membranes (P) and the soluble fraction (S) were analyzed by immunoblotting. Fig. 4, top panel, shows that PLCγ2 was mostly, if not entirely, soluble in the absence of exogenous Rac GTases and in the presence of wild-type Rac2. Very interestingly, expression of Rac2G12V caused the appearance of a considerable amount of immunoreactive PLCγ2 in the particulate fraction (Fig. 4, top panel). Note that both wild-type Rac2 and Rac2G12V were present in the particu-
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FIGURE 3. Tyrosine phosphorylation of PLCγ2 in positions 753, 759, 1197, and 1217 is not required for Rac2G12V to mediate stimulation of inositol phosphate formation in COS-7 cells expressing PLCγ2. A, COS-7 cells were cotransfected as indicated at the abscissa with 250 μg each of either empty vector (Mock) or vector encoding either wild-type PLCγ2 (PLCγ2) or the PLCγ2 mutants PLCγ2Y753F, PLCγ2Y759F, PLCγ2Y1197F, and PLCγ2Y1217F (PLCγ2Y753F, PLCγ2Y759F, PLCγ2Y1197F, and PLCγ2Y1217F) together with 50 ng each of either empty vector (Control) or vector encoding wild-type Rac2 (Rac2) or constitutively active Rac2G12V (Rac2G12V). The total amount of DNA was maintained constant at 1.0 μg in each transfection by adding empty vector. The formation of inositol phosphates was measured as described in the legend to Fig. 1. B, COS-7 cells were cotransfected as described above with either empty vector (Mock) or vector encoding wild-type PLCγ2 together with vector encoding wild-type Rac2 or constitutively active Rac2G12V. Cells from one well were lysed in 100 μl of SDS-PAGE sample preparation buffer. The lysate was subjected to SDS-PAGE, and immunoblotting was performed using antibodies reactive against PLCγ2.

PLCγ isozymes by Rho GTPases in a cell-free system. To this end, the PLCγ isozymes and Rho GTPases were separately produced in baculovirus-infected insect cells and functionally reconstituted to study the effect of Rho GTPase activation by the poorly hydrolyzable GTP analogue GTPγS on the ability of the PLCγ isozymes to hydrolyze PtdInsP2. Fig. 5 shows that both PLCγ1 and PLCγ2 were produced in baculovirus-infected insect cells and were present in large excess of endogenous phospholipases C in the soluble fraction of infected cells. The enzymatic activity of the two recombinant PLCγ isozymes was dependent on and markedly stimulated by Ca2+ (Fig. 5, right panel). The concentration dependence on free Ca2+ was similar for the two PLCγ isozymes with half-maximal and maximal effects at ∼175 μM and 10 μM free Ca2+, respectively. In the experiment shown in Fig. 6, samples containing either PLCγ1 or PLCγ2 and displaying similar phospholipase C activities at 1 μM free Ca2+ (Fig. 6, left panel) were reconstituted with a detergent extract prepared from membranes of baculovirus-infected insect cells expressing Rac2 and then assayed for phospholipase C activity in the presence of 30 nM free Ca2+ and 1 mM sodium deoxycholate (Fig. 6, right panel). Under these conditions, addition of GTPγS caused a marked (∼5.2-fold) increase in inositol phosphate formation by the preparation containing PLCγ1, but did not affect inositol phosphate formation by the preparation containing PLCγ2. There was no effect of GTPγS on phospholipase C activity when the two PLCγ isozymes were produced in baculovirus-infected insect cells expressing Rac2 and then assayed for phospholipase C activity in the presence of 0.65 mM GDP, half-maximal and maximal effects of GTPγS were observed at ∼1.6 and 100 μM, respectively (Fig. 7, left panel).

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FIGURE 5. Expression of recombinant PLCγ1 and PLCγ2 in baculovirus-infected insect cells. Left panel, aliquots of the soluble fractions of Sf9 cells infected with baculovirus encoding PLCγ1 (closed squares) or PLCγ2 (closed circles) were incubated for 45 min at 30 °C in 10 μM free Ca2+ in the absence of sodium deoxycholate with phospholipid vesicles containing PtdInsP2. Aliquots of the soluble fraction of Sf9 cells infected with baculovirus encoding β-galactosidase (open circle) and of the soluble fraction of non-infected Sf9 cells (open square) were analyzed for comparison. The reaction was terminated by the addition of chloroform/methanol/concentrated HCl, and the mixture was analyzed for inositol phosphates. See “Experimental Procedures” for experimental details. Inset, aliquots of soluble fractions of Sf9 cells infected with baculovirus encoding PLCγ1 (lane 1), PLCγ2 (lane 2), β-galactosidase (lane 3), and of the soluble fraction of non-infected insect cells (lane 4) containing 10 μg of protein/sample were subjected to SDS-PAGE, and immunoblotting was performed using antibodies reactive against PLCγ1 and PLCγ2. (Top panel) or PLCγ2 (bottom panel). Right panel, aliquots of the soluble fractions of Sf9 cells infected with baculovirus encoding PLCγ1 (closed squares) or PLCγ2 (closed circles) containing 15 and 30 ng of protein/sample, respectively, were incubated for 30 min at 25 °C at increasing concentrations of free Ca2+ in the absence of sodium deoxycholate with phospholipid vesicles containing PtdInsP2. Aliquots of the soluble fraction of non-infected Sf9 cells (Control, open diamonds) were analyzed for comparison. To avoid potential changes in the pH of the incubation medium caused by the addition of CaCl2, the incubation was performed in the presence of 50 mM Tris maleate/NaOH, pH 7.3, instead of 50 mM HEPES/NaOH, pH 7.2.

FIGURE 6. Isozyme-specific stimulation of PLCγ2 by Rac2. Left panel, aliquots of soluble fractions of Sf9 cells infected with baculovirus encoding PLCγ1 (closed squares) or PLCγ2 (closed circles) containing 90 and 200 ng of protein/sample, respectively, were incubated for the times indicated at the abscissa at 25 °C with phospholipid vesicles containing PtdInsP2. The incubation was performed in the presence of 1 μM free Ca2+ and in the absence of sodium deoxycholate. Aliquots of the soluble fraction of non-infected Sf9 cells (Control, open diamonds) containing 200 ng of protein/sample were analyzed for comparison. Right panel, aliquots of soluble fractions of Sf9 cells infected with baculovirus encoding PLCγ1 or PLCγ2 containing 180 and 400 ng protein/sample, respectively, were reconstituted with aliquots (5 μl/sample) of a detergent extract prepared from membranes of Sf9 cells infected with baculovirus encoding Rac2 and incubated for 30 min at 25 °C in the presence of 100 μM GDP or GTPγS with phospholipid vesicles containing PtdInsP2. The incubation was performed in the presence of 30 mM free Ca2+ and 1.5 mM sodium deoxycholate.

FIGURE 7. Concentration dependence on GTPγS and nucleotide specificity of the stimulation of PLCγ2 by Rac2. Aliquots of soluble fractions of Sf9 cells infected with baculovirus encoding PLCγ2 containing 2.2 μg of protein/sample were reconstituted with aliquots (5 μl/sample) of a detergent extract prepared from membranes of Sf9 cells infected with baculovirus encoding Rac2 and incubated for 45 min at 30 °C with phospholipid vesicles containing PtdInsP2. The incubation was performed at increasing concentrations of GTPγS (left panel) or at concentrations of 100 μM of the purine nucleotides indicated at the abscissa (right panel) in the presence of 30 mM free Ca2+ and 1.0 mM sodium deoxycholate. The concentration of GDP carried over into the incubation medium from the detergent extract was 0.65 μM.

Thus, maximal activation of Rac2 with GTPγS caused an -15-fold stimulation of inositol phosphate formation by PLCγ2 in this experiment. Among the purine nucleoside di- and triphosphates tested, only the poorly hydrolyzable guanine nucleoside triphosphate analogues GTPγS and GppNHp caused a robust stimulation of inositol phosphate formation in the presence of Rac2 and PLCγ2. ADP, GDP, and GTPβS
caused no change in this activity. Only minimal, if any, effects were observed upon addition of ATP, ATPγS, and GTP (Fig. 7, right panel). To examine the specificity of PLCγ2 stimulation by Rac GTPases family members, the recombinant Rho GTPases were produced in baculovirus-infected insect cells, extracted from the membrane of infected cells with detergent-containing buffer, and reconstituted with samples containing PLCγ2. The amounts of GTPγS-binding proteins present in the detergent extracts were assayed by [35S]GTPγS binding. Fig. 8, left panel, shows that the Rho GTPases Cdc42, RhoA, Rac1, Rac2, and Rac3 were present in the detergent extracts used in this experiment at different concentrations (Cdc42 > RhoA > Rac3 > Rac1 ~ Rac2). When equal volumes of these detergent extracts were reconstituted with PLCγ2, addition of GTPγS instead of GDP caused an ~6.5-fold stimulation of inositol phosphate formation in the presence of either Rac1 and Rac2 and an ~4.0-fold stimulation in the presence of Rac3. In marked contrast, there was no effect of GTPγS in the presence of detergent extracts prepared from insect cells expressing β-galactosidase, Cdc42, or RhoA (Fig. 8, right panel). In additional experiments (data not shown), we found that GTPγS caused an ~73- and 3.7-fold activation of inositol phosphate formation upon reconstitution of the latter two extracts with samples containing recombinant PLCβ2 (10) and PLCe (22), respectively, confirming that Cdc42 and RhoA were present in these extracts as functional proteins. There was no effect of GTPγS on inositol phosphate formation when the detergent extracts used in Fig. 8 were reconstituted with soluble fractions of insect cells expressing β-galactosidase or PLCγ2 (not shown). Given the fact that Cdc42, RhoA, and Rac3 were present in the detergent extracts at ~8.0-, 4.2-, and 2.3-fold excess, respectively, of Rac1 and Rac2 (Fig. 8, left panel), these results collectively suggest that PLCγ2 is specifically activated by Rac GTPases and that the rank order of Rac GTPases to mediate this activation is Rac1 ~ Rac2 > Rac3.

**DISCUSSION**

The observation that inactivation of the genes encoding the Rho guanine nucleotide exchange factors (Rho GEFs) Vav1 and Vav2 (51, 52) or all three Vav proteins (53) causes a marked reduction of the BCR-mediated increase in [Ca2+]i, has previously lead to the suggestion that Rho GTPases are critically involved in regulating Ca2+ signaling in B cells. Vav1 is known to activate Rac1, Rac2, and RhoG, as well as RhoA and Cdc42, and Vav2 to act on RhoA, RhoB, and RhoG, as well as Rac1 and Cdc42. Vav3 preferentially activates RhoA, RhoG, and, to a lesser extent, Rac1 (reviewed in Ref. 54). BCR ligation on intact B cells has been shown to cause activation of Rho GTPases, e.g. Rac1 (55, 56). The observations that certain Rho GTPases, e.g. Rac1 and Cdc42, activate PI3K(s) in vitro (reviewed in Ref. 54) and that PtdIns3P activates PLCγ1 and PLCγ2 (57) has been taken to suggest that Rho GEFs and Rho GTPases may activate PLCγ isozymes in intact cells indirectly through enhanced formation of PtdIns3P, followed by PH domain- and/or SH2 domain-mediated translocation of the PLCγ isozymes to the plasma membrane or to specific plasma membrane microdomains referred to as lipid rafts or glycolipid-enriched microdomains, GEMs (36). Very recently, RhoA has been shown to be activated, again indirectly and downstream of PI3K(s), in response to BCR stimulation and to promote activation of PLCγ2, Ca2+ mobilization, and B cell proliferation by augmenting the synthesis of the PLC substrate PtdIns3P (58).

The results of this study suggest that Rac GTPases may also control the enzymatic activity and/or the subcellular localization of the PLCγ2 isozyme independently of enhanced formation of PtdIns3P and PtdIns3P2. Thus, both PLCγ1 and PLCγ2 have previously been shown to be activated by PtdIns3P and PI3K(s) (57). As such, Rac GTPases may activate PLCγ1 and PLCγ2 independently of enhanced formation of PtdIns3P and PI3K(s) (57). As such, Rac GTPases may activate PLCγ1 and PLCγ2 independently of enhanced formation of PtdIns3P and PI3K(s). Thus, the observation that PLCγ2 activity is increased in cells treated with brefeldin A, which disrupts the Golgi apparatus, suggests that PLCγ2 activity is increased in cells (59).

The functional defects observed in mice deficient in PLCγ2 are not restricted to B cells but are also observed in platelets, natural killer cells, monocytes/macrophages, and mast cells (60, 61). Thus, although both PLCγ1 and PLCγ2 are present in rat RBL-2H3 tumor mast cells, and are activated in response to cross-linking of the high affinity IgE receptor FceRI (62), primary mast cells from PLCγ2−/− mice show a marked reduction in FceRI-mediated degranulation (60, 61). Of note, treatment of RBL-2H3 cells with Clostridium difficile toxin B, which inactivates

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Rho and Rac GTPases as well as Cdc42, and with Clostridium sordellii lethal toxin, which inactivates Rac GTPases, possibly Cdc42, but not Rho GTPases, caused a complete abrogation of degranulation and an inhibition of intracellular Ca2+ mobilization that normally follows FcεRI ligation. In contrast, treatment of cells with Clostridium botulinum exoenzyme C3 or the fusion toxin C21N-C3, which selectively inactivate Rho GTPases, was without effect (63, 64). Along the same lines, constitutively active mutants of Cdc42 and Rac1 have previously been shown to stimulate exocytosis of secretory granules in RBL-2H3 cells by stimulating InsP3 formation and Ca2+ mobilization upon antigen stimulation of FcεRI (65). Furthermore, constitutively active Cdc42 and Rac1 reconstituted FcεRI-mediated Ca2+ mobilization and degranulation in B6A4C1 mutant RBL cells that are defective in antigen-stimulated PLCγ isoyme activity. Overexpression of PLCγ2 together with either activated Cdc42 or Rac1 synergistically stimulated degranulation (66). Interestingly, bacterially expressed glutathione S-transferase fusion proteins of constitutively active Cdc422046 and, to a lesser extent, wild-type Cdc42 interacted in vitro with a protein immunoreactive with antibodies raised against amino acids 1249–1262 of bovine PLCγ2. There was no effect, however, of activated Cdc42 on PLCγ activity upon reconstitution of the two purified proteins in a cell-free system (65). At first glance, these findings are not easily consistent with the findings reported here. However, PLCγ2 was apparently not examined in the latter studies, and the results presented here do not exclude the possibility that activated Cdc42 and Rac1 interact with PLCγ and that Cdc42 interacts with PLCγ2 without enhancing the catalytic activities of the PLCγ isoymes. Moreover, activated Cdc42 appeared to interact with a species of PLCγ that migrated slightly faster on SDS-polyacrylamide gels than the majority of recombinant PLCγ2, suggesting that it may be a modified form of the enzyme that binds most effectively to activated Cdc42 (65).

The site(s) of interaction between activated Rho GTPases and PLCβ2 has been mapped to the amino-terminal 138 or 144 amino acids of PLCβ2, containing the putative PH domain (aa 11–135) of the enzyme (12–14). Activated Rho GTPases appear to directly and specifically interact with this portion of PLCβ2, with affinities (Kd, in micromolar range) (14). The mode of interaction of activated Rac GTPases with PLCγ2 and the structural requirements of this interaction are currently unknown. Importantly, the PLCγ isoymes carry two putative PH domains, one at an amino-terminal position corresponding to the position of the putative PH of PLCβ2 (PLCγ2, aa 18–144; PLCγ2, aa 11–133) and a second, split PH domain located between the two catalytic subdomains X and Y (PLCγ2, PH-n, aa 482–527, PH-c, aa 872–937; PLCγ2, PH-n, aa 468–513, PH-c, aa 849–914) (reviewed in Ref. 67). The split PH domain of PLCγ2 has been shown to be involved in protein-protein interactions (68, 69). It is thus tempting to speculate that activated Rac GTPases interact with PLCγ2 either via the amino-terminal or via one or both components of the split PH domain. Interestingly, both PLCγ isoymes have been shown to mediate agonist-induced Ca2+ entry, at least in part by a phospholipase C activity-independent manner (59, 70). Very recent evidence suggests that PH-c of PLCγ2 controls cell surface expression of the canonical transient receptor potential channel 3 (TRPC3) by interacting with a complementary partial PH-like domain present within its amino-terminal portion (71). Whether activated Rac GTPases mediate translocation of TRP channels to the plasma membrane via PLCγ2 remains an intriguing question to be clarified by future experimentation, in particular since there is precedence for an enhancement of rapid vesicular translocation and insertion of TRP channels by activated Rac1 (72).

Although the changes of B cell development and signaling exhibited by mice deficient in either Rac2 (34, 35) or PLCγ2 (60) are complex, certain features were observed in both cases, suggesting that the functions of Rac GTPases and PLCγ2 in B cells may overlap. These include a paucity of mature B cells, such as B1 B cells, in the periphery, reduced serum concentration of IgM, and a decreased ability to respond to T cell-dependent antigens in vivo (34, 35, 60). While it seems clear that activation of Rac1 and Rac2 is not required for BCR-mediated activation at high levels of BCR cross-linking (34, 35), it is likely that Rac GTPases are important under more limiting conditions, i.e. when the antigen is present at low abundance or interacts with the BCR with low affinity, i.e. only for short periods of time (34). Under these circumstances, efficient mechanisms are required to amplify the extracellular signal at the level of the plasma membrane. Interestingly, B cells have been shown to acquire antigen from antigen-presenting cells after formation of synapses, which are characterized by a central supramolecular activation cluster-containing antigen-bound BCR and PLCγ2 at high densities. The cSMACs are surrounded by peripheral rings, referred to as pSMACs, containing high concentrations of integrins such as LFA-1 (73, 74). The interaction of LFA-1 with its main ligand, ICAM-1, which is present on leukocytes, follicular dendritic cells, dendritic cells, and vascular endothelial cells (74), has been shown to lower the threshold of B cell activation (75). Although the relationship between lipid rafts and B cell synapses is still tenuous (76), the observation that Rac GTPases may be targeted to the plasma membrane by integrins via lipid rafts and represent major mediators of integrin signaling (reviewed in Refs. 77 and 78) raises the intriguing possibility that Rac GTPases coordinate, both temporally and spatially, BCR-mediated PLCγ2 activation with integrin engagement and thus allow B cells to recognize antigens even at low concentrations in a context-dependent way.

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REFERENCES
1. Irvine, R. F. (2003) Nat. Rev. Mol. Cell. Biol. 4, 586–590
2. Berriug, M. J. (2005) Annu. Rev. Physiol. 67, 1–21
3. Ohno, S., and Nishizuka, Y. (2002) J. Biochem. (Tokyo) 132, 509–511
4. Nishizuka, Y. (2003) J. Biochem. (Tokyo) 133, 155–158
5. McLaughlin, S., Wang, J., Gambhir, A., and Murray, D. (2002) Annu. Rev. Biophys. Biomol. Struct. 31, 151–175
6. Yin, H. L., and Janmey, P. A. (2003) Annu. Rev. Physiol. 65, 761–789
7. Suh, B. C., and Hille, B. (2005) Curr. Opin. Neurobiol. 15, 370–378
8. Cho, W., and Stahelin, R. V. (2005) Annu. Rev. Biophys. Biomol. Struct. 34, 199–151
9. Rebecchi, M. J., and Pentsyia, S. N. (2000) Physiol. Rev. 80, 1291–1335
10. Rhee, S. G. (2001) Annu. Rev. Biochem. 70, 281–312
11. Ilbenninger, D., Schwald, F., Finmer, D., Binder, W., Maier, G., Dietrich, A., and Gierschik, P. (1998) EMBO J. 17, 6241–6249
12. Ilbenninger, D., Walliser, C., Nürnberg, B., Díaz Lorente, M., and Gierschik, P. (2003) J. Biol. Chem. 278, 3006–3014
13. Ilbenninger, D., Walliser, C., Strobel, J., Gunzler, O., Niew, H., Gaździk, V., Klooß, Y., Gierschik, P., and Henis, Y. I. (2003) J. Biol. Chem. 278, 8645–8652
14. Snyder, J. T., Singer, A. U., Wing, M. R., Harden, T. K., and Sundek, J. (2003) J. Biol. Chem. 278, 21099–21104
15. Wilde, J. I., and Watson, S. P. (2001) Cell Signal. 13, 691–701
16. Katan, M., Rodriguez, R., Matsuda, M., Newbatt, Y., and Aherne, G. W. (2003) Adv. Enzyme Regul. 43, 77–85
17. Sekiya, F., Ban, H. T., and Rhee, S. G. (1999) Chem. Phys. Lipids 98, 3–11
18. Osbourn, A. M., and Pawelczak, T. (2003) Acta Biochim. Pol. 50, 1097–1110
19. Caprini, M., Gomis, A., Cabedo, H., Planells-Cases, R., Belmonte, C., Viana, F., and Ferrer-Montiel, A. (2003) EMBO J. 22, 3004–3014
20. Guo, Y., Rebecchi, M., and Scarlata, S. (2005) J. Biol. Chem. 280, 1438–1447
21. Sinfu, R. S., Clough, R. R., and Bhullar, R. P. (2005) J. Biol. Chem. 280, 21933–21941
