Rac-mediated Stimulation of Phospholipase Cγ2 Amplifies B Cell Receptor-induced Calcium Signaling

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Background: Phospholipase Cγ2 (PLCγ2) is stimulated by Rac GTPases through direct protein-protein interaction.

Results: The Rac-PLCγ2 interaction markedly enhances B cell-receptor-mediated Ca2+ mobilization and nuclear translocation of the Ca2+-regulated transcription factor NFAT in B cells.

Conclusion: Rac-mediated stimulation of PLCγ2 activity amplifies B cell receptor-induced Ca2+ signaling.

Significance: A specific Rac-resistant PLCγ2 variant is used to determine the physiological cell signaling relevance of a functional Rac-PLCγ2 interaction in an appropriate cellular context.

The Rho GTPase Rac is crucially involved in controlling multiple B cell functions, including those regulated by the B cell receptor (BCR) through increased cytosolic Ca2+. The underlying molecular mechanisms and their relevance to the functions of intact B cells have thus far remained unknown. We have previously shown that the activity of phospholipase Cγ2 (PLCγ2), a key constituent of the BCR signalosome, is stimulates by activated Rac through direct protein-protein interaction. Here, we use a Rac-resistant mutant of PLCγ2 to functionally reconstitute cultured PLCγ2-deficient DT40 B cells and to examine the effects of the Rac-PLCγ2 interaction on BCR-mediated changes of intracellular Ca2+ regulation and of Ca2+-regulated and nuclear-factor-of-activated-T-cell-regulated gene transcription at the level of single, intact B cells. The results show that the functional Rac-PLCγ2 interaction causes marked increases in the following: (i) sensitivity of B cells to BCR ligation; (ii) BCR-mediated Ca2+ release from intracellular stores; (iii) Ca2+ entry from the extracellular compartment; and (iv) nuclear translocation of the Ca2+-regulated nuclear factor of activated T cells. Hence, Rac-mediated stimulation of PLCγ2 activity serves to amplify B cell receptor-induced Ca2+ signaling.

Inositol phospholipid-specific phospholipases C (PLC) catalyze the formation of inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG) from plasma membrane lipid substrate phosphatidylinositol 4,5-bisphosphate (PtdInsP2) (1). Both the rise of the former two and the decline of the latter may serve as intracellular signals to regulate a myriad of cell functions (2). In B lymphocytes, receptors for cell surface immunoglobulins such as the B cell receptors (BCR), cleavage fragments of the third complement component (CD19/CD21) (3), bacterial, viral, or autoimmunity host DNA (toll-like receptors) (4), and even certain G-protein-coupled chemokine receptors (5) mediate activation of PLCγ2, one of the two human PLCγ isozymes. The activity of PLCγ2 controls many B cell functions, such as protein kinase signaling, nucleocytoplasmic trafficking of transcription factors, proliferation, differentiation, cytoskeletal reorganization, cell adhesion and migration, immunological synapse formation, affinity maturation, autoimmunity, homing to and retention in tissue microenvironments, survival, and susceptibility to transformation (6, 7).

Inactivation of the PLCγ2 gene in the mouse caused specific deficits in most cell types of hematopoietic origin, except for T cells (8, 9). Mice lacking PLCγ2 showed reduced numbers of mature conventional B cells, a block in pro-B cell differentiation, B1 B cell deficiency, absence of IgM receptor-mediated Ca2+ responses, and B cell-mitogen-induced cell proliferation. PLCγ2 also plays important roles in pre-BCR-mediated early B cell development, in BAFF receptor-mediated survival, and in activation of light-chain loci for recombination as well as recep-

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Rac and PLCγ2 Amplify BCR-mediated Ca2+ Signaling

Mutational activation of Rac GTPases in B cells results in deregulation of B cell functions (10–12). Mutationally activated forms of PLCγ2 have been identified in mice subjected to N-ethyl-N-nitrosourea mutagenesis (Ali15 and Ali14) and, more recently, in patients with inherited forms of autoinflammation and immunodeficiency (13–16). These defects also lead to deregulation of B cell functions.

Several of the changes described for PLCγ2−/− B cells, e.g. defective Ca2+ signaling, failure to proliferate in response to immunoglobulin receptor stimulation, impediment of B cell development, and failure to mount humoral responses to TD and TI antigens, were also observed in mice carrying deletions in all three genes encoding Vav guanine nucleotide exchange factors of Rho GTPases, Vav1, -2, and -3 (17). These results were difficult to interpret mechanistically because Vav proteins elicit both RhoGEF-dependent and -independent effects (18). However, some of the B cell defects were also observed in mice lacking either Rac2 (19) or both Rac1 and Rac2 (20), including a reduced ability of BCR or CD19 (co)ligation to increase [Ca2+]i, suggesting that at least some of the B cell defects commonly observed in PLCγ2 and Vav1/2/3-null mice were due to loss of Rac activation. At that time, the available evidence suggested that Rac GTPases might activate PLCγ2 indirectly by enhancing the activity of phosphatidylinositol 4-phosphate 5-kinase (21), thus increasing the level of PtdInsP2, the substrate of both PLCγ2 and phosphoinositide 3-kinase (20). Enhanced availability of substrate to the former and enhanced formation of PtdInsP2 by the latter were expected to activate PLCγ2 (22).

We have previously shown that Rac GTPases exhibit Rac2-specific protein-protein interaction (23). Neither enhanced formation of PtdInsP2 nor PtdInsP3 nor protein tyrosine phosphorylation are involved in this effect. Unlike activation of PLCβ2, which is mediated by Rac interacting with the N-terminal PH domain of this effector, activation of PLCγ2 involves binding of Rac to the bipartite, split PH domain (spPH) juxtaposed between the two halves, X and Y, of the PLCγ2 catalytic domain (24). The three-dimensional structures of the heterodimeric complex between PLCγ2, spPH and GTPγS-activated Rac2, monomeric spPH, and monomeric Rac2 liganded with either GTPγS or GDP allowed us to elucidate the conformational changes that accompany the formation of the signaling active PLCγ2-spPH/Rac2 heterodimer (25). A residue unique for spPH of PLCγ2, but not PLCγ1, Phe-897, was found to be particularly important for the functional and structural PLCγ2-spPH/Rac2 interaction. Replacement of Phe-897 to the corresponding glutamine residue of PLCγ1, F897Q, did not affect the overall three-dimensional structure of the PLCγ2 spPH domain, but it specifically blocked the interaction of the mutant domain with activated Rac2 (24, 25).

Rac GTPases are present at many intracellular crossroads of B cell signaling. They receive inputs from numerous cell surface receptors to regulate and integrate a host of intracellular signaling proteins, including PLCγ2, and many proteins involved in cytoskeletal organization (26). This has made it intrinsically difficult to judge the pertinence of the PLCγ2-Rac interaction observed in cell-free experiments and overexpression studies to cell signaling in more physiologically relevant cellular contexts. In this work, we have used DT40 B cells genetically deficient in PLCγ2 for functional reconstitution with the Rac-resistant PLCγ2 mutant F897Q to study the effects of a specific loss of the PLCγ2-Rac interaction on BCR-mediated cell signaling. The results reveal that loss of this functional interaction causes a marked decrease of BCR-mediated Ca2+ release from intracellular stores, Ca2+/ entry from the extracellular compartment, and nuclear translocation of the Ca2+-regulated transcription factor NFAT. Some of these changes have previously been observed in Rac-deficient mice (19, 20). Hence, the results may provide a mechanistic explanation for findings on the role of Rac GTPases in B cell signaling obtained in vivo that have as yet remained largely unexplained. In addition, these insights into BCR-mediated cell signaling may also apply to the mechanisms of action of other B cell receptors such as CD19/CD21, to other cells of hematopoietic origin, e.g. platelets, and to human diseases, such as certain immunodeficiencies. To our knowledge, this is the first time that a Rho-resistant but otherwise normal Rho effector was reintroduced into a genetically Rho effector-deficient background to determine the relevance of the functional Rho effector interaction in a biologically highly relevant context.

Experimental Procedures

Antibodies and Reagents—Mouse monoclonal antibody reactive against the c-Myc epitope (9B11, catalogue no. 2276) was from Cell Signaling. Mouse monoclonal antibody reactive against β-actin (AC-15, catalogue no. A3854), poly-U-lysine (catalogue no. P6282), and ionomycin (catalogue no. I-0634) was from Sigma. Anti-phosphotyrosine antibody (catalogue no. 05-321, 4G10) was purchased from Millipore. Mouse anti-chicken IgM (M-4, catalogue no. 8300-01) was obtained from SouthernBiotech. Alexa Fluor® 488 goat anti-mouse antibody (catalogue no. A-11029), fluo-4 acetoxyethyl ester (catalogue no. F-14201), Pluronic®-F127 (catalogue number P-3000MP), and thapsigargin (catalogue no. T-7459) were from Molecular Probes® (Life Technologies, Inc.). Trypsin (catalogue no. 1418475001) was from Roche Applied Sciences, and puromycin was from InvivoGen.

cDNA Cloning—Because the 5’ end of the mRNA encoding chicken PLCγ2 was unknown at the time, 5’ rapid amplification of cDNA ends (27) was used to gather this information and produce full-length PLCγ2 cDNAs from reverse-transcribed DT40 cell mRNA. Two presumably allelic variants were found, which are identical at the protein level to each other and to database entry XP_414166, except for a Gln to His divergence at position 865. Based on the higher frequency (7/10) of His-865 among PCR products of DT40 cell mRNA, this haplotype was used herein for further studies. A histidine is present at this position in PLCγ2 of numerous species ranging from fish, such as coelacanth, to mammals, such as cattle or sheep. The plasmid pCDNA3.1-c-tfp-RFP611 encodes amino acids 1–400 of mouse NFAT1c fused to a pseudo-monomeric tandem dimer red fluorescent protein, td-RFP611 (28).
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and 5′-TTTACGACACTTTGGTACCACCTTGTGAGTCTTCTACTTATAGTTGC-3′ (antisense). The chicken PLCγ2 deletion mutants PLCγ2ΔPCI and PLCγ2ΔPCI\(^{F987Q}\) lacking the phospholipase C inhibitor (PCI) peptide (amino acids 727–734) were constructed by site-directed mutagenesis using the primers 5′-GAGAAAGCACCCTGGCTGTGACAGTAGAGAGCCTG-3′ (sense) and 5′-GCTCTGAGTCACGGAGCAGGGTGCTCTTC-3′ (antisense). For expression in COS-7 cells, the cDNAs of C-terminally c-Myc epitope-tagged wild-type (WT) PLCγ2\(\), PLCγ2\(^{F987Q}\), PLCγ2ΔPCI, and PLCγ2ΔPCI\(^{F987Q}\) were ligated into the BamHI/NotI site of the expression vector pExpress, in which the expression of a puromycin. For FRAP experiments, human PLCγ2\(\), PLCγ2\(^{F987Q}\), PLCγ2ΔPCI, and PLCγ2ΔPCI\(^{F987Q}\) were ligated into the BamHI/NotI site of pcDNA3.1(+). For production of recombinant baculoviruses, the cDNAs of c-Myc epitope-tagged PCR amplified (17058 bases) containing no inserts. The F897Q variant of human PLCγ2 was constructed by inserting the PLCγ2\(\)\(-\)TTCACGGACACTTTGGTACCATTCCTGCAGTTGCTTCTC-3′ cDNA into the KpnI site of pVL1393. To create stably transfected DT40 cell clones, cDNAs encoding C-Myepitope-tagged PLCγ2 and PLCγ2\(^{F987Q}\) were ligated into the BamHI/NotI site of pVL1393. To create stably transfected DT40 cell clones, cDNAs encoding C-Myc epitope-tagged PLCγ2 and PLCγ2\(^{F987Q}\) were ligated into the BglII/Smal site of the expression vector pExpress, in which the expression of a cloned cDNA is controlled by the chicken β-actin promoter and an SV40 poly(A) signal (29). The pExpress cDNA expression cassette was then combined with the vector pLoxPuro by using the restriction site SpeI generating an expression vector, in which only the selectable marker is flanked by mutant loxP sites (29). For elect! }
(Fig. 4A and supplemental Video S1). Then, baseline-corrected and normalized Ca\(^{2+}\) response traces were subjected to further analysis using algorithms developed in-house and implemented in MATLAB (MathWorks). A Ca\(^{2+}\) response peak was defined by a transient positive divergence from the baseline with a minimum of 10% in intensity of the maximum observed in the presence of ionomycin. The number of responding cells was calculated as the number of traces with at least one Ca\(^{2+}\) response peak and expressed as the percentage of the total number of traces in each experiment. Latency was defined as a mean time lapse between the time of ligand addition and the time corresponding to the maximum of the first Ca\(^{2+}\) response peak. Cells that did not show any detectable Ca\(^{2+}\) spike during the initial observation time for the release of Ca\(^{2+}\) from internal stores, 365.1 s, were considered to display maximal latency equal to the time of observation. The integrated intensity was calculated as the mean area under the curve of all individual traces corresponding to single cells. The peak frequency was calculated for cells showing discrete Ca\(^{2+}\) spikes with baseline resolution, i.e. after addition of anti-IgM at concentrations of 4, 40, and 400 ng/ml, and expressed in millihertz (10\(^{-3}\) Hz). The same cells were used to calculate the peak amplitude as the average intensity of the Ca\(^{2+}\) peaks in percentage of the ionomycin maximum. The integrated intensities, peak frequencies, and peak amplitudes of nonresponding cells were set to zero.

**Analysis of Nuclear NFAT1c Translocation**—To study the BCR-mediated, Ca\(^{2+}\)-dependent translocation of the transcription factor NFAT into the nucleus, 30 μg of cDNA encoding NFAT1c-td-RFP611 was transfected into \(10^6\) PLC\(_{\gamma2}\)-generated DT40 B cells per cuvette that had or had not been reconstituted with either WT or F897Q mutant PLC\(_{\gamma2}\) by nucleofection (Amaxa\textsuperscript{®} Nucleofector\textsuperscript{®} Technology, Cell Line Nucleofector\textsuperscript{®} kit T, program B-023). Forty eight h after nucleofection, 6 \(\times\) 10\(^{5}\) DT40 B cells per channel were allowed to adhere to poly-l-lysine-coated ibidi\textsuperscript{®} 6-channel microscopy slides. After two washing steps with buffer B, the cells were treated for 60 min with either 40 μg/ml of anti-IgM or 2 μm ionomycin in buffer B containing 1 mM Ca\(^{2+}\). The translocation of NFAT1c-td-RFP611 was analyzed at room temperature on a single cell level by spinning disc confocal microscopy over a time period of 60 min using a 532-nm excitation laser for fluorescence imaging.

**Indirect Immunofluorescence Staining**—For immunofluorescence staining, 3 \(\times\) 10\(^5\) DT40 cells per channel were allowed to adhere to tissue culture-treated 6-channel microscopy slides (ibiTreat, ibidi\textsuperscript{®} 6-slide VI0.4, ibidi, catalogue no. 80606), as described above. The cells were then fixed by incubation for 15 min in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. After washing the cells twice with PBS, the nonreacted aldehyde groups were quenched by treatment for 10 min with 50 mM NH\(_4\)Cl in PBS. The cells were then washed four times with PBS, and nongspecific protein-binding sites in the channels were blocked by incubation for 1 h with buffer C (PBS, pH 7.4, 5% (v/v) FCS, 0.02% (w/v) sodium azide, 0.01% (w/v) saponin, 0.1% (v/v) Triton X-100). The cells were then incubated for 45 min with buffer C containing the secondary goat anti-mouse IgG antibody conjugated with Alexa Fluor 488 (1:1000). After four further washing steps with buffer D, the expression of PLC\(_{\gamma2}\) protein was analyzed on a single cell level by spinning disk confocal microscopy employing a 473-nm excitation laser for fluorescence and a halogen lamp for transmission light imaging. All steps were done at room temperature.

**Analysis of PLC\(_{\gamma2}\) Phosphorylation by Immunoprecipitation**—H\(_2\)O\(_2\) enhances tyrosine phosphorylation of PLC\(_{\gamma2}\) mediated by the tyrosine kinases Syk and Btk (31). Aliquots (12 \(\times\) 10\(^6\) each) of PLC\(_{\gamma2}\)- or PLC\(_{\gamma2}\) F897Q-expressing DT40 cells were treated for 5 min with 2 mM H\(_2\)O\(_2\) in 400 μl of PBS, pH 7.4, at 37°C in a water bath. After addition of 4 ml of ice-cold PBS and centrifugation at 300 \(\times\) g for 5 min, the pellet was resuspended in 400 μl of ice-cold lysis buffer (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40, 0.2 mM Na\(_2\)VO\(_4\), phosphatase inhibitor mixture 2 (1:100; v/v) (Sigma), 0.1 mM PMSF, 10 μM leupeptin, 2 μM pepstatin, 2 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin, 3 mM benzamidine, 1 mM DTT, 16 μg/ml 1-chloro-3-otosylamido-7-amino-2-heptanone, and 16 μg/ml 1-1-tosylamide-2-phenylethyl chloromethyl ketone), and the cells were lysed by incubation for 30 min at 4°C. After centrifugation at 100,000 \(\times\) g for 1 h at 4°C, aliquots of the supernatants (1 mg of protein each) were added to anti-c-Myc antibody-protein A complexes, which had been prepared by preincubuation of anti-c-Myc antibody (1:1000 (v/v) in 500 μl of PBS) with 30 μl of protein A-agarose beads (50% (v/v) slurry) overnight at 4°C and three washes with lysis buffer. After incubation for 2 h at 4°C, the beads were washed three times with lysis buffer and once with PBS and then subjected to SDS-PAGE and Western blotting using anti-phosphoysrinosine or anti-c-Myc antibody (1:1000 (v/v) each).

**FRAP Experiments**—FRAP studies (32, 33) were conducted as described earlier (34). The experiments were performed 24–26 h post-transfection on COS-7 cell transfected with PLC\(_{\gamma2}\)-GFP derivatives. All experiments were conducted at 22°C in Hanks’ balanced salt solution supplemented with 20 mM HEPES, pH 7.2. The monitoring argon ion laser beam (488 nm, 1.2 microwatts; Innova 70C, Coherent) was focused through the microscope (AxioImager.D1, Carl Zeiss MicroImaging) to a Gaussian spot with a radius \(\omega = 0.77 \pm 0.03 \mu m\) (\(\times63/1.4 NA\) oil-immersion objective) or 1.17 ± 0.05 μm (\(\times40/1.2 NA\) water immersion objective). Experiments were conducted with each beam size (beam size analysis is described in Refs. 35, 36). The ratio between the illuminated areas (\(\omega^2(\times40)/\omega^2(\times63)\)) was 2.28 ± 0.17 (\(n = 59\)). After a brief measurement at the monitoring intensity, a 5-milliwatt pulse (4–6 ms or 10–20 ms for the \(\times63\) and \(\times40\) objectives, respectively) bleached 50–70% of the fluorescence in the spot. Fluorescence recovery was followed by the monitoring beam. The apparent characteristic fluorescence recovery time (\(\tau\)) and the mobile fraction (\(R_m\)) were derived from the FRAP curves by nonlinear regression analysis, fitting to a lateral diffusion process with a single \(\tau\) value (37). The significance of differences between \(\tau\) values measured with the same beam size was evaluated by Student’s t test. To compare ratio measurements (\(\tau(\times40)/\tau(\times63)\) and \(\omega^2(\times40)/\omega^2(\times63)\)), we employed bootstrap analysis,
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which is preferable for comparison between ratios (38), as
described by us earlier (36), using 1000 bootstrap samples.

Miscellaneous—SDS-PAGE and immunoblotting were per-
formed according to standard protocols using antibodies reac-
tive against the c-Myc epitope for wild-type and mutant PLCγ2.
Immunoreactive proteins were visualized using the ECL West-
ern blotting detection system (GE Healthcare). All experiments
were performed at least three times. Similar results and identi-
cal trends were obtained each time. Data from representative
experiments are shown as means ± S.E. of triplicate determi-
nations, if not stated otherwise in the figure legends. Unless
stated otherwise, the significance of differences was assessed by
using either the unpaired t test with two-tailed p values or
repeated measures analysis of variance with Tukey’s post test
(Fig. 7B), both contained in GraphPad InStat®, version 3.10.
Statistically significant effects are denoted by ***, p < 0.001; **,
0.001 < p < 0.01; and *, 0.01 < p < 0.05. Nonsignificant (ns)
changes are denoted by ns, 0.05 < p. In Figs. 1, D and E, and 6,
A–C, the data were fitted by nonlinear least squares curve fit-
tting to three or four parameter dose-response equations using
GraphPad Prism®, version 5.04. In certain cases, the global
curve fitting procedure contained in Prism® was used to deter-
mine whether the best fit values of selected parameters differed
between data sets. The simpler model was selected unless the
extra sum of squares F-test had a p value of less than 0.05. In Fig.
6A, right panel, the data were fitted to the equation of a bell-
shaped dose response curve provided by Prism®, with manual
adjustments.

Results

Point Mutation F897Q Renders the DT40 Cell PLCγ2 Ortho-
logue Specifically Resistant to Stimulation by Activated Rac2—
Mutational and structural analyses have shown that the stimu-
lation of human PLCγ2 by activated Rac GTPases is due to an
interaction of the Rac switch I and II regions with specific resi-
dues in the C-terminal half of the PLCγ2 spPH domain (24, 25).
Fig. 1A illustrates the importance of the PLCγ2 residue Phe-
897, contained within the C-terminal α helix of spPH, in this
respect. Phe-897 forms the core of a hydrophobic pocket on
PLCγ2 spPH, which interacts with several hydrophobic resi-
dues of the Rac2 switch I and II regions, such as Val-36 (I),
Phe-37 (I), Trp-56 (II), Leu-67 (II), and Leu-70 (II). Rac2-PLCγ2
complex formation is thought to have two consequences as
follows: (i) conversion of GTP-bound Rac2 from conforma-
tional state 1 to state 2. These states were initially described for
H-Ras as having low versus high affinity, respectively, toward
Ras effectors (39). (ii) Stabilization of the Phe-897 side chain
was in one of the two conformational states observed in the
crystal structures of free spPH (25). Replacement of Phe-897 in
human PLCγ2 spPH by the corresponding human PLCγ1 resi-
due glutamine is expected to reduce the hydrophobic moment-
tum of the Rac2 binding pocket on PLCγ2 spPH and, possibly,
interfere with the reorientation of the Leu-67 side chain upon
complex formation (Fig. 1A). Consistent with this view, the
F897Q substitution blocked activation of human PLCγ2 by con-
stitutively active Rac2 and abolished binding of GTPγS-acti-
vated Rac2 to PLCγ2 spPH, while leaving the overall fold of
PLCγ2 spPH unaffected (24, 25).

To minimize the differences between the cellular system to
be functionally reconstituted and analyzed here and unmodi-
died DT40 B cells, a cDNA encoding chicken PLCγ2 was pro-
duced using mRNA from DT40 cells as a template. The
encoded protein shares 83% identical residues with human
PLCγ2. Within the C-terminal helices of the two spPH
domains, 14 of 17 residues, including Phe-897, are identical,
and three are conserved. The experiments shown in Fig. 1, B
and C, examined the consequences of the F897Q mutation on
the ability of the DT40 PLCγ2 orthologue (henceforth referred
to as PLCγ2) to interact with activated Rac2. Fig. 1B shows that
constitutively active Rac2G12V, but not WT Rac2, caused a
marked (~19-fold) stimulation of PLCγ2 activity in COS-7
cells, but it was ineffective in control cells lacking PLCγ2 and
in cells expressing the F897Q mutant. These observations were
confirmed in a cell-free system (Fig. 1C). The F897Q mutation
did not affect activation of PLCγ2 by calcium ions or by loss of
autoinhibition (Fig. 1, D and E) and had no influence on the
extent of PLCγ2 protein tyrosine phosphorylation in response to
H2O2 (Fig. 1F).

To measure the effect of the F897Q mutation on the interac-
tions of PLCγ2 with the plasma membrane in intact cells, FRAP
experiments were performed on GFP-tagged WT and the
F897Q mutant PLCγ2. PLCγ2-GFP fluorescence recovery occurred
with a fluorescence recovery time (τ) of 0.079 s (Fig.
2A, left panel). Coexpression of constitutively active Rac2G12V
enhanced the interaction of the enzyme with the plasma mem-
brane, resulting in an ~1.5-fold increase in τ of PLCγ2-GFP to
0.115 s (Fig. 2A, right panel). Interestingly, FRAP beam size
analysis experiments showed that the WT enzyme and the var-
iant F897Q did not differ in their modes of membrane interac-
tion, as the fluorescence recovery of both PLCγ2-GFP and
PLCγ2F897Q-GFP occurred by a mixture of binding to and dis-
sociation from the membrane, referred to as exchange, and of
stable association with the membrane, resulting in lateral diffu-
sion. In the presence of Rac2G12V, the fluorescence recovery
of WT PLCγ2-GFP was shifted toward lateral diffusion, as the
ratio between the fluorescence recovery times with the two
beam sizes (~40 and ~63 objectives) was 2.3 (Fig. 2, B and C).
This ratio is indistinguishable from that expected for recovery
by pure lateral diffusion (2.28 ± 0.17, the ratio between the
areas illuminated by the two beam sizes employed). Conse-
quently, in the presence of Rac2G12V, the interaction of PLCγ2-
GFP with the plasma membrane is dominated by lateral diffu-
sion, suggesting that the exchange rate becomes much slower
than the lateral diffusion rate (i.e. a shift to stable membrane
interactions). In marked contrast, Rac2G12V had no effect at all
on the mode of membrane association of the mutant
PLCγ2F897Q-GFP, which correlates well with our observations
that Rac2G12V cannot interact with and fails to stimulate
PLCγ2F897Q. We conclude that PLCγ2 is not compromised in
its overall activity and in its mode of membrane interaction by
the F897Q mutation, in contrast to its specific loss of regulation
by activated Rac.

Functional Reconstitution of Wild-type and F897Q Mutant
PLCγ2 into PLCγ2−/− DT40 B Cells—Next, genetically PLCγ2-
deficient DT40 cells were stably reconstituted with either iso-
genic WT PLCγ2 or the PLCγ2F897Q mutant such that the
resultant cell clones were indistinguishable in terms of enzyme expression and subcellular distribution (Fig. 3). Spinning disc confocal fluorescence microscopy was then used to determine the regulatory influence of Rac on the kinetic parameters of BCR-mediated changes in \([Ca^{2+}]\) at the level of individual DT40 B cells. Cells were loaded with the fluorescent Ca\(^{2+}\) indi-
cator fluo-4, and authentic PLC\(_{\gamma2}^{+/-}\) DT40 cells were compared with PLC\(_{\gamma2}^{-/-}\) cells, before and after stable reconstitution of the latter with either WT or F897Q mutant PLC\(_{\gamma2}\), for their ability to respond to BCR ligation with increases in \([Ca^{2+}]_{i}\). In accordance with earlier studies (40), cells were first treated with 40 ng/ml anti-IgM in the absence of extracellular Ca\(^{2+}\) to measure the BCR-mediated Ca\(^{2+}\) release from intracellular stores. After some time, the effect of the same concen-

**FIGURE 2.** FRAP beam size analysis demonstrates that wild-type and F897Q mutant PLC\(_{\gamma2}\) display similar membrane interactions, but only that of wild-type PLC\(_{\gamma2}\) is augmented by Rac2G12V. A, COS-7 cells were cotransfected with a vector encoding GFP-tagged wild-type PLC\(_{\gamma2}\) (WT) together with either empty vector (left panel) or vector encoding Rac2G12V (WT + Rac2G12V; right panel). The typical FRAP curves shown were obtained using a ×63 objective. The solid lines represent the best fit obtained by nonlinear regression analysis. The best fit \(\tau\) values are depicted in each panel. The mobile fractions (Rf) were above 0.93 in all cases and therefore are not shown. B and C, FRAP beam size analysis. Transfection was as in A, except that in some studies the GFP-tagged mutant (F897Q) replaced its wild-type counterpart (WT). Bars represent the means ± S.E. of 30–60 measurements. The studies employed ×40 and ×63 objectives, yielding a beam size ratio of 2.28 ± 0.17 (n=59). Thus, this \(\tau(\times40)/\tau(\times63)\) ratio is expected for FRAP by lateral diffusion (C, upper dashed line). A \(\tau\) ratio of 1 (C, lower dotted line) indicates recovery by exchange (35). In the absence of Rac2G12V, both the \(\tau\) values (comparing values measured with the same beam size (B) and the \(\tau(\times40)/\tau(\times63)\) ratios (C) of WT and F897Q mutant PLC\(_{\gamma2}\) were similar (\(p>0.4\) in all cases; Student’s \(t\) test), suggesting similar lateral diffusion in and exchange rate from the plasma membrane. Although coexpression of Rac2G12V had no significant effect on either the \(\tau\) values or \(\tau\) ratio of the F897Q mutant (\(p>0.4\)), it significantly enhanced the membrane association of wild-type PLC\(_{\gamma2}\), reflected in a slower \(\tau(\times40)\) (*) and in a significant increase of its \(\tau\) ratio (*, \(p<0.02\); bootstrap analysis) from an intermediate value (characterizing recovery by a mixture of lateral diffusion and exchange) to 2.3, similar to the value expected for pure lateral diffusion.
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Individual cells (Fig. 4A). The latter values were subsequently used to normalize the individual intensity time traces. Sixty cells from each cell type were analyzed individually in each experiment (Fig. 4B). Each of the curves shown represents the fluorescence intensity time trace of a single cell in the observation area. Fig. 4B, upper left panel, shows that addition of anti-IgM to WT DT40 caused oscillations of \([\text{Ca}^{2+}]\), in most but not all cells. In responding cells, the oscillations commenced after a lag time of \(\approx 2 \) min. Although some cells displayed a less oscillatory and more monophasic increase in \([\text{Ca}^{2+}]\), upon addition of extracellular \(\text{Ca}^{2+}\) accompanied by a loss of spiking, most responding cells showed a relatively homogeneous oscillatory behavior in the absence and presence of extracellular \(\text{Ca}^{2+}\). In \(\text{PLC}\textsubscript{\textgamma} \textsubscript{2}^{-/-}\) cells, by contrast, none of the cells responded to addition of anti-IgM (400 ng/ml), regardless of whether \(\text{Ca}^{2+}\) was absent from or present in the incubation medium (Fig. 4B, upper right panel). Addition of 4 ng/ml trypsin to these cells in the presence of extracellular \(\text{Ca}^{2+}\) caused the appearance of a single \([\text{Ca}^{2+}]\) peak, presumably by activation of G-protein-coupled PAR2 receptors endogenously present in DT40 cells, followed by activation of endogenous PLC\(\beta\) (41). In this case, almost all cells responded, and the lag time was much shorter (-10 s). Fig. 4B, lower panels, shows that although both PLC\(\gamma\textsubscript{2}\) and PLC\(\gamma\textsubscript{2}\textsubscript{F897Q}\) were able to reconstitute oscillatory \(\text{Ca}^{2+}\) responses to PLC\(\gamma\textsubscript{2}^{-/-}\) DT40 cells following BCR ligation, substantial quantitative differences were apparent already at the level of visual assessment of the two sets of time traces. Specifically, the proportion of cells responding to anti-IgM as well as both the peak amplitudes and the frequencies of the spikes were considerably lower, and the mean latencies of the overall responses were distinctly longer for cells expressing PLC\(\gamma\textsubscript{2}\textsubscript{F897Q}\) rather than WT PLC\(\gamma\textsubscript{2}\). A more detailed, quantitative characterization of this difference will be presented below in Figs. 5 and 6. A quantitative comparison of the \([\text{Ca}^{2+}]\) transients observed in PLC\(\gamma\textsubscript{2}^{-/-}\) DT40 cells versus PLC\(\gamma\textsubscript{2}^{-/-}\) cells reconstituted with WT PLC\(\gamma\textsubscript{2}\) in response to 400 ng/ml anti-IgM is shown in Fig. 4C (upper panels). There were no significant differences between the two cell types in the proportions of responding cells, integrated peak intensities, peak frequencies, and amplitudes (Fig. 4C, lower panels). Thus, the PLC\(\gamma\textsubscript{2}\)-deficient cells reconstituted with WT PLC\(\gamma\textsubscript{2}\) bear a close resemblance to their native counterparts. Fig. 4D shows that PLC\(\gamma\textsubscript{2}^{-/-}\) cells reconstituted with WT versus F897Q mutant PLC\(\gamma\textsubscript{2}\) do not appreciably differ in \([\text{Ca}^{2+}]\), responses triggered by addition of thapsigargin, an inhibitor of the sarco-/endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase (40, 41). Thus, the F897Q mutation and, by extension, interaction of PLC\(\gamma\textsubscript{2}\) with activated Rac have no effect on BCR-independent \([\text{Ca}^{2+}]\), mobilization in DT40 B cells.

Concentration Dependence on Anti-IgM of \([\text{Ca}^{2+}]\). Response Impairment in DT40 B Cells Expressing Wild-type Versus Rac-insensitive F897Q Mutant PLC\(\gamma\textsubscript{2}\).—To determine the influence of the Rac-PLC\(\gamma\textsubscript{2}\) interaction on \([\text{Ca}^{2+}]\), changes in DT40 B cells in response to increasing extents of BCR ligation, PLC\(\gamma\textsubscript{2}^{-/-}\) DT40 cells stably reconstituted with either WT or F897Q mutant PLC\(\gamma\textsubscript{2}\) were treated with increasing concentrations of anti-IgM in the absence of extracellular \(\text{Ca}^{2+}\), followed by the same concentration of anti-IgM in the presence of 1 mM extracellular \(\text{Ca}^{2+}\) to also allow for the entry of extracellular \(\text{Ca}^{2+}\) into the cells. Finally, the \(\text{Ca}^{2+}\) ionophore ionomycin was added to normalize the fluorescence intensities obtained for the

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**FIGURE 3.** Stable reconstitution of PLC\(\gamma\textsubscript{2}^{-/-}\) DT40 B cells with wild-type versus F897Q mutant PLC\(\gamma\textsubscript{2}\). PLC\(\gamma\textsubscript{2}-\)deficient DT40 cells (89) were stably reconstituted with either isogenic wild-type or F897Q mutant PLC\(\gamma\textsubscript{2}\). Aliquots of the lysates (50 \(\mu\)g of protein) of native DT40 cells (PLC\(\gamma\textsubscript{2}^{-/-}\)), PLC\(\gamma\textsubscript{2}-\)deficient DT40 cells (PLC\(\gamma\textsubscript{2}^{-/-}\)), and from three independent clones PLC\(\gamma\textsubscript{2}^{-/-}\) DT40 cells stably expressing similar quantities of either wild-type (A–C) or F897Q mutant PLC\(\gamma\textsubscript{2}\) (a–c) were subjected to SDS-PAGE and immuno blotting (upper panel). The same membrane was subsequently probed with an anti-\(\beta\)-actin antibody to control for equal loading of samples (lower panels). All six clones were used for experimentation in this study, with no differences detected between clones A–C and a–c, respectively. B, cells from the clones A and c were analyzed by indirect fluorescence staining (left panels). Right panels, corresponding phase contrast images. C, mean fluorescence intensities of the three images each, as shown in B, were corrected for background staining. Similar results were obtained for other pairs of clones.

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**TABLE 1.** Characteristics of PLC\(\gamma\textsubscript{2}^{-/-}\) DT40 B cells reconstituted with wild-type or F897Q mutant PLC\(\gamma\textsubscript{2}\). Wild-type PLC\(\gamma\textsubscript{2}\)-deficient DT40 cells (89) were stably reconstituted with either isogenic wild-type or F897Q mutant PLC\(\gamma\textsubscript{2}\). Aliquots of the lysates (50 \(\mu\)g of protein) of native DT40 cells (PLC\(\gamma\textsubscript{2}^{-/-}\)), PLC\(\gamma\textsubscript{2}-\)deficient DT40 cells (PLC\(\gamma\textsubscript{2}^{-/-}\)), and from three independent clones PLC\(\gamma\textsubscript{2}^{-/-}\) DT40 cells stably expressing similar quantities of either wild-type (A–C) or F897Q mutant PLC\(\gamma\textsubscript{2}\) (a–c) were subjected to SDS-PAGE and immuno blotting (upper panel). The same membrane was subsequently probed with an anti-\(\beta\)-actin antibody to control for equal loading of samples (lower panels). All six clones were used for experimentation in this study, with no differences detected between clones A–C and a–c, respectively. B, cells from the clones A and c were analyzed by indirect fluorescence staining (left panels). Right panels, corresponding phase contrast images. C, mean fluorescence intensities of the three images each, as shown in B, were corrected for background staining. Similar results were obtained for other pairs of clones.

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**FIGURE 4.** Stable reconstitution of PLC\(\gamma\textsubscript{2}^{-/-}\) DT40 B cells with wild-type versus F897Q mutant PLC\(\gamma\textsubscript{2}\). PLC\(\gamma\textsubscript{2}-\)deficient DT40 cells (89) were stably reconstituted with either isogenic wild-type or F897Q mutant PLC\(\gamma\textsubscript{2}\). Aliquots of the lysates (50 \(\mu\)g of protein) of native DT40 cells (PLC\(\gamma\textsubscript{2}^{-/-}\)), PLC\(\gamma\textsubscript{2}-\)deficient DT40 cells (PLC\(\gamma\textsubscript{2}^{-/-}\)), and from three independent clones PLC\(\gamma\textsubscript{2}^{-/-}\) DT40 cells stably expressing similar quantities of either wild-type (A–C) or F897Q mutant PLC\(\gamma\textsubscript{2}\) (a–c) were subjected to SDS-PAGE and immuno blotting (upper panel). The same membrane was subsequently probed with an anti-\(\beta\)-actin antibody to control for equal loading of samples (lower panels). All six clones were used for experimentation in this study, with no differences detected between clones A–C and a–c, respectively. B, cells from the clones A and c were analyzed by indirect fluorescence staining (left panels). Right panels, corresponding phase contrast images. C, mean fluorescence intensities of the three images each, as shown in B, were corrected for background staining. Similar results were obtained for other pairs of clones.

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**TABLE 2.** Characteristics of PLC\(\gamma\textsubscript{2}^{-/-}\) DT40 B cells reconstituted with wild-type or F897Q mutant PLC\(\gamma\textsubscript{2}\). Wild-type PLC\(\gamma\textsubscript{2}-\)deficient DT40 cells (89) were stably reconstituted with either isogenic wild-type or F897Q mutant PLC\(\gamma\textsubscript{2}\). Aliquots of the lysates (50 \(\mu\)g of protein) of native DT40 cells (PLC\(\gamma\textsubscript{2}^{-/-}\)), PLC\(\gamma\textsubscript{2}-\)deficient DT40 cells (PLC\(\gamma\textsubscript{2}^{-/-}\)), and from three independent clones PLC\(\gamma\textsubscript{2}^{-/-}\) DT40 cells stably expressing similar quantities of either wild-type (A–C) or F897Q mutant PLC\(\gamma\textsubscript{2}\) (a–c) were subjected to SDS-PAGE and immuno blotting (upper panel). The same membrane was subsequently probed with an anti-\(\beta\)-actin antibody to control for equal loading of samples (lower panels). All six clones were used for experimentation in this study, with no differences detected between clones A–C and a–c, respectively. B, cells from the clones A and c were analyzed by indirect fluorescence staining (left panels). Right panels, corresponding phase contrast images. C, mean fluorescence intensities of the three images each, as shown in B, were corrected for background staining. Similar results were obtained for other pairs of clones.
extracellular Ca$^{2+}$). Cells were treated with 4 μM ionomycin at the end of each experiment for baseline correction and normalization of the [Ca$^{2+}$], responses in single cells. Fig. 5, left panels, shows that oscillatory [Ca$^{2+}$], responses developed in cells reconstituted with WT PLC$\gamma_2$, at anti-IgM concentrations between 4 and 400 ng/ml. At 40–400 ng/ml anti-IgM, the responses were similar in both their frequencies and intensities, in the absence or presence of extracellular Ca$^{2+}$. At 4 μg/ml anti-IgM (Fig. 5, next-to-lowest left panel), individual Ca$^{2+}$ oscillations appeared to coalesce into single major asymmetric peaks in many traces. These peaks reached their maximum intensities within seconds after anti-IgM addition in the absence of extracellular Ca$^{2+}$ to gradually decline to base levels over the next 6 min. Addition of extracellular Ca$^{2+}$ to anti-IgM at this time point led to a second, more symmetric [Ca$^{2+}$], wave, reaching a maximum after about 1.5 min and declining with similar kinetics thereafter. At still higher concentrations of anti-IgM, 40 μg/ml, the pattern was qualitatively similar but somewhat reduced in quantitative terms, both in the absence and presence of extracellular Ca$^{2+}$. In cells expressing the Rac-insensitive mutant F897Q of PLC$\gamma_2$, (Fig. 5, right panels), there was a striking loss of the [Ca$^{2+}$], responses, in particular at low concentrations of anti-IgM, e.g. 40 ng/ml. In each case, this effect was clearly evident both with and without extracellular Ca$^{2+}$. At high (4 and 40 μg/ml) concentrations of anti-IgM, there also was a loss of coalescence of individual oscillations in responding cells and a decrease in the overall duration of the [Ca$^{2+}$], response to BCR ligation, in particular in the absence of extracellular Ca$^{2+}$.

The results of further quantitative analyses of the differences between the [Ca$^{2+}$], responses of cells expressing WT versus F897Q PLC$\gamma_2$ are shown in Fig. 6. Thus, in the absence of extra-
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FIGURE 5. Rac-insensitive mutant PLCγ2 F897Q is quantitatively impaired in its ability to restore B cell receptor-mediated Ca²⁺ flux in PLCγ2−/− DT40 cells. PLCγ2−/− cells stably expressing wild-type (left column) or F897Q mutant PLCγ2 protein (right column) were treated with increasing concentrations of anti-IgM. The treatment was performed as indicated by the arrowheads in the following sequence: anti-IgM; no Ca²⁺ → anti-IgM; 1 mM Ca²⁺ → 4 μM ionomycin; 1 mM Ca²⁺. Forty-nine to 60 cells were analyzed in each single experiment.

In the presence of extracellular Ca²⁺, there was a marked reduction in the anti-IgM sensitivity of the latter cells by an order of magnitude, but no change in the maximal extent, when the proportion of responding cells was used as a response parameter (Fig. 6A, left panel). In the presence of extracellular Ca²⁺, the major difference was a distinct loss of the proportion of responding cells, which was similar (~55%) at low and intermediate anti-IgM concentrations, 4–400 ng/ml, and even more striking (~80%) at high anti-IgM concentrations (Fig. 6A, right panel). There was no apparent change in the sensitivity of the cells to anti-IgM. Integration of the fluorescence intensities in single cells over time and evaluation of their concentration dependence on anti-IgM at the level of the means showed a reduction of the maximal mean intensity in cells expressing the F897Q mutant by 64% in the absence of extracellular Ca²⁺ (Fig. 6B). In its presence, this loss amounted to 82% (Fig. 6C).

Fig. 6D shows that resistance of PLCγ2 to regulation by Rac also resulted in a longer latency of the [Ca²⁺]i response to addition of anti-IgM. This became evident at low anti-IgM concentrations (40 ng/ml) and more prominent at all higher concentrations. [Ca²⁺]i peak frequencies and amplitudes were only analyzed for the three lowest ligand concentrations, 4–400 ng/ml, and in the absence of extracellular Ca²⁺, mostly because of peak coalescence at higher anti-IgM concentrations. By assigning a peak frequency of zero to nonresponding cells, we determined a reduction of this parameter by ~86 and ~55% in cells expressing the F897Q mutant rather than WT PLCγ2 (Fig. 6E, left panel). Likewise, decreases of peak amplitudes amounting to ~84 and ~58% were observed for the cells harboring the mutant at 40 and 400 ng/ml anti-IgM, respectively (Fig. 6E, right panel). Collectively, these results indicate that the regulation of PLCγ2 by Rac is not an absolute requirement for BCR-mediated Ca²⁺ release at most BCR ligand concentrations tested herein, both in the absence and presence of extracellular Ca²⁺. However, although the qualitative patterns of [Ca²⁺]i responses to BCR ligation are similar in the absence and presence of PLCγ2 regulation by Rac, there are striking quantitative differences. These are readily evident at the level of the proportion of responding cells and their sensitivity to anti-IgM, the latency of the response after addition of anti-IgM, as well as both the peak frequency and amplitude of the oscillatory [Ca²⁺]i responses.

Dependence of BCR-mediated Nuclear Translocation of the Transcription Factor NFAT1c on Functional Rac-PLCγ2 Interaction—Within minutes after BCR-mediated B cell activation, several transcription factors, including the Ca²⁺-dependent transcription factor NFAT1c, are translocated into the nucleus to induce transcription of regulatory genes involved in B cell-fate decisions (6). To determine the role of a functional Rac-PLCγ2 interaction in this response, a C-terminally truncated NFAT1c construct consisting of the N-terminal transactivation and the calcineurin-binding domain (amino acids 1–400) fused to the red fluorescent protein td-RFP611 was introduced into PLCγ2−/− DT40 B cells and PLCγ2−/− DT40 B cells stably expressing either WT or F897Q mutant PLCγ2, and its nuclear translocation following BCR ligation was analyzed by spinning disk fluorescence microscopy (Fig. 7). Treatment of cells with ionomycin, resulting in PLCγ2-independent nuclear translocation of NFAT1c-td-RFP611 in almost all transfected cells, was used as a positive control (Fig. 7A, lower panels, and B, right panel). In PLCγ2-deficient cells, there was little, if any, nuclear translocation of the fluorescent reporter protein in response to anti-IgM (Fig. 7A, upper panels), consistent with the concept that this event is absolutely dependent on PLCγ2-induced Ca²⁺ mobilization. In cells expressing WT PLCγ2, NFAT1c-td-RFP611 translocated into the nuclei in about 80% of the cells (Fig. 7B, left panel). Importantly, only 16% of the cells expressing the Rac-insensitive PLCγ2 mutant F897Q displayed nuclear NFAT1c-td-RFP611, such that uncoupling of PLCγ2 from Rac caused an 80% decrease of a cellular response directly related to altered gene transcription and, by extension, cell fate decisions in B cells.

Discussion

This study shows that specific interference with a functional Rac-PLCγ2 interaction in intact DT40 B cells precipitates several clear alterations in BCR-mediated Ca²⁺ signaling. First, the
sensitivity of Ca²⁺ release from internal stores to the BCR ligand anti-IgM is reduced by an order of magnitude. Second, the extent of the Ca²⁺ signal in the presence of extracellular Ca²⁺, also involving entry of extracellular Ca²⁺ into the cells, is greatly diminished. Third, Ca²⁺-regulated, NFAT-mediated transcriptional regulation is largely decreased.

Several lines of evidence have already suggested that PLCγ₂ and Rac are both activated by BCR ligation, although a direct interaction of the two signaling molecules has received relatively little attention (6, 26, 42, 43). Thus, several elements of the canonical B cell receptor signaling cascade, e.g., Syk (44), Btk (45), and BLNK (46, 47), are known to physically interact with and activate the Rac activator Vav. BCR cross-linking caused activation of both Rac1 and Rac2 within minutes (48). Rac cooperated with PLCγ₂ in BCR-mediated activation of c-Jun N-terminal kinase, p38 mitogen-activated protein kinase, SRF.
and NFAT (49, 50). Dominant negative Rac1 suppressed these cooperative relationships. These results were interpreted to suggest that the signaling pathways controlling the activities of Rac and PLC\(_{\gamma2}\) are activated by BCR ligation in parallel to converge at points distal to enhanced formation of InsP\(_3\) and DAG. Btk, BLNK, Vav, and PLC\(_{\gamma2}\) form highly coordinated microsignalosomes in a process dependent on Syk and Lyn (51). Formation of these complexes is important for amplification of signaling and, hence, for appropriate B cell activation, in particular at low antigen concentrations. The results presented here strongly suggest that signal amplification is caused within or in the vicinity of these sites by convergence of signals emanating from activated BCR through a direct interaction of activated PLC\(_{\gamma2}\) with Rac (40, 56). Selective and direct inhibition of TRPC3 channels by pyrazole-3 eliminated the Ca\(^{2+}\) influx-dependent PLC\(_{\gamma2}\) plasma membrane translocation and the late oscillatory phase of the BCR-induced Ca\(^{2+}\) response (57). The same TRPC3 inhibitor also suppressed Rac1 activation without affecting total Rac1 protein abundance in cardiomyocytes (58), whereas activated Rac1 enhanced the rapid vesicular translocation and membrane insertion of another transient receptor potential cation channel, TRPC5 (59). Thus, the interaction of PLC\(_{\gamma2}\) with Rac may influence BCR-mediated Ca\(^{2+}\) oscillations in several ways.

Previous studies have shown that the interaction of PLC\(_{\gamma2}\) with Rac\(^{G12V}\) markedly enhances its enzymatic activity in vitro and in intact cells and coincides with a translocation of PLC\(_{\gamma2}\) from the soluble to the particulate fraction of intact cells (23). Here, we demonstrate that binding of activated Rac to WT PLC\(_{\gamma2}\) also changes the mode of plasma membrane association of the latter from a mixture of exchange and lateral diffusion to almost pure lateral diffusion (Fig. 2). This suggests that Rac-PLC\(_{\gamma2}\) interaction reduces the exchange rate between membrane-associated and cytoplasmic WT PLC\(_{\gamma2}\) such that it becomes negligible relative to its lateral diffusion rate, demonstrating a stronger membrane association.

In intact cells, Rac-activated WT PLC\(_{\gamma2}\) mainly travels along the plasma membrane, allowing for a spatio-temporal pattern of encounter with both its lipid substrate and its protein interaction partners, e.g. TRPC3, that is different from that of its unliganded or Rac-resistant counterparts.

The dependence of the Ca\(^{2+}\) oscillations in cells expressing WT PLC\(_{\gamma2}\) on low to intermediate concentrations of anti-IgM (<4 \(\mu\)g/ml) suggests that signal transduction occurs mostly through frequency modulation, although there is also a clear increase in the amplitude with increasing BCR ligation. It is also evident that, in this concentration range of anti-IgM, the spiking behavior of the cells is largely independent of the presence of extracellular Ca\(^{2+}\). The relative independence of oscillation frequency on external Ca\(^{2+}\) suggests that DT40 B cells are highly efficient at recycling their internal Ca\(^{2+}\) (53). In aggregate, these findings indicate that a minimal model based on reversible desensitization of InsP\(_3\) receptors may suffice to explain the oscillatory pattern observed at low to intermediate anti-IgM concentrations (53, 60). According to the minimal model, [Ca\(^{2+}\)]\(_i\) oscillations are driven by a coupled process of Ca\(^{2+}\)-induced activation and obligatory intrinsic inactivation
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of the Ca2+-sensitized state of InsP3 receptors. Ca2+ spikes are initiated by the Ca2+-mediated conversion of low affinity InsP3 receptors from their low to the high affinity type (60). In this paradigm, the decrease in latency and the increase in oscillation frequency observed in Fig. 6, D and E, would be due to the decrease in the time required to generate a sufficient Ca2+ trigger signal to initiate the first or next Ca2+ release spike, respectively. Enhanced and accelerated translocation of PLCγ2 to the plasma membrane containing its phospholipid substrate by activated Rac would allow for a decrease in the time necessary for reaching this threshold level, such that considerably lower levels of activated BCR are required to elicit a given [Ca2+]i response. Of note, enhanced association of PLCγ2 with the plasma membrane following interaction with activated Rac is indeed observed in the FRAP beam size analysis experiments (Fig. 2), and the absence of this effect on the PLCγ2 mutant defective in Rac interactions (F897Q) is in accord with the much lower quantitative effect of anti-IgM in cells expressing this mutant on the Ca2+ oscillations (Fig. 6E). In addition to temporal changes of PLCγ2 activation by Rac, spatial changes may come into play. Given that the Ca2+-constancies are similar for the three InsP3-R subtypes (61), the increase in amplitude observed with increasing anti-IgM concentrations may be due to expression of the receptors at different cellular levels in DT40 B cells, with higher abundance of subtypes with lower InsP3 sensitivity (InsP3-R1 (4.7 μM); InsP3-R2 (0.35 μM); InsP3-R3 (18.6 μM)); EC50 values for InsP3-mediated Ca2+ release in permeabilized cells expressing a single InsP3-R subtype in parentheses (54)).

When extracellular Ca2+ is provided to cells incubated without Ca2+ for several minutes at high concentrations of anti-IgM, the main effect of a loss of Rac regulation by PLCγ2 is a marked reduction in the number of responding cells, which cannot be recuperated by increasing the concentration of anti-IgM. Several scenarios may explain this observation: (i) the interaction of PLCγ2 with activated Rac could strengthen the productive interaction of PLCγ2 with plasma membrane channels such as TRPC3 allowing entry of extracellular Ca2+, either by altered temporal or spatial interaction of PLCγ2 with the membrane (cf. Fig. 2). If TRPC3 channels were involved in Rac activation in B cells, as they appear to be in cardiomyocytes (see above), this would further reinforce this process. (ii) Cells could be desensitized to BCR-mediated Ca2+ signaling with continued anti-IgM exposure. They can become less sensitive to this course of action if the Rac-PLCγ2 interaction is intact. Both homologous and heterologous desensitization of B cell membrane-immunoglobulin-mediated Ca2+ mobilization have long been known to occur within minutes of anti-immunoglobulin exposure (62). Interestingly, anti-IgM-treated B cells are hyperresponsive to AIF4 and mastoparan (63). Although the two agents are known as activators of heterotrimeric G proteins, they also appear to activate Rho GTPases, including Rac (64, 65). If this was to occur in B cells, the results presented here and by Cambier et al. (63) may indicate that anti-IgM-mediated B cell activation affects a function(s) of the macromolecular complex involving PLCγ2, without impinging upon the activation of the enzyme by Rac, and that the complex is protected, at least in part, when the Rac-PLCγ2 interaction is intact. The markedly diminished effect of high concentrations of anti-IgM on [Ca2+]i, observed in cells lacking the functional Rac-PLCγ2 interaction does not appear to require exposure of B cells to anti-IgM in the absence of extracellular Ca2+, because the inhibitory effect was also evident at the level of nuclear NFAT1c-ttd-RFP611 translocation, which was assayed in the continued presence of extracellular Ca2+ (Fig. 7).

Under these conditions, the insensitivity of PLCγ2 to activated Rac led to a marked reduction in BCR-mediated nuclear translocation of NFAT1c-ttd-RFP611 (Fig. 7), strongly suggesting that the changes observed at the level of [Ca2+]i, are effectively transduced to the transcriptional level. In B lymphocytes, the activity of the Ca2+-regulated transcriptional regulators c-Jun N-terminal kinase (JNK), NFkB, and NFAT are differentially regulated by the amplitude and duration of oscillatory Ca2+ signals (66). NF-kB and JNK are selectively activated by a large transient [Ca2+]i, rise, whereas NFAT is activated by a low, sustained Ca2+ plateau. These results and findings obtained in T lymphocytes (67) and basophilic leukemia cells (68) are consistent with the notion that nuclear translocation of NFAT functions as a working memory of Ca2+ signals by decoding Ca2+ oscillations (69). In general, this process appears to be more cost-effective than translation of continuous Ca2+ signals. In this study, nuclear translocation and, hence, activation of NFAT1c-ttd-RFP611 was studied at 40 μg/ml anti-IgM, allowing for similar numbers of responders (~92%) when cells expressing either WT or F897Q mutant PLCγ2 were analyzed for BCR-mediated Ca2+ release from internal stores (Fig. 6A), with an overall reduction of the integrated fluorescence intensity in the latter cells by 64% (Fig. 6B). In the presence of extracellular Ca2+, both the proportion of responding cells and the mean integrated single cell fluorescence intensity were reduced by more than 80% (Fig. 6, A and C). Because NFAT activation is highly dependent on both InsP3-induced intracellular Ca2+ release and store-operated Ca2+ entry mediated by CRAC channels (50, 70), it is likely that reductions of both responses participate in reducing nuclear NFAT translocation. Furthermore, analysis of the Ca2+ oscillatory behavior in the absence of extracellular Ca2+ at 40 μg/ml anti-IgM revealed that the majority of responses (>75%) consisted of more than one Ca2+ spike in cells expressing WT PLCγ2, whereas this parameter was reduced to 35% in cells expressing the F897Q mutant, showing only a single Ca2+ spike in most cases (cf. Fig. 5, lower panels). Thus, reduced [Ca2+]i spiking from intracellular sources may also contribute to the substantial decrease in nuclear NFAT translocation caused by resistance of PLCγ2 to activation by Rac.

Because Rac GTPases are activated in B cells by quite a number of cell surface receptors in addition to the BCR, the signaling pathway convergence shown in this work is very well suited to detect coincident extracellular signals and to ensure signaling reliability by correctly interpreting the emergence of signals according to the particular cellular context and the specific (patho)physiological circumstances (71). Thus, agonist activation of receptors for integrins, chemokines, and pathogen-derived ligands has also been shown to activate Rac, which provides the GTPase with the potential to act as a central B cell signaling hub. For integrins, it is interesting to note that coliga-
tion of the $\beta_2\alpha_1$ integrin LFA-1 and BCR has been shown to cause an ~10-fold reduction in the amount of BCR ligand required for B cells to make a tight contact with target membranes containing the receptor ligands (72). Using a similar experimental design, Henderson et al. (73) showed that Rac2 plays an important role in outside-in signaling from LFA-1 that leads to firm adhesion of murine B cells to ICAM-1. Interestingly, the Rac2 deficiency could be bypassed by treatment of B cells with phorbol ester and ionomycin, thus mimicking enhanced activation of PLC$\gamma_2$. Although the molecular mechanisms of Rac activation by LFA-1 in B cells are unknown, they may be similar to those encountered in T cells (74–76), although a non-Vav RacGEF(s) may be involved in this case (73). Hence, simultaneous activation of both BCR and LFA-1, e.g., at the immunological synapse, may give rise to locally enhanced Rac activation, allowing for an increased potency of BCR ligand to mediate integrin activation, at least in part by enhanced activity of PLC$\gamma_2$ (73, 77).

A positive interaction between Rac and PLC$\gamma_2$ may also be (patho)physiologically relevant in platelets, where PLC$\gamma_2$ is activated downstream of other immunoreceptor tyrosine-based activation motif-coupled receptors, such as the major platelet collagen receptor glycoprotein VI or CLEC-2 (78), and by integrin $\alpha_{\text{II}}\beta_3$ (79). Inactivation of the Rac1 gene in the mouse caused defective thrombus formation on collagen under flow conditions (78), and pharmacological inhibition of Rac in human platelets led to reduced PLC$\gamma_2$ activity and impaired $\alpha_{\text{II}}\beta_3$ fibrinogen receptor stimulation (79). Intriguingly, the functional consequences of Rac1 deficiency were particularly striking at low and intermediate concentrations of GPVI or CLEC-2 receptor agonists (78), suggesting that Rac may enhance the sensitivity of PLC$\gamma_2$ stimulation to extracellular ligands in platelets as well. Hence, pharmacological targeting of Rac1 could be an interesting approach in the development of future antiplatelet drugs.

The regulation of B cells by BCR ligation is intertwined with their regulation by certain chemokines, such as the CXCR4 and CXCR5 agonists CXCL12 and CXCL13, respectively (5). Activated CXCR4 and CXCR5 appear to be coupled to both Btk and PLC$\gamma_2$ and, through a yet unidentified RacGEF (possibly DOCK2), to activation of Rac. Recently, Rac2 has been shown to be critical for LFA-1-mediated adhesion of mouse B cells in response to CXCL12 or CXCL13 (73). It remained unclear whether Rac2 causes enhanced adhesion directly by augmenting inside-out activation of integrins or indirectly by fostering receptor-mediated activation of PLC$\gamma_2$. Recently, pathogenic receptor-mediated signals have been suggested to activate Rac in murine B cells via Toll-like receptor TLR4 and MyD88 (80). Activated TLR4 activation also enhanced CXCR5-mediated Rac activation. Of note, TLR4 was found in other leukocytes to be coupled to activation of PLC$\gamma_2$ (81, 82), raising the possibility that Rac is of regulatory importance in that respect as well.

The enhanced BCR ligand sensitivity observed for cells expressing WT PLC$\gamma_2$ versus its F897Q mutant is reminiscent of the reduced threshold for antigen receptor stimulation observed in human peripheral blood B lymphocytes upon coligation of CD19 with BCR (3). In mouse B cells, the costimulatory effect of CD19 on BCR-mediated increases in $[\text{Ca}^{2+}]_i$ was clearly reduced in B cells from rac2$^{-/-}$ mice (19). Previously, these effects were mainly ascribed to the known interactions of CD19 with the RhogEF Vav and to the positive interaction of Vav, presumably via activated Rac, with phosphatidylinositol 4-phosphate 5-kinase and/or PI3K, followed by indirect activation of PLC$\gamma_2$ (6, 42). Our current results strongly suggest that Rac enhances BCR-mediated Ca$^{2+}$ signaling in B cells by direct interaction with and activation of PLC$\gamma_2$.

Several families have been described with members affected by homozygous mutations in the CD19 gene causing undetectable or substantially decreased levels of CD19 in B cells. In these patients, suffering from an antibody-deficiency syndrome, there were marked alterations in Ca$^{2+}$ mobilization in B cells following anti-IgM treatment (83). Similar defects of Ca$^{2+}$ mobilization were observed in patients with defective CD81 and CD21, which function in a complex with CD225 and CD19 and cooperate with BCR to mediate antigen recognition (84, 85). Thus, the stimulatory interaction of PLC$\gamma_2$ with Rac may not only play a pivotal role in determining the sensitivity of the BCR to stimulation by antigen, but may also contribute to BCR coreceptor signaling and to functional alterations of the latter in human disease such as certain forms of monogenic common variable immunodeficiency (86). The very recent observation of a RAC2 loss-of-function mutation in two siblings with characteristics of a common variable immunodeficiency is consistent with this view (87).

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