Treatment of patients with chronic lymphocytic leukemia (CLL) with inhibitors of Bruton’s tyrosine kinase (BTK), such as ibrutinib, is limited by primary or secondary resistance to this drug. Examinations of CLL patients with late relapses while on ibrutinib, which inhibits BTK’s catalytic activity, revealed several mutations in BTK, most frequently resulting in the C481S substitution, and disclosed many mutations in PLCG2, encoding phospholipase C-γ2 (PLCγ2). The PLCγ2 variants typically do not exhibit constitutive activity in cell-free systems, leading to the suggestion that in intact cells they are hypersensitive to Rac family small GTPases or to the upstream kinases spleen-associated tyrosine kinase (SYK) and Lck/Yes-related novel tyrosine kinase (LYN). The sensitivity of the PLCγ2 variants to BTK itself has remained unknown. Here, using genetically-modified DT40 B lymphocytes, along with various biochemical assays, including analysis of PLCγ2-mediated inositol phosphate formation, inositol phospholipid assessments, fluorescence recovery after photobleaching (FRAP) static laser microscopy, and determination of intracellular calcium ([Ca2+]i), we show that various CLL-specific PLCγ2 variants such as PLCγ2S707Y are hypersensitive to activated BTK, even in the absence of BTK’s catalytic activity and independently of enhanced PLCγ2 phospholipid substrate supply. At high levels of B-cell receptor (BCR) activation, which may occur in individual CLL patients, catalytically-inactive BTK restored the ability of the BCR to mediate increases in [Ca2+]i. Because catalytically-inactive BTK is insensitive to active-site BTK inhibitors, the mechanism involving the noncatalytic BTK uncovered here may contribute to preexisting reduced sensitivity or even primary resistance of CLL to these drugs.

The two members of the γ subfamily of inositol phospholipid–specific phospholipase C (PLC), PLCγ1 and PLCγ2, have recently been found to be involved in a number of ostensibly different human disease states (1). These range from rare hereditary diseases caused by, or associated with, germline mutations in the PLCG2 gene, such as complex immunological diseases with an autoinflammatory hallmark (2, 3) and childhood-onset steroid-sensitive nephrotic syndrome (4), to acquired conditions associated with somatic PLCG1 or PLCG2 mutations, such as angiosarcoma and T-cell lymphomas (PLCG1) (5, 6), and endemic Burkitt lymphoma (PLCG2) (7). Point mutations in PLCG2 have also been reported to modify the risk of developing neurodegenerative diseases like Alzheimer’s disease (8, 9) and to cause resistance of chronic lymphocytic leukemia (CLL) cells to catalytic-site inhibitors of Bruton’s tyrosine kinase (BTK) (10).

BTK is one of the five members of the strongly-conserved Tec family of nonreceptor tyrosine kinases, also including the IL-2–inducible T-cell kinase ITK, TEC proper, RLK, and BMX (11). Although all five Tec variants are expressed in cells of the hematopoietic lineage, BTK and ITK are key regulators of B-cell and T-cell transmembrane signaling, respectively (12). Mutations of the human and mouse BTK and Btk genes functionally affecting BTK were found to be associated with human disease.

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This article contains Figs. S1–S6 and Table S1.

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4 The abbreviations used are: PLC, inositol phospholipid-specific phospholipase C; RAC, Ras-related C3 botulinum toxin substrate; BTK, Bruton’s tyrosine kinase; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; BCR, B-cell receptor; [Ca2+]i, intracellular calcium ion concentration; PtdInsPs, phosphatidylinositol 3,4,5-trisphosphate; PtdInsP3, phosphatidylinositol 4,5-bisphosphate; PLCγ2, chronic lymphocytic leukemia; WM, Waldenström’s macroglobulinemia; FRAP, fluorescence recovery after photobleaching; NFAT, Ca2+-regulated nuclear factor of activated T cells; XLA, human X-linked agammaglobulinemia; ANOVA, analysis of variance; CFSE, 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester; SH, Src homology; PH, pleckstrin homology; v-SRC, rous sarcoma virus tyrosine kinase.
X-linked agammaglobulinemia (XLA) and murine X-linked immunodeficiency (11). BTK and the other Tec family members share an overall similar domain organization that closely resembles the organization of the Src family tyrosine kinases, with an SH3–SH2–protein-tyrosine kinase domain tandem at their C termini, but divergent N-terminal regions (11). In B cells, BTK couples antigen-activated B-cell receptors (BCR) to tyrosine phosphorylation of PLCγ2 at the four canonical tyrosine residues, Tyr-733, Tyr-759, Tyr-1197, and Tyr-1217 (13, 14). The latter modifications activate PLCγ2 to hydrolyze its substrate phosphatidylinositol 4,5-bisphosphate (PtdInsP2) to inositol 1,4,5-trisphosphate and diacylglycerol. All three of the latter act as intracellular second messengers (15, 16), which are involved in controlling key functions of both normal and neoplastic B lymphocytes. The activation of PLCγ2 is greatly facilitated by the adaptor protein BLNK/SLP-65 (17, 18) and requires membrane targeting of BTK via its N-terminal pleckstrin homology domain to phosphatidylinositol 3,4,5-trisphosphate generated from PtdInsP2 by phosphatidylinositol 3-kinases. For this reason, it can be blocked by the R28C and mimicked by the E41K mutation within its PH domain (19, 20). The K430R mutation of BTK, by replacing a crucial lysine coordinating the α- and β-phosphates of the substrate ATP and being present in this position in all protein kinases (21), renders the BTK protein catalytically inert (22).

Previous experimentation has shown that, during B-cell development, BTK partially acts as an adaptor molecule, independent of its catalytic activity (23), and that the tumor suppressor function of BTK identified in pre-B cells is independent of its catalytic activity (24). Specifically, kinase-inactive BTKK430R completely reconstituted the catalytic activity (24). These effects have thus far been attributed to the observation that BTK associates with phosphatidylinositol-4-phosphate 5-kinases (PIPS5ks), the enzymes that synthesize the generic substrate of inositol phospholipid–specific phospholipase C, PtdInsP2 (25).

Irreversible BTK inhibitors such as ibrutinib have demonstrated remarkable activity in a number of B-cell malignancies with approval in CLL, mantle cell lymphoma, marginal zone lymphoma, and Waldenström’s macroglobulinemia (26, 27). As a result, ibrutinib is being used with increasing frequency as frontline therapy, beginning to displace chemoimmunotherapy in this setting (28, 29). Currently, the drugs are also being evaluated for the treatment of other diseases, such as other malignancies and various autoimmune and/or inflammatory diseases (30–33). As is the case for other targeted tumor therapies, BTK inhibitor treatment of CLL is limited by either primary or secondary, i.e., acquired drug resistance (10). Thus, whole-exome sequencing of CLL cells causing late relapses revealed (i) C481S and a small number of other mutations in the same and in other positions in BTK and (ii) a higher variety of mutations in PLCγ2. To date, some 25 point mutations of one or two adjacent residues are known in PLCγ2, some of which cluster in specific regions of the encoded PLCγ2 protein, such as one of the two SH2 domains and the C2 domain (34–36). Collectively, the BTK and PLCγ2 mutations are associated with about 85% of CLL cases with acquired resistance to BTK inhibitors (37).

Although the C481S mutation of BTK eliminates covalent binding of some BTK inhibitors, such as ibrutinib, to their target and hence markedly reduces their affinity to their binding site, the mechanism of resistance caused by the other BTK and the PLCγ2 mutations is less clear. PLCγ2 is immediately downstream of BTK (26) leading to the suggestion that the variant proteins may be constitutively active. However, we have previously found that several PLCγ2 variant enzymes associated with ibrutinib resistance carrying mutations in various regions are not constitutively active when assayed, even as purified proteins, in a cell-free system employing artificial lipid vesicles as substrate (38, 39). The observation that PLCγ2 variants are, instead, hypersensitive in intact cells to the Rho GTPase RAC2, as well as the upstream protein-tyrosine kinases SYK and LYN (39, 40), has led us to suggest that the PLCγ2 mutations found in BTK inhibitor–resistant cells may cause a rerouting of the transmembrane signals emanating from BCR to converge on and activate PLCγ2.

This work was undertaken to study the functional interaction of BTK with PLCγ2, WT, and ibrutinib resistance variants in a system reconstituted in intact cells from the two protein constituents. We chose enhanced inositol phosphate formation as a functional readout of a productive interaction between the two, rather than PLCγ2 phosphorylation, as it is the most relevant functional outcome of stimulated PLCγ2 in terms of B-cell signaling. Also PLCγ2 is phosphorylated at many other residues, for instance Tyr-733 or Tyr-1245, in addition to the four canonical sites utilized by activated BTK (41, 42), which may confound results. To our knowledge, this approach has not been reported previously. Here, we report that several PLCγ2 variants mediating BTK-inhibitor resistance in CLL patients are strikingly hypersensitive to activated and/or membrane-bound BTK and to BTK variants lacking protein-tyrosine kinase activity. Importantly, we demonstrate that the activity of the PLCγ2 variants in the context of catalytically-inactive BTK is not contingent on enhanced formation of the PLC substrate PtdInsP2 via stimulation of PtdInsP2 kinases, as postulated earlier. Hence, stimulation of PLCγ2 variants mediating BTK inhibitor resistance can be explained by their hypersensitivity to the noncatalytic actions of BTK. We suggest that this mechanism contributes to the acquired BTK inhibitor resistance observed in CLL patients with PLCγ2 mutations and, potentially, to preexisting reduced sensitivity or even primary resistance of clonal populations of CLL cells with hyperactivated BCR.

**Results**

PLCγ2S707Y can be functionally reconstituted with and is hypersensitive to activation by BTK

To investigate and compare the functional reconstitution of various PLC isozymes with WT BTK and its predominantly membrane-bound and, hence, active variant E41K, WT PLCγ1, PLCγ2S, PLCβ2, WT, and Δ44 variant PLCδ2, and S707Y variant PLCγ2 were co-expressed with the two BTK variants in COS-7
Figure 1. Analysis of the functional reconstitution of PLCγ2 and BTK. A COS-7 cells were transfected as indicated with 250 ng/well of either empty vector (Control) or 150 ng/well of vector encoding either WT PLCγ2 (γ2 WT), WT PLCγ2 (γ2 WT), WT PLCβ1 (β WT), or WT PLCδ1 (δ WT), 50 ng/well of vector encoding PLCγ2,707Y (γ2707Y) or PLCδ1,Δ44 (Δ44), and 100 ng/well of vector encoding either WT BTK (BTKWT) or a constitutively-active version of BTK (BTKE41K). B, cells from one well each functionally analyzed in A were washed with 0.2 ml of Dulbecco’s PBS and then lysed by addition of 100 μl of SDS-PAGE sample preparation buffer. Aliquots of the samples were subjected to SDS-PAGE, and immunoblotting was performed using an antibody reactive against the c-Myc epitope present on WT PLCγ2, WT PLCγ2, PLCγ2,707Y, WT PLCδ1, and PLCδ1,Δ44 (a–d and f–g), antibody reactive against PLCδ1 (e), antibody reactive against BTK (a–g), or antibody reactive against β-actin (a–g). C COS-7 cells were transfected as indicated with 50 ng/well of vector encoding WT PLCγ2 (γ2 WT) or PLCγ2,707Y (γ2707Y) and increasing amounts (0–450 ng/well) of vector encoding BTK (BTKWT) (left panel) or BTKE41K (right panel). A and C 24 h after transfection, the cells were incubated for 18 h with [3H]inositol, and inositol phosphate formation was then determined. D, cells from one well each functionally analyzed in C were washed with 0.2 ml of Dulbecco’s PBS and then lysed by addition of 100 μl of SDS-PAGE sample preparation buffer. Aliquots of the samples were subjected to SDS-PAGE, and immunoblotting was performed using a antibody reactive against the c-Myc epitope present on WT PLCγ2, and PLCγ2,707Y (a–c), antibody reactive against BTK (d–f), or antibody reactive against β-actin (g–i). E COS-7 cells were transfected as indicated with 150 ng/well of vector encoding WT PLCγ2 (γ2 WT) and 100 ng/well of vector encoding BTKE41K or BTKE41K/C481S. Twenty four hours after transfection, the cells were incubated for 18 h with myo-[2-3H]inositol and either solvent (0.1% (v/v) DMSO) or increasing amounts of ibrutinib (0.1 nM to 10 μM), vecabrutinib (0.1 nM to 10 μM), or acalabrutinib (1 nM to 3 μM). Inositol phosphate formation was then determined. The results were normalized to percent of maximal inositol phosphate formation, which was 10,012, 9747, and 15,178 cpm for ibrutinib, vecabrutinib, and acalabrutinib, respectively. The IC50 values of ibrutinib, vecabrutinib, and acalabrutinib were determined by nonlinear curve fitting and are shown above the graph in nM. F COS-7 cells were transfected as indicated with 1.33 μg/well of vector encoding WT PLCγ2 (γ2 WT) and 0.66 μg/well of empty vector (Control) or vector encoding BTKWT or BTKC481S. Twenty four hours after transfection, the cells were incubated for 18 h, as indicated with either solvent (0.1% (v/v) DMSO), acalabrutinib (1 μM), or vecabrutinib (1 μM). Cells were lysed, and immunoblotting was performed as indicated using an antibody reactive against the c-Myc epitope present on WT PLCγ2, antibody reactive against BTK, antibody reactive against β-actin, antibody reactive against phosphorylated tyrosine on PLCγ2 at position 759 (pY-PLCγ2), and antibody reactive against phosphorylated tyrosine on BTK at position 223 (pY-BTK). A, C, E, and F show representative results from three independent experiments each as mean values of three technical replicates.

cells radiolabeled with [3H]inositol for measurement of [3H]inositol phosphate formation (Fig. 1A). WT and constitutively-active Δ44 variants of the evolutionarily divergent PLC isoform PLCδ1, carrying a deletion of the autoinhibitory X-Y linker (43), were analyzed for comparison. PLCδ1 is not known to be regulated by heterotrimeric GTP-binding proteins or members of the Tec kinase family and is thus considered to be distinct from a mechanistic point of view. There was little effect
of WT BTK on inositol phosphate formation in the absence of exogenous PLC enzymes and in the presence of any of the WT PLCs. In contrast, in the presence of PLCγ2/S707Y, WT BTK caused a 4.6-fold increase in inositol phosphate formation. BTK/E41K caused a minor increase in inositol phosphate formation in the absence of exogenous PLCs but triggered much more marked increases in the presence of WT PLCγ1, PLCγ2, PLCβ2, and, most strikingly, of PLCγ1/S707Y. Neither WT nor Δ44 variant PLCδ1 activity was affected by WT or E41K variant BTK, suggesting that the stimulatory effects observed for the other PLC isozymes were not due to enhanced supply of their common substrate PtdInsP2. Fig. 1B shows that the differences in inositol phosphate formation observed in Fig. 1A were not explained by differences in protein expression. Note that there was no difference in the expression of WT PLCγ2 and PLCγ1/S707Y when directly compared on the same immunoblots (cf. Fig. 3D and Fig. S3).

Fig. 1C shows the dependence of WT versus S707Y variant PLCγ2 activity on increasing expression levels of WT versus E41K variant BTK after correction of inositol phosphate formation for background activity observed in the absence of exogenous PLCγ enzyme. In contrast to WT PLCγ2 activity, which was unaffected by WT BTK even at the highest expression level, PLCγ1/S707Y variant activity was markedly stimulated by WT BTK in a concentration-dependent fashion. Furthermore, increased expression of BTK/E41K caused striking increases in the activities of both WT and S707Y variant PLCγ2, although the variant was several-fold more sensitive to BTK/E41K. The E41K mutation also rendered BTK more potent in activating PLCγ2/S707Y (cf. Fig. 1C, left versus right panel). There were no differences in protein expression that would explain the functional differences shown in Fig. 1C (cf. Fig. 1D).

To determine whether the productive interaction between WT PLCγ2 and BTK/E41K (Fig. 1C, right panel) was contingent on the protein-tyrosine kinase activity of BTK/E41K, the effects of BTK protein-tyrosine kinase inhibitors, ibrutinib, acalabrutinib, and vecabrutinib, were investigated (Fig. 1E). In addition, the effect of the BTK C481S mutation was tested by incorporating this mutation into a BTK/E41K/C481S compound variant. The C481S mutation is known to cause resistance to the two irreversible covalent BTK inhibitors, ibrutinib and acalabrutinib, with limited, if any, impact on vecabrutinib activity, a novel noncovalent and reversible BTK inhibitor (44). Fig. 1E shows that both the E41K single and the E41K/C481S double variants caused a similar ∼38-fold increase in PLCγ2 activity. All three drugs showed potent inhibition of PLCγ2 activity in the E41K single variant cells. However, ibrutinib and acalabrutinib reduced inositol phosphate formation by only 50% in PLCγ2 and BTK/E41K/C481S cells. Estimated IC50 values of these covalent inhibitors were ∼3530-fold (ibrutinib) and 410-fold (acalabrutinib) higher in cells expressing the BTK/C481S acquired resistance mutation than in cells without this mutation. In contrast, this difference was only about 4.8-fold for vecabrutinib, which does not require BTK Cys-481 interaction for activity. We also tested the effects of acalabrutinib and vecabrutinib at a single concentration, 1 μM, on protein tyrosine phosphorylation of WT PLCγ2 and either WT BTK or its C481S variant. Fig. 1F shows that acalabrutinib was capable of substantially reducing both the autophosphorylation of WT BTK and the BTK-mediated phosphorylation of PLCγ2, but not of BTK/C481S and PLCγ2, respectively, whereas vecabrutinib was strongly inhibitory in the presence of either WT or C481S variant BTK together with PLCγ2. Collectively, the results shown in Fig. 1, E and F, clearly support the view that activation of WT PLCγ2 by BTK/E41K in the reconstituted systems requires the tyrosine kinase activity of BTK.

PLCγ2/S707Y is stimulated by catalytically-inactive BTK independently of tyrosine phosphorylation and increased PtdInsP2 substrate supply

To interrogate the pharmacology of the BTK-mediated activation of WT and S707Y variant PLCγ2, both PLCγ2 variants were coexpressed with WT or variant BTK to examine the effects of 1 μM acalabrutinib on inositol phosphate formation (Fig. 2A). Acalabrutinib caused an almost complete loss of the PLCγ2 activity in BTK/E41K cells but, as expected, had a much lower impact in BTK/E41K/C481S cells. Surprisingly, acalabrutinib was much less effective in the context of PLCγ2/S707Y-expressing BTK/E41K cells, showing only ∼25% inhibition. Acalabrutinib had no activity at all in the PLCγ2/S707Y-BTK/E41K/C481S cells. Several point variants of BTK were coexpressed with PLCγ2/S707Y to further investigate the functional differences between WT and S707Y variant PLCγ2 shown in Fig. 2A and to also extend this analysis to WT PLCγ1 (Fig. 2B). Point mutations of BTK, Y223F, or Y551F, which prevent tyrosine phosphorylation of BTK in cis or in trans by upstream protein kinases, and the covalent inhibitor binding site mutation C481S had only small, if any, effects on the ability of WT BTK to mediate PLCγ2/S707Y activation. As expected, modulating the plasma membrane interaction through either inhibitory (R28C) or stimulatory (E41K) mutations caused considerable decreases and increases, respectively, in the reconstitutive activity of BTK. For both WT PLCγ1 and PLCγ2 enzyme activation was only observed for the E41K variant of BTK. Most interestingly, however, when BTK protein-tyrosine kinase activity was blocked by the K430R mutation, the BTK-mediated stimulation of PLCγ2/S707Y was only reduced by ∼50%. This result, together with those shown in Fig. 2A, right panel, raised the distinct possibility that PLCγ2/S707Y is sensitive to activation by BTK even in the absence of its tyrosine kinase activity.

To further challenge this hypothesis, WT and S707Y variant PLCγ2 were coexpressed with increasing concentrations of BTK/K430R and its membrane-targeted variant BTK/E41K/K430R (Fig. 2C, left panel). Although the activity of WT PLCγ2 remained unaffected by the two BTK variants, inositol phosphate formation of the S707Y variant was markedly and concentration-dependently increased. Membrane targeting of even the catalytically incompetent BTK caused an almost 10-fold shift to the left of the concentration–response relationship. The functional effects observed in Fig. 2C were unrelated to changes in PLCγ2 expression (Fig. 2D). Analysis of protein tyrosine phosphorylation showed that the two BTK variants, K430R and E41K/K430R, similar to their catalytically-competent counterparts, WT and E41K, in the presence of ibrutinib or acalabrutinib were unable to mediate phosphorylation of the PLCγ2 residues Tyr-759 or Tyr-1217, i.e. known BTK phosphor-
Importantly, stimulation of PLCγ2, but not WT PLCγ2, was confined to the catalytically-inactive variant K430R and was also observed for another variant devoid of catalytic activity, R525Q (13). This stimulation was even seen for a BTK deletion variant (Δ amino acids 402–659) lacking the protein-tyrosine kinase domain (Fig. 2E). Fig. 2F shows that the effects of BTK variants on WT and variant PLCγ2 activity observed in Fig. 2E were not explained by differences in protein expression. We then determined how the differential sensitivity of the variant PLCγ2/S707Y to the actions of both the catalytic and the noncatalytic functions of BTK translates into the concentration dependence of acalabrutinib-mediated BTK inhibition. It is clear from these results that the inhibitor did not affect the strikingly enhanced inositol phosphate formation in the presence of BTKE41K/K430R and PLCγ2/S707Y (Fig. 2G). Furthermore, acalabrutinib reduced the activities of the BTKE41K/WT PLCγ2 and BTKE41K/PLCγ2/S707Y pairs with very similar, if not identical, Ig0 values (~20 nm), but did so in a markedly different extent. Although enhanced activity was almost fully abrogated in the presence of WT PLCγ2, it was only partially reduced (~23%) in the presence of PLCγ2/S707Y, most interestingly to a level very similar to the one observed for catalytically-incompetent BTKE41K/K430R and PLCγ2/S707Y. Notably, this level of activity, which was unaffected by acalabrutinib over the whole range of concentrations, was still ~14-fold higher than that observed in the presence of PLCγ2/S707Y alone. Therefore, the acalabrutinib resistance of the BTKE41K/PLCγ2/S707Y pair is very likely based on the BTK variant exerting tyrosine phosphorylation-independent, stimulatory effects on PLCγ2/S707Y.

To address the possibility that the stimulatory effect of membrane-targeted, catalytically-inactive BTKE41K/K430R on inositol phosphate formation by PLCγ2/S707Y is due to enhanced supply of the enzyme phospholipid substrate directly, the effects of WT as well as E41K, K430R, and E41K/K430R variant BTK on the abundance of the inositol phospholipids, PtdIns, PtdIns(4,5)P2, and PtdInsP2, in COS-7 cells was examined (Fig. 3A). No effect of any of the BTK variants on the abundance of these phospholipids was observed. To further examine potential effects of BTK on substrate availability rather than enzyme activity of PLCγ2, a close congener of PLCγ2/S707Y, PLCγ1/S729Y, which shares enhanced activity in intact cells with its PLCγ2 counterpart (Fig. 3B), was coexpressed with WT as well as variant BTK and functionally compared with PLCγ2/S707Y. As shown in Fig. 3C, there was a marked difference between the two PLCγ2 variants vis à vis BTK, in that PLCγ2/S729Y largely behaved like WT PLCγ1 and PLCγ2 and displayed a functional phenotype very different from that of PLCγ2/S707Y, which was not due to differences in protein expression (Fig. 3D). These results are difficult, if not impossible, to explain by an effect of BTK on substrate availability to the two PLCγ2 variants in the reconstituted system. Interestingly, the PLCγ2-specific functional phenotype is not restricted to the S707Y variant. Instead, it is also observed for many of the PLCγ2 variants that have previously been associated with ibrutinib resistance of CLL cells: D334H (catalytic subdomain X), R665W, S707F, and S707P (all C-terminal SH2 domain), L845F (C-terminal half of split PH domain), D993H (catalytic subdomain Y), and M1141R (C2 domain) (34, 45, 46). All listed variants were found to be sensitive to stimulation by catalytically-inert BTK (activity ratios BTKE41K/K430R: BTKE41K = 43.5%: Table 1). Notably, the highest activity ratios, maximal sensitivities for catalytically-inactive BTK, with no difference between BTKE41K and BTKE41K/K430R were observed for the PLCγ2 variants L845F and M1141R. Note that there are no differences in variant PLCγ2 protein expression in the presence of BTKE41K or BTKE41K/K430R. Therefore, the differences in activity ratios BTKE41K/K430R:BTKE41K analyzed for each row of data sets cannot be explained by differences in PLCγ2 and/or BTK protein expression (Fig. S4).

Functional phenotype of PLCγ2/S707Y vis à vis BTK is instigated by mutational reactivation of PLCγ2

To determine whether the enhanced sensitivity of PLCγ2/S707Y to catalytically-inert BTK is related to its augmented basal activity in intact cells, PLCγ2/S707Y was compared with two other variants of PLCγ2 also showing this property, the phosphomimetic variant PLCγ2E4, carrying glutamic acid residues in all four canonical positions of BTK-mediated tyrosine phos-
phorylation, Tyr-753, Tyr-759, Tyr-1197, and Tyr-1217, and the N-myr–PLCɛ/H92532 carrying an artificial N-terminal signal for myristoylation. The inset of Fig. 4 shows that the two variants caused specific enhancements of inositol phosphate formation in intact cells (PLCγ2,4F < PLCγ2,4E < PLCγ2,S707Y). Importantly, a very similar, if not identical, order was observed for the sensitivity of the five PLCγ2 variants to membrane-targeted BTKE41K, both catalytically-competent and -incompetent. Hence, preactivation of PLCγ2 appears to predispose the enzyme to further activation by catalytically-inert BTK.

Next, fluorescence recovery after photobleaching (FRAP) static laser microscopy, employing a fixed-localization Gaussian beam and photon counting to follow fast recoveries (see under “Experimental procedures”) (47), was used to investigate the effects of BTKE41K and BTKE41K/K430R on the membrane interaction and diffusion of GFP-tagged WT and S707Y variant PLCγ2 in intact cells. A significant fraction of PLCγ2 is cytoplasmic and can therefore recover in FRAP by either lateral

Figure 3. Activation of PLCγ2,S707Y by catalytically-inert BTK is not mediated by augmented PLC substrate availability. A, COS-7 cells were transfected as indicated with 2 μg/well of empty vector or 0.5 μg/well of vector encoding WT BTK (WT) or BTK variants K430R, E41K, or E41K/K430R. Twenty four hours after transfection, the cells were incubated for 18 h with myo-[2-3H]inositol. Then, the amounts of radioactively labeled PtdIns (PI), PtdInsP (PIP), and PtdInsP2 (PIP2) in the intact COS-7 cells were determined by TLC analysis as described under “Experimental procedures.” Inset, cells from one well each were lysed and subjected to SDS-PAGE. Subsequent immunoblotting was performed using an antibody reactive against BTK or antibody reactive against β-actin. B, COS-7 cells were transfected as indicated with 500 ng of empty vector (Co.) or increasing amounts (15, 50, 150, or 500 ng) of vector encoding WT PLCγ1, PLCγ1,S729Y (γ1,S729Y), WT PLCγ2 (γ2,WT), or PLCγ2,S707Y (γ2,S707Y). C, COS-7 cells were transfected as indicated with 50 ng/well of vectors as described in B, and 100 ng/well of vector encoding WT BTK (WT) or BTK variants K430R, E41K, or E41K/K430R. Analysis of inositol phosphate formation was done as in Fig. 1. D, cells from one well each functionally analyzed in C were washed with 0.2 ml of Dulbecco’s PBS and then lysed by addition of 100 μl of SDS-PAGE sample preparation buffer. Aliquots of the samples were subjected to SDS-PAGE, and immunoblotting was performed using an antibody reactive against the c-Myc epitope present on WT PLCγ1, WT PLCγ2, PLCγ1,S729Y, and PLCγ2,S707Y, antibody reactive against BTK, or antibody reactive against β-actin. A–C show representative results from three independent experiments each as mean values of three technical replicates.
diffusion along the membrane surface or by exchange between membrane-associated and cytoplasmic populations. To evaluate the relative contribution of lateral diffusion and exchange (where the faster process contributes more), we previously developed the FRAP beam-size test (48, 49). This method is based on performing the FRAP experiment using two different laser beam sizes; if the fluorescence recovery is by exchange, the characteristic recovery time ($\tau$) reflects the chemical relaxation time, which is independent of the beam size (48). Under such conditions, the ratio between the $\tau$ values measured with the two beam sizes generated in the current experiments by the two objectives equals $1 (\tau_{40}/\tau_{63} = 1)$. For lateral diffusion, $\tau$ is the characteristic diffusion time $\tau_{D}$, which is proportional to the area bleached by the beam ($\tau_{D} = \omega^2/4D$, where $\omega$ is the Gaussian radius of the beam, and $D$ is the lateral diffusion coefficient). The results obtained with WT PLC-$\gamma_2$ (Fig. 5) are in line with recovery by lateral diffusion, because the $\tau_{40}/\tau_{63}$ ratio (Fig. 5C, leftmost bar) is not significantly different from 2.28, which is the ratio between the beam sizes generated with these two objectives. However, the $D$ value calculated from the FRAP studies, 3.1 $\mu$m$^2$/s, is much faster than the $D$ value of a lipid probe DiIC$_{16}(3)$ (1,1'-dihexadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate) in the plasma membrane of the same cells (1 $\mu$m$^2$/s) (50). This suggests that PLC-$\gamma_2$ membrane interactions are characterized by “gliding” along the inner membrane surface (i.e. it dissociates from the membrane but re-associates before it can diffuse away into the cytoplasm). The current results are similar to our earlier FRAP measurements of WT PLC-$\gamma_2$ with the smaller beam size ($\times63$) (49), but we find a somewhat slower recovery with the beam generated by the $\times40$ objective. Slower recovery suggests stronger interactions with the membrane (because free GFP in the cytosol recovers at a rate faster by at least an order of magnitude). This difference may reflect a higher level of RAC2 activity in the current measurements, because overexpression of constitutively-active RAC2 increased to a similar level the $\tau_{40}$ of WT PLC-$\gamma_2$ (49). The effects of coexpressing BTKE41K or BTKE41K/K430R on the membrane interactions of WT PLC-$\gamma_2$–GFP are depicted in Fig. 5B, left panel. Both BTK variants induced increases in the $\tau_{40}$ values of WT PLC-$\gamma_2$–GFP and in the $\tau_{63}$ values; here, the effect of BTKE41K/K430R was weaker. Most importantly, neither of the BTK variants significantly affected the $\tau_{40}/\tau_{63}$ ratio of WT PLC-$\gamma_2$–GFP (Fig. 5C, left), which remained only insignificantly different from the beam-size ratio.

**Table 1**

Activation of PLC-$\gamma_2$ by catalytically-inert BTK is not limited to PLC-$\gamma_2$S707Y but is also observed for other PLC-$\gamma_2$ variants mediating ibrutinib resistance in CLL cells

COS-7 cells were transfected as indicated with 150 ng/well vector encoding WT PLC-$\gamma_2$ (PLC-$\gamma_2$WT), 50 ng/well vector encoding the PLC-$\gamma_2$ variants D334H, R665W, S707Y, S707F, L845F, D939H, and M1141R; 15 ng of vector encoding PLC-$\gamma_2$S707P; and 100 ng/well of vector encoding BTKWT or BTK variants K430R, E41K, or E41K/K430R. The ratios of the PLC activities determined in the presence of BTKE41K/K430R and BTKE41K are specified in the right column. The data depicted in the table show representative results from three independent experiments each as mean values of three technical replicates.

<table>
<thead>
<tr>
<th>BTK</th>
<th>Inositol phosphate formation</th>
<th>Ratio (E41K/K430R): E41K</th>
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<td>PLC-$\gamma_2$WT</td>
<td>67 86 67</td>
<td>2358 121</td>
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<td>PLC-$\gamma_2$D334H</td>
<td>40 1097 236</td>
<td>10089 4390</td>
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<tr>
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<td>17442 8132</td>
</tr>
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<td>PLC-$\gamma_2$S707F</td>
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<td>20150 11215</td>
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<td>728 2991 2563</td>
<td>17734 11584</td>
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<td>PLC-$\gamma_2$L845F</td>
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<td>10808 7892</td>
</tr>
<tr>
<td>PLC-$\gamma_2$M1141R</td>
<td>1966 5858 5395</td>
<td>14268 14187</td>
</tr>
</tbody>
</table>

Figure 4. Preactivation of PLC-$\gamma_2$ sensitizes the enzyme to further activation by catalytically-inert BTK. COS-7 cells were transfected as indicated with 50 ng/well of vector encoding WT PLC-$\gamma_2$ (γWT), PLC-$\gamma_2$4Y→F (γ4F), PLC-$\gamma_2$4Y→E (γ4E), PLC-$\gamma_2$N-myr (γN-myr) (left panel), or PLC-$\gamma_2$S707Y (γS707Y) (right panel) and 100 ng/well of vector encoding WT BTK (WT) or BTK variants K430R, E41K, or E41K/K430R. Inset, COS-7 cells were transfected as indicated with 500 ng/well of vector to examine the influence of the PLC-$\gamma_2$ mutations on basal inositol phosphate formation in intact transfected cells. Analysis of inositol phosphate formation was done as in Fig. 1. The graph shows representative results from three independent experiments as mean values of three technical replicates.
throughout (2.28). This suggests that the mechanism of the FRAP remains by gliding, albeit with somewhat enhanced interactions with the membrane, as suggested by the larger $\tau$ values. We then compared the membrane interactions of PLC$_{\gamma_2}$S707Y–GFP with those of the WT enzyme (Fig. 5B, right panel). The FRAP results for this variant in the absence of BTK resembled those obtained for WT PLC$_{\gamma_2}$–GFP, suggesting recovery by gliding lateral diffusion. However, expression of BTKE41K or BTKE41K/K430R had a markedly different effect than in the case of WT PLC$_{\gamma_2}$. Here, only the $\tau(\times 40)$ values of PLC$_{\gamma_2}$S707Y–GFP became larger (slower recovery kinetics), resulting in a highly-significant deviation of the $\tau(\times 40)/\tau(\times 63)$ ratio from the 2.28 beam-size ratio expected for recovery by lateral diffusion (Fig. 5C, right panel). The $\tau$ ratio dramatically decreased, to 3.6–3.8, suggesting that the recovery becomes slower than expected for lateral diffusion with increasing distances. Such a phenomenon, termed subdiffusion or hop diffusion, may arise due to sensing of membrane heterogeneity by the diffusing molecule, which may reflect phase separation, potentially assisted by the cytoskeleton (51, 52).

Figure 5. BTK mediates subdiffusion of PLC$_{\gamma_2}$S707Y but not of WT PLC$_{\gamma_2}$. COS-7 cells were transfected with a vector encoding PLC$_{\gamma_2}$–GFP (γ$_2$WT) or PLC$_{\gamma_2}$S707Y–GFP (γ$_2$S707Y) together with either empty vector or a vector encoding BTKE41K or BTKE41K/K430R. FRAP studies were conducted 24–26 h post-transfection at 22 °C. A, typical FRAP curves obtained using ×63 (smaller beam size) or ×40 (larger beam size) objectives. The time segments showing pre-bleach fluorescence intensity, the bleach point, and post-bleach recovery are indicated by arrows. Solid lines show the best fit of a nonlinear regression analysis (102), with the resulting $R_1$ and mobile fraction ($R_f$) values. $R_f$ values were high in all cases (0.98–1.00) and are given in Table S1.

B, γ$_{\text{WT}}$ and γ$_{\text{S707Y}}$. The number of measurements for each condition was as follows: (i) PLC$_{\gamma_2}$ × 63 objective, $n = 28$; (ii) × 40 objective, $n = 62$; (iii) PLC$_{\gamma_2}$ with BTKE41K, × 63, $n = 24$; (iv) × 40, $n = 22$; (v) PLC$_{\gamma_2}$ with BTKE41K/K430R, × 63, $n = 27$; (vi) × 40, $n = 29$; (vii) PLC$_{\gamma_2}$S707Y–GFP, × 63, $n = 30$; (viii) × 40, $n = 80$; (ix) PLC$_{\gamma_2}$S707Y–GFP with BTKE41K, × 63, $n = 63$; (x) × 40, $n = 58$; (xi) PLC$_{\gamma_2}$S707Y–GFP with BTKE41K/K430R, × 63, $n = 58$; and (xii) × 40, $n = 51$. The studies employed × 40 and × 63 objectives, whose beam size measurements ($n = 59$) yielded a $\omega^2(\times 40)/\omega^2(\times 63)$ ratio of 2.28 ± 0.15. Thus, a similar $\tau(\times 40)/\tau(\times 63)$ ratio is expected for FRAP by lateral diffusion. A $\tau$ ratio of 1 is expected for recovery by exchange (48). The $R_1$ values were high in all cases (0.98–1.00) and are given in Table S1. B displays the $\tau$ values, and C displays the $\tau(\times 40)/\tau(\times 63)$ ratios. $B$, asterisks indicate significant differences between the sample without BTK and the samples with the two BTK variants; the comparison was done separately for the $\tau(\times 63)$ values and the $\tau(\times 40)$ values, comparing the same PLC$_{\gamma_2}$–GFP protein without BTK and with one of the BTK variants (n.s., not significant; **, $p < 5 \times 10^{-3}$; ***, $p < 10^{-3}$; ****, $p < 10^{-10}$; one-way ANOVA and Bonferroni post hoc test). C, bootstrap analysis (see under “Experimental procedures”; 1000 bootstrap resampling values for both $\tau$ and beam-size values) shows that the $\tau$ ratio of PLC$_{\gamma_2}$ (WT or the S707Y variant) in the absence of BTK fits FRAP by lateral diffusion. In the presence of BTK (either E41K or E41K/K430R), the $\tau$ ratio of PLC$_{\gamma_2}$S707Y, but not of WT PLC$_{\gamma_2}$, becomes significantly higher than the 2.28 beam size ratio ($\tau(\times 40)/\tau(\times 63)$, bootstrap analysis and Student’s two-tailed test). The source data for the FRAP studies containing statistics (B and C) are provided in Table S1.
**PLCγ2 activation by noncatalytic BTK**

Figure 6. Stimulation via RAC2 augments the responsiveness of the PLCγ2 enzyme to catalytically-inert BTK. **A**, COS-7 cells were transfected as indicated with 50 ng/well of vector encoding WT PLCγ2 (γ2WT), PLCγ2S707Y (γ2S707Y), and PLCγ2S707Y containing a Phe → Gln mutation at position 897, providing resistance toward RAC2 stimulation (F897Q) and 100 ng/well of vector encoding WT BTK (WTB) or BTK variants K430R, E41K, or E41K/K430R. **B**, lower panel, COS-7 cells were transfected as indicated with 50 ng/well of vector encoding WT PLCγ2 (γ2WT) and BTKE41K/K430R or 25 ng/well of vector encoding PLCγ2S707Y (γ2S707Y) or increasing amounts (0–100 ng/well) of vector encoding RAC2G12V. Upper panel, COS-7 cells were transfected as indicated with 250 ng/well of vector encoding PLCδ1Δ44 (δ1Δ44) or PLCγ2ΔSH (γ2ΔSH). The ED50 values of vector encoding RAC2G12V for the stimulation of WT and variant PLCγ2 activity obtained by nonlinear curve fitting are shown *above the graphs* in ng/well. **C**, cells from one well each functionally analyzed in **B** were washed with 0.2 ml of Dulbecco’s PBS and then lysed by addition of 100 μl of SDS-PAGE sample preparation buffer. Aliquots of the samples were subjected to SDS-PAGE, and immunoblotting was performed using an antibody reactive against the c-Myc epitope (g–h) present on WT PLCγ2 and PLCγ2S707Y, antibody reactive against BTK (c–d), antibody reactive against RAC2 (e–f), or antibody reactive against β-actin (g–h). **D**, COS-7 cells were transfected as indicated with 50 ng/well of vector encoding WT PLCγ2 (γ2WT) and RAC2G12V or increasing amounts (0–350 ng/well) of vector encoding BTKE41K/K430R. Analysis of inositol phosphate formation was done as in Fig. 1. A, B, and D show representative results from three independent experiments each as mean values of three technical replicates.

**Activated RAC2 allows catalytically-inert BTK to activate WT PLCγ2**

The previous demonstration that PLCγ2 variants mediating ibrutinib resistance in CLL, PLCγ2R665W, and PLCγ2L845F are hypersensitive to activated RAC endogenously present in COS-7 cells (38) prompted us to determine the contribution of RAC to the effects of catalytically-competent and -incompetent BTK on PLCγ2S707Y. Fig. 6A shows that blocking the functional interaction of PLCγ2S707Y with activated RAC in variant PLCγ2F897Q caused conspicuous losses of inositol phosphate formation, which were particularly striking for the catalytically-inert BTK variants K430R (~85%) and E41K/K430R (~58%). Because the F897Q mutation, located in the C-terminal half of the split PH domain of PLCγ2, is known to neither affect the overall three-dimensional structure of the PH domain nor the catalytic activity of PLCγ2 *in vitro* (49, 53, 54), these results indicate that activated RAC may facilitate BTK-mediated PLCγ2 stimulation, in particular through the noncatalytic functions of BTK. Fig. 6B shows that the cellular abundance of exogenous, activated RAC2G12 exerts impressive effects on the activation of both WT and S707Y variant PLCγ2 by catalytically-inert BTKE41K/K430R. Thus, PLCγ2S707Y not only shows higher activity in the presence of RAC2G12V and catalytically-inactive BTKE41K/K430R, but also reveals a higher sensitivity to RAC2G12V in the presence of the latter. The basal activities of the constitutively-active PLC variants PLCγ2ΔSH (lacking the complete autoinhibitory SH2–SH2–SH3 tandem (55)) and PLCδ1Δ44 are shown in Fig. 6B, top panel, to demonstrate that the plateau observed for PLCγ2S707Y in the presence of BTKE41K/K430R at high RAC2G12V levels is not limited due to exhaustion of the enzyme substrate PtdInsP2. Increasing concentrations of RAC2G12V had no effect on the expression of PLCγ2 and BTKE41K/K430R (Fig. 6C).
PLCγ2 activation by noncatalytic BTK

Strikingly, however, RAC2G12V appeared to unmask a conspicuous sensitivity of WT PLCγ2 to stimulation by the tyrosine phosphorylation–independent functions of BTK. This strikingly permissive effect of RAC is substantiated by the results shown in Fig. 6D, demonstrating insensitivity of PLCγ2 to BTKK430R/H11011 in the absence of RAC2G12V, but with an ~25-fold stimulation of inositol phosphate formation in its presence. The latter results indicate that coactivation of BTK and RAC by upstream regulators may render even WT PLCγ2 sensitive to the noncatalytic functions of BTK and, by extension, render WT BTK and WT PLCγ2 at least partially resistant to active-site inhibitors of BTK.

The ability of catalytically-inert BTK to mediate BCR-depen- dent Ca2+ mobilization in intact DT40 B-lymphoma cells is conditional on the degree of BCR stimulation

To investigate the functional significance of the catalytic functions of BTK, the WT enzyme and its catalytically-inactive variant BTKK430R were stably expressed in BTK-deficient DT40 B-lymphoma cells. Fig. 7A shows that BTK−/− cells lack immunoreactive BTK protein and that the two reconstituted cells lines, BTK−/− + BTKWT and BTK−/− + BTKK430R, express BTK at levels very similar, if not identical, to each other and to unmodified DT40 BTK+/+ cells. Fig. 7B and C shows the changes of intracellular free Ca2+ in response to increasing concentrations of anti-IgM, as determined by flow cytometry

Figure 7. Ability of catalytically-inert BTK to mediate intracellular Ca2+ mobilization depends on the level of BCR stimulation. A, DT40 cells (1.5 × 10⁶) were lysed as described under “Experimental procedures,” and aliquots of the samples were subjected to SDS-PAGE, and immunoblotting was performed using an antibody reactive against both human and chicken BTK, antibody reactive against human and chicken PLCγ2, or antibody reactive against β-actin. One empty lane was present between each of the four lanes from the same immunoblot with the same length of exposure time. These lanes were removed by splicing. The positions of the splice sites are indicated by gray dotted lines and arrowheads. B, WT DT40 cells (BTK−/−), DT40 BTK−/− (BTK−/−), and DT40 BTK−/− cells expressing either human WT (BTK−/− + BTKWT) or human K430R BTK (BTK−/− + BTKK430R) were loaded with Indo-1-AM. After pre-warming to 37 °C, the cells were applied to the fluorescence analyzer and treated with anti-IgM followed by ionomycin as described under “Experimental procedures.” C, DT40 BTK−/− cells expressing either human WT (BTK−/− + BTKWT) or human K430R BTK (BTK−/− + BTKK430R) were either left unstained (BTK−/− + BTKWT) or stained with CFSE (BTK−/− + BTKK430R). After mixing the two populations at a 1:1 ratio, the cells were incubated for 1 h at 37 °C in the presence of either solvent (0.1% (v/v) of DMSO, Co.), or 1 μM ibrutinib (Ibru.), or acalabrutinib (Aca.) followed by washout. Afterward, cells were incubated with indo-1-AM, and the changes in Ca2+ were analyzed as described above. The individual points shown in A and C correspond to the times in full seconds versus the medians of all fluorescence ratios representing [Ca2+]i, following their correction for basal fluorescence intensity in the absence of anti-IgM and normalization according to the maximal responses in BTK−/− cells expressing WT BTK when the concentration of anti-IgM was increased to values higher than 30 ng/ml. Specifically, the values were 32.6, 42.9, 35.9, and 28.0% of the maximal Ca2+ responses in BTK−/− cells expressing WT BTK when the concentration of anti-IgM was increased to values higher than 30 ng/ml. Specifically, the values were 32.6, 42.9, 35.9, and 28.0% of the maximal Ca2+ responses in BTK−/− cells expressing WT BTK when the concentration of anti-IgM was increased to values higher than 30 ng/ml. Specifically, the values were 32.6, 42.9, 35.9, and 28.0% of the maximal Ca2+ responses in BTK−/− cells expressing WT BTK when the concentration of anti-IgM was increased to values higher than 30 ng/ml. Specifically, the values were 32.6, 42.9, 35.9, and 28.0% of the maximal Ca2+ responses in BTK−/− cells expressing WT BTK when the concentration of anti-IgM was increased to values higher than 30 ng/ml. Specifically, the values were 32.6, 42.9, 35.9, and 28.0% of the maximal Ca2+ responses in BTK−/− cells expressing WT BTK when the concentration of anti-IgM was increased to values higher than 30 ng/ml. Specifically, the values were 32.6, 42.9, 35.9, and 28.0% of the maximal Ca2+ responses in BTK−/− cells expressing WT BTK when the concentration of anti-IgM was increased to values higher than 30 ng/ml.

The changes of intracellular free Ca2+ in response to increasing concentrations of anti-IgM, as determined by flow cytometry

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followed by statistical analysis of the results to allow for direct, quantitative comparison between the different experimental conditions. The most-right panel of Fig. 7B shows how the maximal Ca$^{2+}$ concentration in response to 2 µm ionomycin was used to correct the traces shown in the other panels for differences in fluorescent dye loading. The corrected traces were then normalized to the maximal Ca$^{2+}$ concentrations observed in BTK$^{-/-}$ + BTKWT cells (100%). Fig. 7B shows that there was little, if any, effect of anti-IgM in BTK$^{-/-}$ + BTKK430R cells compared with BTK$^{-/-}$ cells upon addition of anti-IgM at the lowest concentration, 30 ng/ml. Raising the concentration of anti-IgM over 100 and 300 ng/ml to 1000 ng/ml led to a gradual, distinct increase in the ability of the receptor ligand to increase free Ca$^{2+}$. In addition, there was a progressive loss of the differences in the lag time of the Ca$^{2+}$ responses from about ~35 s at 100 ng/ml anti-IgM and ~20 s at 300 ng/ml, such that there were only relatively minor differences in the kinetics of the Ca$^{2+}$ responses at 1000 ng/ml anti-IgM between the two genetically-modified cell lines and unmodified BTK$^{+/+}$ cells, both in terms of the lag time and the maximal Ca$^{2+}$ concentrations. The results show that the ability of catalytically-inert BTK to mediate BCR-mediated increases in intracellular Ca$^{2+}$ is conditional on the concentration of the BCR ligand, anti-IgM, ranging from no to very sizable responses between 30 and 1000 ng/ml of anti-IgM.

Because the actions of catalytically-inert BTKK430R are insensitive to catalytic site BTK inhibitors like ibrutinib or acalabrutinib (see above), the relative inhibitory effect of these drugs was tested as a function of the degree of BCR activation by anti-IgM. Fig. 7C, most left panel, shows that ibrutinib (1 µM) caused an ~67% decrease in the maximum Ca$^{2+}$ response in cells expressing WT BTK at the lowest anti-IgM concentration, 30 ng/ml, very close to the Ca$^{2+}$ concentrations observed for cells expressing BTKK430R. In addition, there was a slight inhibitory effect of the drug in the latter cells, presumably due to the known off-target effects of ibrutinib (56). Therefore, acalabrutinib, with less off-target effects than ibrutinib (56), was used. It is evident from the comparison shown in Fig. 7C that, at increasing anti-IgM concentrations, not only was there a gradual increase in the ability of catalytically-inert BTK to reconstitute BCR-mediated Ca$^{2+}$ increases, as before, but that, at the same time, there was a quantitative loss to BTK catalytic site inhibition, down to only ~25% in the presence of the maximal anti-IgM concentration tested, 10 µg/ml. In addition, less inhibition at high-ligand concentrations also coincided with a continuous loss of the lag time of the Ca$^{2+}$ response in BTK$^{-/-}$ + BTKWT cells in the absence versus presence of the BTK inhibitor. In other words, both the inhibitory efficacy and the kinetic effects on [Ca$^{2+}$], of BTK catalytic site inhibitors were found to be both conditional on and inversely related to the degree of BCR stimulation.

Discussion

Although the molecular basis of clinical resistance to BTK inhibitors, such as ibrutinib and acalabrutinib, caused by mutations at position Cys-481 of BTK is relatively clear, the mechanism of resistance caused by the other BTK and the PLCG2 mutations is less understood. Although PLCG2 mutations have been suggested to constitute, as a whole, gain-of-function mutations (40, 45, 57), we have previously found that several PLC$_2$ variant enzymes carrying mutations in various regions are not constitutively active when assayed in cell-free systems (38, 39). The observation that these PLC$_2$ variants are, instead, hypersensitive in intact cells to the Rho GTPase RAC2 led us to suggest that the PLCG2 mutations found in BTK inhibitor–resistant CLL cells may cause a rerouting of the transmembrane signals emanating from BCR and converging on as well as activating PLC$_2$ (38, 39). In addition, Liu et al. (40) showed that PLC$_2$R665W is sensitive to activation by the proximal tyrosine kinases SYK and LYN even in BTK-deficient chicken DT40 B cells. Even in the presence of BTK, PLC$_2$R665W appeared to be resistant to ibrutinib in this system.

Here, we have established a reconstituted system allowing us to monitor the functional interaction of WT and variant forms of BTK and PLC$_2$ in intact cells, placing special emphasis on a comparison between WT PLC$_2$ and variants known to mediate clinical BTK inhibitor resistance in CLL patients. Although BTK has long been known to mediate protein tyrosine phosphorylation of PLC$_2$ following coexpression in cells or cell-free preparations devoid of BLNK (58–60), information on the structural and functional requirements of this interaction to occur and result in enhanced PLC$_2$ enzyme activity is limited. This is presumably due to the known requirement of the adapter protein BLNK to observe BCR-mediated, BTK-dependent activation of PLC$_2$ in B cells (61), together with the known difficulties to functionally reconstitute all three components outside the B cells context.

Our findings reveal that functional interaction of BTK and PLC$_2$ in a non-B-cell environment is conditional on the introduction of mutations into the two coding regions and on the level of protein expression. Thus, although there was no productive interaction between the two WT components even at the highest expression level of BTK, mutational alteration of either PLC$_2$(S707Y) or BTK (E41K), or both, caused profound increases in PLC$_2$ activity. The results obtained on WT PLC$_2$ are consistent with the view that one of the roles of BLNK is to provide membrane targeting to PLC$_2$ to make it more susceptible to BTK-mediated protein tyrosine phosphorylation. In fact, a membrane-targeted variant of PLC$_2$ was sensitive to BCR stimulation even in the absence of BLNK (62, 63). According to this view, membrane targeting of BTK, such as introduced by the E41K mutation, would serve the same purpose. Extrapolating this concept to the altered functional properties of PLC$_2$S707Y, which is highly sensitive to stimulation by even WT BTK, could indicate that this PLC$_2$ variant may also be different from the WT enzyme in its membrane interaction.

The marked sensitivity of the functional BTKE41K–PLC$_2$WT interaction to BTK inhibitors of all three genera-
PLCγ2,S707Y, but not of PLCγ2 WT, in the absence of apparent protein tyrosine phosphorylation of the PLCγ2 variant. Importantly, this activity was also observed for another known catalytically-inactive variant of BTK, BTKE41K/R525Q (13), and even by a deletion variant of BTK lacking its catalytic domain.

In the absence of alternative regulatory pathways between BTK and PLCγ2, as is the case in the cell system employed here for transient functional reconstitution, activation of PLCγ2, both WT and the various point variants analyzed herein, are, on first approximation, expected to be similarly sensitive to inhibition by active-site inhibitors of BTK. However, as shown in Fig. 2, A and G, they are not. PLCγ2,S707Y was inhibited by acalabrutinib only to the extent of its activity that depended on the kinase activity of BTK,E41K, whereas the remaining activity, which results from BTK catalysis-independent functions, was completely acalabrutinib-resistant. This drug-resistant portion may even be higher for other PLCγ2 point variants with a higher BTK,E41K/K430R:E41K ratio, such as the variants L845F or M1141R (Table 1). This mechanism provides an explanation, at least in part, for active-site BTK inhibitor resistance in tumor cells expressing PLCG2 point variants, without a requirement for constitutively-enhanced intrinsic activity, which is consistent with previous studies of the R665W and S707Y PLCγ2 variant proteins in vitro (38, 39).

Precactivation of PLCγ2, regardless whether by phosphomimetic mutations or by membrane targeting at its N terminus (64), rendered the variants hypersensitive to activation by both catalytically-active and -inactive BTK (cf. Fig. 4). This effect was particularly striking for the variant carrying the myristoylation tag of Rous sarcoma virus v-SRC at its N terminus, PLCγ2,N-myr, which had a functional phenotype vis à vis BTK WT, and variants, very similar to PLCγ2,S707Y. Importantly, PLCγ1 phosphorylation and Ca2+ release were previously promoted in Xenopus laevis egg extracts by lipid rafts prepared from COS-7 cells expressing the Xenopus SRC protein, suggesting that COS-7 cells contain lipid raft microdomain structures and that SRC kinase is targeted to these structures (65, 66). Hence, the N-terminal SRC-derived myristoylation tag was likely to promote lipid raft targeting of PLCγ2,N-myr to COS-7 cell lipid rafts. Membrane-targeting tags of lipid raft–associated proteins FcγRIII (62, 67, 68) or the Src family member LYN (63) have previously been attached to PLCγ2, and found to render BCR-mediated stimulation of PLCγ2 in DT40 cells independent of the adaptor protein BLNK. The similarity of the functional phenotypes vis â vis BTK WT, as well as variants, between PLCγ2,N-myr and PLCγ2,S707Y (cf. Fig. 4) and the hop diffusion in the membrane detected by FRAP for PLCγ2,S707Y in the presence of BTK,E41K or BTK,E41K/K430R (cf. Fig. 5), suggests that the latter protein functionally resembles a lipid raft–associated protein, possibly with acquired BLNK independence of activation by BTK. With regard to the FRAP results, an “anchored membrane-protein picket model” has been proposed by Fujiwara et al. (51), in which transmembrane proteins referred to as “pickets” are anchored to the membrane skeleton to act as posts along the membrane skeleton fence. The anchored protein pickets, observed in all examined cell types, including COS cells, were suggested to act as effective diffusion barriers due to steric hindrance as well as circumferential slowing (packing and frictional) effects even for phospholipids in the outer leaf of the membrane. According to this model, the long-range lateral diffusion of diffusible molecules in compartmentalized membranes is largely limited by their hop rate across the compartment boundaries. Importantly, compartmentalization of the cell membrane has also been suggested to occur for glycosylphosphatidylinositol-linked proteins and to be involved in modulating the size and lifetime of lipid rafts. Collectively, the results suggest that the point mutation S707Y promotes association with compartmentalized plasma membrane sub-domains, possibly with lipid rafts. The importance of the latter for BCR-mediated signal transduction in B cells has previously been emphasized (69–72).

We have shown earlier that RAC, by physically interacting with the split PH domain of PLCγ2, amplifies BCR-induced Ca2+ signaling and markedly increases the sensitivity of B cells to BCR ligation, BCR-mediated Ca2+ release from intracellular stores, Ca2+ entry from the extracellular compartment, and nuclear translocation of the Ca2+-regulated nuclear factor of activated T cells (49). We also showed that various PLCγ2 variants are strikingly hypersensitive to activation by RAC2, such that even WT RAC2 suffices to activate the variant enzymes upon its introduction into intact cells (38, 39). These findings are highly consistent with the cooperative interaction between RAC2 and BTK as stimulators of PLCγ2 shown here, in particular with the striking permissive effect of activated RAC on the ability of catalytically-inert BTKE41K/K430R to activate even WT PLCγ2 (cf. Fig. 6, A and D). This result may indicate an “unleashing”, via VAV and RAC, of even WT PLCγ2 activity by the catalysis-independent functions of BTK. This effect could act in cis, i.e. within the BCR signal transduction pathway in a positive feed-forward regulatory loop, or in trans, by BCR coreceptors or integrins also capable of activating RAC (38), both enhancing BCR-mediated PLCγ2 activation. In either case, the sensitivity of BCR-mediated inositol phosphate formation to catalytic site–targeted inhibitors of BTK would be dependent on the relative penetration of the pathways mediated by BTK independently of its protein kinase activity in the various physiological and pathophysiological environments of either normal or leukemic B cells. Of note, both VAV and RAC have been shown to localize to lipid rafts in B cells (70, 73).

An important extracellular variable encountered by B cells in different tissue environments is the abundance of the BCR antigen stimulus and, hence, the degree of BCR activation. The result shown herein indicate that this degree takes a profound influence on both the kinetics of the [Ca2+]i response and on the portion that may be contributed by the protein-tyrosine kinase–independent functions of BTK, which are resistant to catalytic site-targeted BTK inhibitors. Remarkably, in DT40 cells, this portion increased considerably when the concentration of anti-IgM was increased over the range of concentrations commonly used in the functional analysis of these cells.

At first glance, these findings may appear to contradict earlier seminal findings, such as the almost complete loss of the reconstitutive activity of catalytically-inert BTK variant R525Q in BTK−/− DT40 cells (K. /btk−), which was tested in response to type M4 anti-IgM at a nominal concentration of 2 µg/ml (13). Although it seems possible, as pointed out earlier (74), that
the R525Q mutation used by Takata and Kurosaki (13) affects the catalytic capacity of the BTK kinase differently, the three kinase-inactive BTK variants analyzed herein, including the variant R525Q and even lacking the kinase domain, behaved very similarly in our experiments (cf. Fig. 2E). Notably, however, unlike Lys-430, Arg-525 is not conserved in the protein kinase superfamily and is already absent in this position in most of the human Src subfamily members, including LYN. Finally, in our hands, the anti-IgM concentration response of DT40 cells is sensitive to freezing and storage of the cells and, possibly, storage of the ligand at 4 °C, as well as somewhat variable with the particular batch of the ligand. For example, freshly-transfected cells that had not been frozen as such were used with freshly-prepared ligand in Fig. 7B. In Fig. 7C, the anti-IgM-mediated [Ca\(^{2+}\)]\(i\) increases were already reduced, e.g. at 1 μg/ml anti-IgM, after one freeze-thaw cycle. Therefore, it seems possible that the observations in Fig. 7C did in fact correspond to less-responsive cells and/or a less potent anti-IgM preparation. In vivo analysis of genetically-modified mice and experiments performed ex vivo on cultured cells from these animals have previously shown that the catalytically-inactive BTK variant K430R could either fully, only partially, only modestly, or not at all fulfill the functions of WT BTK, depending on the particular function tested (23, 24). These results have previously been explained by the finding, in primary and A20 cultured B cells, that BTK, regardless of its catalytic capabilities, associates with PIP5Ks, the enzymes that synthesize PtdInsP\(_2\) (25, 75). The findings were taken by the authors (25) to suggest that both forms of BTK act as shuttles to bring PIP5Ks to the plasma membrane and specifically to lipid rafts to enhance the availability of PtdInsP\(_2\) as a substrate for both PI3 kinases and PLC\(\gamma_2\) with subsequently enhanced membrane targeting of BTK (via its PH domain) and inositol phosphate formation, respectively. Although such a mechanism(s) appears persuasive, it is important to note that increased formation of PtdInsP\(_2\) by protein kinase–deficient BTK has not been shown in Saio et al. (25), neither in anti-IgM–stimulated DT40 cells nor in PIP5K\(\beta\)–overexpressing A20 cells. In addition, the observation herein that a number of PLC\(\gamma_2\) point mutations associated with ibrutinib resistance of CLL cells cause markedly enhanced inositol phosphate formation in transfected cells (cf. Table 1), arguing strongly against a shortage of substrate PtdInsP\(_2\) at the site of catalysis. Furthermore, even activated BTKE41K did not alter the activity of WT or mutually preactivated PLC\(\delta_1\) in the experimental system utilized here (cf. Fig. 1A). There was also no change in the levels of the major inositol phospholipids in response to expression of various variant forms of BTK, including those capable of stimulating PLC\(\gamma_2\)S707Y (cf. Fig. 3A). Finally, and perhaps most convincingly, there was a striking PLC isozyme specificity of the stimulatory effect of catalytically-inactive BTK, even within the PLC\(\gamma\) subfamily, where PLC\(\gamma_7\)S729Y, the direct structural and functional counterpart of PLC\(\gamma_2\)S707Y (39), did not show activation by BTKE41K/K430R (cf. Fig. 3C). Note that both PLC\(\gamma_1\) and PLC\(\delta_1\) associate with lipid raft membrane microdomains (76, 77), and should, as such, be similarly if not equally sensitive, as compared with PLC\(\gamma_2\), to an enhanced availability of the PtdInsP\(_2\) substrate.

Tomlinson et al. (74) showed earlier that an inducible form of kinase-inactive BTK, in contrast to its kinase-active counterpart, was unable to induce an increase in [Ca\(^{2+}\)]\(i\), in BTK-deficient DT40 cells on its own. However, in the context of BCR activation by 2 μg/ml anti-IgM, there was virtually no difference in the kinetics of [Ca\(^{2+}\)]\(i\), between BTK–/– cells expressing kinase-active versus -inactive BTK. Hence, BCR-mediated activation of PLC\(\gamma_2\) via kinase-inactive BTK involves an additional mechanism. In the context of the results shown here (cf. Fig. 6, A–D), it is likely that RAC activated by the BCR, e.g. via VAV, permits the implementation of such a mechanism. We previously noted major losses of the proportion of singly analyzed DT40 cells carrying the RAC-resistant PLC\(\gamma_2\) variant F897Q in response to intermediate and high concentrations of anti-IgM, without an apparent change in the anti-IgM sensitivity of the cells. In addition, the integrated single-cell fluorescence intensities, as well as peak latencies, amplitudes, and frequencies were markedly (mostly up to >80%) reduced in these cells under these conditions (49). We therefore suggest that, following intermediate to high degree BCR activation by antigens, activated RAC physically interacts with WT PLC\(\gamma_2\) to sensitize the enzyme to the noncatalytic actions of BTK.

Considering the BCR-coupled multiprotein transmembrane-signaling chain as one functional unit coupling a ligand-binding receptor to a second messenger–generating enzyme such as PLC\(\gamma_2\), catalytic site–targeted BTK inhibitors can be categorized as variably effective, either irreversible (e.g. ibrutinib and acalabrutinib) or reversible (e.g. vecabrutinib) allosteric inhibitors: allosteric because they act at a site distinct from the (orthosteric) receptor–ligand-binding site. Based on the results presented herein, we suggest that inhibition by ibrutinib and acalabrutinib is only fully expressed at low degrees of BCR activation where only the catalysis-dependent effects of BTK are in effect. At high degrees of BCR activation, more and more of the catalysis-independent BTK functions come into effect, such that the BTK inhibitors acquire the properties of only partially-effective inhibitors. Whether or not the remaining relative inhibition suffices to block specific (patho)biologic functions of BCR depends on the “functional reserve” of the particular B-cell response distal to the increase in [Ca\(^{2+}\)]\(i\). In other transmembrane-signaling systems, such as those relying on enhanced intracellular formation of cAMP, this term refers to the fact that many receptor agonists are more powerful than they need to be, in that maximal responses of cellular functions are elicited at submaximal, in some cases only minimal degrees of receptor activation or second messenger generation (78, 79). For example, it is well-known that in adipocytes stimulated by lipolytic hormones the levels of cAMP are far in excess of those required for maximal lipolytic rates, even in the presence of inhibitors (80–82). This large “overshoot” of second messenger is also observed, e.g. in the heart and in adrenocortical cells, in many other biological systems with no direct correlation of second messenger concentration with the metabolic or physiological process under investigation (83, 84). Under these conditions, both orthosteric and allosteric inhibitors need to be capable of blocking a substantial portion, if not a great majority, or even all agonist effects to be efficacious (85, 86). Thus, as long as the BCR operates under a functional reserve arrangement in a given
B cell, the changes in BTK inhibitor efficacy shown herein (cf. Fig. 7C) may take profound effects on and limit their therapeutic efficiency in cells expressing both WT BTK and WT PLCγ2, in particular at high degrees of BCR activation, as determined by high affinity and/or high abundance of the antigen.

In CLL cells, BCR activation may occur through engagement of the receptor by (auto)antigens (87, 88) or in a cell-autonomous way by BCR recognition of conserved epitopes within specific regions of the Ig heavy and light chains (89, 90). Either way, there are substantial differences in the degrees of this activation among samples of different patients, which in turn translates (at least in part) to the clinical heterogeneity of the disease (90, 91). Importantly, BTK inhibitor treatment of CLL has been reported to be limited by primary drug resistance in 13–30% (10). Partial efficacy of the drugs caused by the activation of PLCγ2 through catalysis-independent mechanisms of BTK provides a potential mechanism of this therapeutic problem. Novel drugs capable of interfering with the protein–protein interaction(s) of BTK with PLCγ2 may provide a way to address this treatment drawback in the future. Finally, clinical heterogeneity, referred to as “leaky” or “mild” phenotype in the less affected patients, is also known in XLA, even in patients from single pedigrees. It has previously been difficult or even impossible to establish a clear genotype–phenotype relationship for this disease caused by a large number of distinct mutations in BTK (RRID:SCR_013101). In some cases, these mutations either abrogate the catalytic functions of BTK (92) or cause a loss of its catalytic domain (93). We suggest that the noncatalytic functions of the variant BTK enzymes, possibly triggered in a sibling-specific fashion by high BCR activation, may contribute to this heterogeneity.

**Experimental procedures**

**Materials**

The rabbit polyclonal antibody 3872 reactive against human and chicken PLCγ2, the rabbit polyclonal antibody 3871 reactive against human PLCγ2 phosphorylated at tyrosine 1217, the rabbit polyclonal antibody 3874 reactive against human PLCγ2 phosphorylated at tyrosine 759, and the rabbit polyclonal antibody 5082 reactive against human BTK phosphorylated at tyrosine 223 were obtained from Cell Signaling Technology. The rabbit polyclonal antiserum reactive against human RAC2 (sc-96), the mouse mAb reactive against human BTK (sc-81735), and the mouse mAb (PY99) reactive against phosphotyrosine-containing proteins (sc-7020) were purchased from Santa Cruz Biotechnology. The rabbit polyclonal antibody reactive against human and chicken BTK (F52632) was obtained from NSI Bioreagents. CFSE (eBioscience™, 65-0850-84) and Indo-1-AM (I0634, Sigma) were added for 10 min at 37 °C, and 10% CO2, and incubated for 45 min at 37 °C and 10% CO2 with 5 μM Indo-1-AM. The calcium measurements were performed on a BD FACS Celesta™. In brief, 10⁶ cells per sample in 0.5 ml of FACS buffer were pre-warmed at 37 °C for 10 min. Next, baseline was measured for 2 min before addition of the ligand anti-chicken IgM. After an additional 4 min, 2 μM ionomycin (10634, Sigma) was added to the samples to obtain the maximal Ca²⁺/free. Cells were kept at 37 °C during the measurements. The fluorescence ratio of Indo-1-violet (Ca²⁺/bound) to Indo-1-blue (Ca²⁺/free) was measured by using FACSDiva™ software (BD Biosciences). In some cases, two cell populations were analyzed simultaneously by staining half of the cells for 10 min at 37 °C and 10% CO2, with 20 nM CFSE, followed by washing of the cells (10⁷ cells/ml) with 10 volumes of PBS. For BTK inhibitor treatment, unstained and CFSE-labeled DT40 cells were treated for 1 h at 37 °C with continuous shaking at a density of 10⁶ cells/ml with 1 μM inhibitor or solvent in RPMI 1640 medium containing supplements. The inhibitor was washed out before adding the Ca²⁺ indicator. For data evaluation, the experimental results contained in FCS files generated by the FACSDiva™ software (BD Biosciences) were imported into the FlowPy Python tool.

**Construction of vectors**

The 4E variant (Y753E, Y759E, Y1197E, and Y1217E) of PLCγ2 was generated according to the 4F variant. The cDNA encoding PLCγ2-N-myristoylated human BTK was generated by N-terminal insertion of the first 15 amino acids from Rous sarcoma virus SRC (MGSSKSKPKDPSQR) (95) and a small linker (PGIQ) providing a BamHI site into PLCγ2-pcDNA3.1(+) BTKΔkinase, lacking amino acids 402–659, was generated with help of in vitro mutagenesis by insertion of a stop codon (TAG) in front of the kinase domain. The primer sequences and PCR protocols are available from the authors upon request. The cDNAs of n-FLAG epitope-tagged human BTK and its point variants R28C, E41K, Y223F, and K430R were provided by C. Brunner, Department of Otorhinolaryngology, Head and Neck Surgery, Ulm University Medical Center. The BTK variant Y551F was generated by O.-O. Wolz from the Department of Immunology, University of Tübingen. The generation of all other cDNAs has been detailed in Refs. 43, 49, 96.

**Generation of stably-transfected DT40 cell clones, DT40 cell culture, and preparation of DT40 cell lysates**

This was essentially done as described in Ref. 49. Proteomic analysis of whole-cell extract proteins, fractionated by SDS-PAGE, was used to verify the expression of WT and K430R variant BTK in the respective cell clones as described previously (97). MS/MS spectra were correlated with the UniProt chicken database complemented with the sequences of human WT and K430R variant BTK. Carbamidomethylated cysteine was considered as a fixed modification along with oxidation of methionine, and acetylated protein N termini as variable modifications. False discovery rates were set on both peptide and protein levels to 0.01.

**Calcium flux measurements**

Cells were suspended at a density of 10⁷ cells/ml in FACS buffer (143 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 20 mM HEPES/NaOH, pH 7.4, 1 mM CaCl₂, 5.6 mM glucose) and incubated for 45 min at 37 °C and 10% CO₂ with 5 μM Indo-1-AM. The calcium measurements were performed on a BD FACS Celesta™. In brief, 10⁶ cells per sample in 0.5 ml of FACS buffer were pre-warmed at 37 °C for 10 min. Next, baseline was measured for 2 min before addition of the ligand anti-chicken IgM. After an additional 4 min, 2 μM ionomycin (10634, Sigma) was added to the samples to obtain the maximal Ca²⁺/free. Cells were kept at 37 °C during the measurements. The fluorescence ratio of Indo-1-violet (Ca²⁺/bound) to Indo-1-blue (Ca²⁺/free) was measured by using FACSDiva™ software (BD Biosciences). In some cases, two cell populations were analyzed simultaneously by staining half of the cells for 10 min at 37 °C and 10% CO₂, with 20 nM CFSE, followed by washing of the cells (10⁷ cells/ml) with 10 volumes of PBS. For BTK inhibitor treatment, unstained and CFSE-labeled DT40 cells were treated for 1 h at 37 °C with continuous shaking at a density of 10⁶ cells/ml with 1 μM inhibitor or solvent in RPMI 1640 medium containing supplements. The inhibitor was washed out before adding the Ca²⁺ indicator. For data evaluation, the experimental results contained in FCS files generated by the FACSDiva™ software (BD Biosciences) were imported into the FlowPy Python tool.
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(RRID:SCR_018195) and then transferred to Excel spreadsheets. Following binning of the time attributes of the individual data points to integers (full seconds), the data of one experiment were assembled into a matrix M such that the first row listed the time attributes and the columns j in rows i + 1 tabulated the fluorescence ratios. The resulting matrices were typically made up of about 540 columns and up to about 1650 rows. In experiments analyzing two cell populations at the same time, DT40 cells expressing W T BTK (unstained) and DT40 cells expressing BTKK430R (stained) (Fig. 7 C), the matrix was fractionated into two parts, M1 and M2, according to the fluorescence intensities determined for intracellular CFSE. The individual points shown in Fig. 7, B and C, correspond to the times in full seconds versus the medians of all fluorescence ratios representing [Ca2+]j, following their correction for basal fluorescence intensity in the absence of anti-IgM and normalization according to the maximal [Ca2+]j concentrations for normalization to allow for quantitative comparison between experiments.

Cell culture and transfection of COS-7 cells, extraction, and analysis of inositol phospholipids

This was done as described previously (38, 39, 53, 55, 99).

Radiolabeling of inositol phospholipids and analysis of inositol phosphate formation

Four hours after transfection, COS-7 cells were washed once with 0.3 ml/well of Dulbecco’s PBS (PAA Laboratories) and then incubated for 18 h in 0.2 ml/well DMEM containing supplements, as described previously (38), supplemented with 2.5 μCi/ml myo-[2-3H]inositol (NET1156005MC, PerkinElmer Life Sciences), and 10 mM LiCl. The cells were then washed once with 0.2 ml/well Dulbecco’s PBS and lysed by addition of 0.2 ml/well 10 mM ice-cold formic acid. The analysis of inositol phosphate formation was performed as described previously (53) except that 15 ml of scintillation fluid Quicksafe A from Zinsser Analytic was used. To examine BTK inhibition via ibrutinib, acalabrutinib, or vecabrutinib, COS-7 cells were radiolabeled for 18 h in 0.2 ml/well 10 mM ice-cold formic acid. The analysis of inositol phospholipids and analysis of inositol phospholipids to allow for quantitative comparison between experiments.

Phosphoblot analysis

COS-7 cells were seeded into 6-well plates and transfected. After an additional medium change, the cells were incubated for a further 18 h at 37 °C and 10% CO2. The medium was removed, and the cells were washed with 0.5 ml of ice-cold TBS and then lysed by addition of 250 μl of phospholysis buffer (25 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5% (v/v) NP40, 0.2% (v/v) Triton X-100, 5% (v/v) glycerol, pH 7.5) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 2 μM pepstatin A, 2 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin, and 3 mM benzamidine) and phosphatase inhibitors (2 mM sodium orthovanadate, 9.5 mM sodium fluoride, 10 mM β-glycerophosphate, and 10 mM tetradsodium pyrophosphate). After lysis, 250 μl of 2× SDS-PAGE sample preparation buffer were added, and aliquots of the samples were subjected to SDS-PAGE followed by immunoblotting.

FRAP experiments

FRAP studies (100, 101) were conducted as described earlier (49), using a fixed-localization Gaussian laser beam focused to a very small spot on the membrane for both bleaching and monitoring, continuously measuring the fluorescence intensity at the spot by photon counting (47). This method gives a much better time resolution (down to a few milliseconds) than FRAP of larger regions-of-interest in a confocal microscope, which employs nonsynchronous stepwise bleaching and monitoring, which take significantly longer (47). The improved time resolution is critical in the case of fast recovery contributed by exchange and/or gliding, as is the case in this study. The experiments were performed 24–26 h post-transfection on COS-7 cells transfected with PLCγ2–GFP derivatives. All experiments were conducted at 22 °C, in Hanks’ balanced salt solution supplemented with 20 mM HEPES/NaOH, 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 ω = 0.77 ± 0.03 μm (×63/1.4 NA oil-immersion objective) or 1.17 ± 0.05 μm (×40/1.2 NA water immersion objective). Experiments were conducted with each beam size (beam size analysis is described in Refs. 48, 49). The ratio between the illuminated areas (ω2(×40)/ω2(×63)) was 2.28 ± 0.15 (n = 59; S.D. calculated using bootstrap analysis as described below). After a brief measurement at the monitoring intensity, a 5-milikwatt pulse (4–6 or 10–20 ms for the ×63 and ×40 objectives, respectively) bleached 50–70% of the fluorescence in the illuminated spot. Fluorescence recovery was followed by monitoring beam. The apparent characteristic fluorescence recovery time (τ) and the mobile fraction (Rf) were derived from the FRAP curves by nonlinear regression analysis, fitting to a lateral diffusion process with a single τ value (102). The significance of differences between sets of τ values measured with the same beam size was evaluated by one-way ANOVA and Bonferroni post hoc test. To compare the τ(×40)/τ(×63) ratio of a given PLCγ2 variant with the τ2(×40)/τ2(×63) beam-size ratio (comparing a single pair of data sets), statistical significance was evaluated by two-tailed Student’s t test using bootstrap analysis with 1000 bootstrap samples (103), which is preferable for comparison between ratios, as described by us previously (104).

Miscellaneous

SDS-PAGE and immunoblotting were performed according to standard protocols using antibodies reactive against the c-Myc epitope for WT and variant PLCγ1, PLCγ2, and PLCδ1, or antibodies reactive against BTK, PLCβ1, RAC2, and β-actin. Immunoreactive proteins were visualized using the Pierce ECL Western blotting detection system (catalog 32106, Thermo Scientific).
Fisher Scientific). Samples to be analyzed by Western blotting were taken, quasi as a fourth replicate, from the same plate as and immediately adjacent to the samples taken in triplicate for functional analysis. Using this protocol and paying meticulous attention to experimental detail, we were able to demonstrate for all results obtained in transiently-transfected COS-7 cells throughout this work that the differences observed in inositol phosphate formation were not explained by or related to differences in the respective protein expression (cf. Figs. 1, B and D, 2, D and F, 3D, and 6C and Figs. S1–S6). All experiments were performed at least three times. Similar results and identical trends were obtained each time. Data from representative experiments are shown as mean values of triplicate determinations. In the experiments shown in Figs. 1C, 2, A–C and E, 3C, 4, and 6A and Table 1, background inositol phosphate formation in response to PLC and/or BTK expression was determined in parallel on cells transfected with empty vector and subtracted from the corresponding values, with appropriate consideration of error propagation for differences and quotients to determine the standard deviations of means (S.D.) of triplicate determinations (105). Although the S.D. values of these technical replicates are not shown in Figs. 1, A, C, and E, 2, A–C, E, and G, 3, A–C, 4, and 6, A, B, and D, and Table 1, their relationships to the corresponding means were calculated in aggregate for all mean values shown in these representations (n = 500) and found to be distributed with a median of 9.93%, with 95% confidence limits of 8.97% and 10.93%. In the experiments displayed in Figs. 1, C and E, 2, C, E, and G, 6, B and D, and 7, B and C, the data were fitted by nonlinear least-squares curve fitting using all replicate values to three- or four-parameter dose-response equations using GraphPad Prism®, version 4.03. In certain cases, the global curve–fitting procedure contained in Prism was used to determine 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.

Data availability
All data are contained within the manuscript. Raw data as well as computer subroutines used for data processing and equations used to calculate propagation of experimental errors are available on request from Peter Gierschik (peter.gierschik@uni-ulm.de).


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