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A High-Throughput Assay to Identify Allosteric Inhibitors of the PLC-γ Isozymes Operating at Membranes

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Supporting Information

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Figures S1–S4 (PDF)

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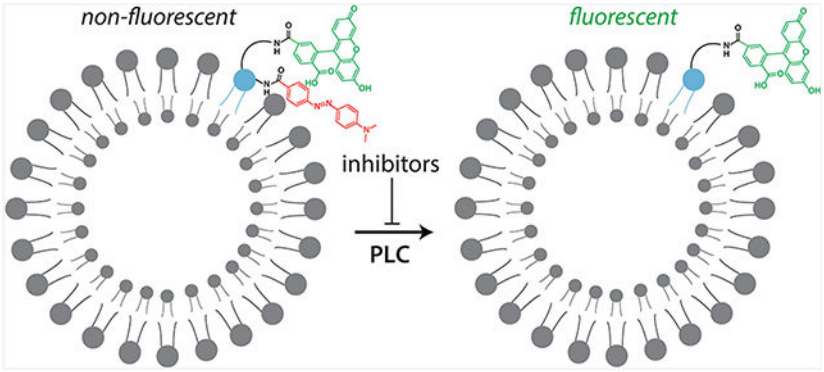
Abstract

The two phospholipase C-γ (PLC-γ) isozymes are major signaling hubs and emerging therapeutic targets for various diseases, yet there are no selective inhibitors for these enzymes. We have developed a high-throughput, liposome-based assay that features XY-69, a fluorogenic, membrane-associated reporter for mammalian PLC isozymes. The assay was validated using a pilot screen of the Library of Pharmacologically Active Compounds 1280 ($LOPAC_{1280}$) in 384-well format; it is highly reproducible and has the potential to capture both orthosteric and allosteric inhibitors. Selected hit compounds were confirmed with secondary assays, and further profiling led to the interesting discovery that adenosine triphosphate potently inhibits the PLC-γ isozymes through noncompetitive inhibition, raising the intriguing possibility of endogenous, nucleotide-dependent regulation of these phospholipases. These results highlight the merit of the assay platform for large scale screening of chemical libraries to identify allosteric modulators of the PLC-γ isozymes as chemical probes and for drug discovery.

Graphical Abstract



The two PLC-γ isozymes (PLC-γ1 and -γ2) are a major nexus for signals originating from numerous receptor tyrosine kinases and immune receptors, including the B and T cell receptors.[1] Like the other PLCs in humans, PLC-γ1 and PLC-γ2 have a conserved catalytic domain and hydrolyze the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3).[2,3] Increased concentrations of DAG and IP_3 result in activated protein kinase C (PKC) isozymes and the release of Ca^{2+} from internal stores, respectively. This bifurcated pathway regulates numerous cellular processes such as proliferation and differentiation, chemotaxis, and the fusion of granulocytic vesicles with the plasma membrane. Unlike the other PLCs, the PLC-γ isozymes have several regulatory domains that mediate their phosphorylation-dependent activation.[4] Aberrant regulation of the PLC-γ isozymes has been found in various diseases such as cancer, rheumatoid arthritis, and Alzheimer's disease. A case in point is that ≤37% of patients with adult T cell lymphoma have gain-of-function mutations in PLC-γ1.[5] Consequently, the PLC-γ isozymes have emerged as promising new therapeutic targets.

Despite the appreciation that PLC enzymes are widely studied and involved in various pathologies, there are few reported inhibitors (Figure 1) of these phospholipases and none are useful for selectively regulating the PLC-γ isozymes. For example, the aminosteroid U73122 was originally reported to directly inhibit PLCs and is widely used to this effect.[6] Unfortunately, it was later demonstrated that U73122 does not inhibit the phospholipase activity of several purified PLCs, including PLC-γ1, PLC-β2, and PLC-δ1.[7] This discrepancy likely arises from the fact that most experiments with U73122 monitor events secondary to the activation of PLCs such as intracellular Ca^{2+} release that likely do not faithfully report direct inhibition of PLCs. This is consistent with the finding that U73122 prevented Ca^{2+} release by directly inhibiting various Ca^{2+} pumps.[8-11] In addition, the maleimide group within U73122 makes it highly reactive so that most U73122 modifies membrane components and does not effectively enter cells.[12] The U73122 that is internalized reacts with and inhibits a variety of unrelated enzymes, including the lipid-modifying enzymes, phosphatidylinositol-4-phosphate kinase and 5-lipoxygenase.[13,14] Finally, U73122 was reported to sequester the PLC substrate, PIP_2.[15] Thus, U73122 is a singularly poor reagent for probing signaling by PLC isozymes. Likewise, D609[16] contains a reactive dithiocarbonate that modifies multiple proteins, including sphingomyelin synthase[17] and phospholipase A2.[18] In addition, D609 chelates Zn^{2+} and has glutathione mimetic properties.[19,20] Although its antiviral and antitumor effects are attributed to inhibition of phosphatidylcholine (PC)-dependent PLC, the direct inhibition of a purified, mammalian phospholipase C by D609 has not been established. Less frequently used to inhibit PLC enzymes are edelfosine,[21] CCT129957,[22] and small peptides.[23] Unfortunately, these compounds also suffer from indirect effects that are mistakenly attributed to PLCs. Small peptides have the additional limitation of being highly cell impermeable.

As an improvement, ATA, 3013, and 3017 were demonstrated as direct inhibitors of PLCs, both with purified enzymes and in intact cells.[24] However, these compounds are paninhibitors and unlikely to be useful for specifically regulating the PLC-γ isozymes given the diverse and sometimes opposing roles of different PLC isoforms in biology and disease.[25-27] In addition, ATA is not cell permeable and inhibits multiple enzymes other than PLCs.[28-30] The moderate potency and solubility of 3013 and 3017 are also reasons for caution when using them to modulate the phospholipase activity of PLCs. Consequently, there is a substantial need for the development of small molecules that directly and selectively modulate the PLC-γ isozymes.

The catalytic core of PLCs is highly conserved. Therefore, inhibitors that target the active site are unlikely to be selective among different PLC isozymes. However, in contrast to the other PLCs, the PLC-γ isozymes possess an array of regulatory domains that are autoinhibitory until phosphorylated at specific tyrosines during regulated activation. Recently, Sondek and co-workers determined the first structure of an essentially full-length, autoinhibited PLC-γ1 that offers an excellent framework for explaining activation due to phosphorylation or mutation.[31] In this framework, there are three states of PLC-γ1 in equilibrium (Figure 2A). In the autoinhibited state, the regulatory array sits atop the core domains to prevent membrane engagement and PIP_2 hydrolysis. This state is greatly favored until phosphorylation of Tyr783 or mutations shift the equilibrium toward an open state that is subsequently stabilized at membranes to produce the fully active form.

This framework underpins the rationale and promise for identifying selective, allosteric inhibitors of the PLC-γ isozymes. For example, there are many concavities between the regulatory and core domains that could readily interact with compounds to prevent the necessary rearrangements needed for phospholipase activation (Figure 2B). Alternatively, surfaces of PLC-γ1 needed to interact with membranes could also bind compounds to inhibit phospholipase activity. In both scenarios, inhibitors would not target the conserved catalytic core and so would be much more likely to be isozyme specific. We also expect these types of inhibitors to be much more frequent than orthosteric inhibitors because the potential surface area for interaction with allosteric inhibitors is immense relative to the surface area of the active site.

We previously developed XY-69 (Figure 2C) to robustly monitor PLC activity at membranes in real time.[32] XY-69 is a PIP_2 analogue with fluorescein introduced at the *sn*-1 position and a 4-(dimethylaminoazo)benzene-4-carboxylic acid (DABCYL) moiety incorporated into the 6-hydroxyl position of the headgroup. This arrangement effectively quenches the intrinsic fluorescence of fluorescein by the DABCYL moiety. However, once XY-69 is cleaved by PLCs, the fluorophore/quencher pair separate, resulting in a dramatic increase in the quantum yield of the fluorescein group. XY-69 was also designed to retain a long $C_{15}H_{31}$ acyl chain at the *sn*-2 position to favor the partitioning of XY-69 into lipid membranes. Therefore, much like PIP_2 and DAG, XY-69 and the fluorescent product of XY-69 hydrolysis remain in lipid membranes. Importantly, XY-69 reconstituted into liposomes readily reports the activation of PLC-γ1 upon tyrosine phosphorylation or mutations[31] with either purified proteins or cell lysates (Figure S1).

We therefore propose to develop a high-throughput assay based on XY-69 embedded in liposomes to identify allosteric inhibitors of PLC-γ1 that are also likely to be selective. In contrast, a previously developed high-throughput assay using WH-15 (Figure 2D),[24] a water-soluble analogue of PIP_2, is expected to identify mainly orthosteric inhibitors of PLC-γ1. Consequently, the complementary use of both membrane-embedded XY-69 and soluble WH-15 will facilitate the rapid identification of selective, allosteric inhibitors of PLC-γ1 (Figure 2E) using a workflow that can also be applied to PLC-γ2.

MATERIALS AND METHODS

General.

XY-69 and purified PLC-γ1 (D1165H) protein were produced as previously reported. All other reagents, including liver phosphatidylethanolamine (PE) and phosphatidylinositol 4,5-bisphosphate (PIP_2), were purchased from commercial sources and used directly. All buffer stock solutions were prefiltered with a 0.45 μm PES syringe tip filer and stored at 4 °C unless otherwise indicated. All microplate assays were performed using ProxiPlate-384 Plus F black plates.

High-Throughput Screen of the LOPAC$_{1280}$ Library.

The assay plate was prepared by transferring individual compounds (100 nL, 1 mM in DMSO) to a small-volume 384-well microplate using a Mosquito HTS nanoliter dispensing

instrument. Appropriate controls, including DMSO, EGTA (500 mM, pH 10.3), and ATA (5 mM in DMSO), were added to empty wells prior to the high-throughput screen. Liposomes containing liver PE (440 μM), brain PIP_2 (40 μM), and XY-69 (1 μM) were generated by mixing the lipids, drying the mixture under a stream of nitrogen, and resuspending the dried lipid mixture in HEPES (20 mM, pH 7.4) through sonication. Such vesicles were then mixed with 6× assay buffer to form a liposome solution. In parallel, purified PLC-γ1 (D1165H) protein was diluted to 2.5 nM with a buffer containing HEPES (20 mM, pH 7.4), NaCl (50 mM), dithiothreitol (DTT, 2 mM), and fatty acid-free BSA (1 mg/mL). The diluted PLC solution (4 μL) was then added to each well of the assay plate at room temperature. After 15 min, the vesicle solution (6 μL) was added to initiate the enzymatic reaction. After 1 h at room temperature, the quenching buffer (5 μL) containing EGTA (50 mM, pH 10.3) was added. Thirty minutes later, the assay plate was analyzed by fluorescence intensity (λ_{ex} = 485 nm, and λ_{em} = 520 nm) with a plate reader.

IC_{50} Measurement with XY-69.

Assay buffer was prepared with HEPES (20 mM, pH 7.4), KCl (70 mM), $CaCl_2$ (2.35 mM), EGTA (3 mM), and DTT (2 mM). Individual hit compounds were prepared as 10 mM stock solutions in DMSO or H_2O according to the manufacturer's instructions. A 5 μL aliquot of either Hit-1 or Hit-3 was diluted with assay buffer to make a working stock of 500 μM inhibitor. This working stock was used to make 1:3 serial dilutions containing 5% (v/v) DMSO that were each added to wells of a microplate in quadruplicate aliquots of 2 μL. Solutions of purified PLC protein were diluted to the appropriate concentrations with assay buffer containing fatty acid-free BSA (1 mg/mL). Two microliters of the PLC solution was added to each well of inhibitor solution, and the mixtures were incubated at room temperature for 15 min. Liposomes containing liver PE (367 μM), brain PIP_2 (33.3 μM), and XY-69 (0.83 μM) were generated by mixing the lipids, drying the mixture under a stream of nitrogen, and resuspending the dried lipid film using sonication in HEPES (20 mM, pH 7.4), followed by mixing with a 6× concentrated assay buffer to give the vesicle solution containing HEPES (38 mM, pH 7.4), KCl (70 mM), $CaCl_2$ (2.35 mM), EGTA (3 mM), and DTT (2 mM). To initiate the enzymatic reaction, 6 μL of the liposome solution was added to each PLC/inhibitor mixture. The microplate was immediately placed in the plate reader, and the fluorescence intensity was recorded every minute. The linear range of each reaction curve was used to calculate the initial reaction rate for each inhibitor concentration, and the resulting plot was analyzed using GraphPad Prism to calculate the IC_{50} value.

The final assay mixtures contained various concentrations of inhibitors (100, 33.3, 11.1, 3.70, 1.23, 0.411, 0.137, 0.046, 0.015, or 0.005 μM) or inhibitor-free assay buffer for controls of 100% enzyme activity. Baseline correction was performed using replicates that excluded both the inhibitor and PLC (BSA only). The final concentrations for wild-type PLC-γ1, PLC-γ1 (D1165H), PLC-γ1 (P867R), and wild-type PLC-γ2, -β2, -β3, and -δ1 were 2.0, 0.5, 2.0, 2.0, 0.5, 10, and 0.003 nM, respectively. The volumes of all final assay mixtures were 10 μL, and all of the mixtures contained PE (220 μM), PIP_2 (20 μM), XY-69 (0.5 μM), 1% (v/v) DMSO, HEPES (30 mM, pH 7.4), KCl (70 mM), $CaCl_2$ (2.35 mM), EGTA (3 mM), DTT (2 mM), and fatty acid-free BSA (0.2 mg/mL). For experiments

containing lower concentrations of PIP_2 (4 or 2 μM), phosphatidylinositol (PI) was included (16 or 18 μM, respectively) to balance the total final lipid content back to 240 μM.

Kinetic Studies of Inhibition of XY-69 Hydrolysis by ATP.

ATP dilutions were prepared using a cholate micelle buffer containing HEPES (50 mM, pH 7.4), KCl (70 mM), $CaCl_2$ (3 mM), EGTA (3 mM), and 0.5% (w/v) sodium cholate and then added to a microplate in quadruplicate aliquots of 2 μL. Purified, wild-type PLC-γ1 was diluted with micelle buffer that included fatty acid-free BSA (1 mg/mL). The diluted enzyme solution (4 μL) was added to each well, and the microplate was incubated at room temperature for 15 min. Subsequently, XY-69 at various concentrations in micelle buffer (4 μL) containing DTT (5 mM) was added to initiate the reaction. The microplate was immediately placed in the plate reader, and the fluorescence intensity was recorded every 30 s. All final assay mixtures contained wild-type PLC-γ1 (0.8 nM), 1% (v/v) DMSO, HEPES (50 mM, pH 7.4), KCl (70 mM), $CaCl_2$ (3 mM), EGTA (3 mM), DTT (2 mM), 0.5% (w/v) sodium cholate, and fatty acid-free BSA (0.4 mg/mL) in a volume of 10 μL. Kinetic assays were performed with final ATP concentrations of 0, 1.5, and 5 μM in combination with 0.5, 0.75, 1.0, 1.5, 5, and 10 μM XY-69. The initial velocities were calculated as the fluorescence intensity increase per minute, and a double-reciprocal plot was used to analyze the data.

Measurement of the Binding Affinity of ATP with Wild-Type PLC-γ1.

ITC studies were carried out on an Auto-iTC200 instrument at 25 °C. Briefly, purified PLC-γ1 (D1165H) was dialyzed against ITC buffer {50 mM HEPES (pH 7.4), 150 mM NaCl, 1 μM $CaCl_2$, and 2 mM TCEP [tris(2-carboxyethyl)phosphine]}, and ATP was dissolved in the same ITC buffer. To initiate titrations, ATP (500 μM) was injected into a sample cell containing PLC-γ1 (D1165H) (50 μM, 200 μL) while being stirred at 850 rpm. An initial injection (0.2 μL) was followed by 19 serial injections (2 μL each) with a 3 min interval between injections to allow for signals to return to baseline. Data were analyzed by integrating the peaks using Origin software (MicroCal). Heats of dilution were subtracted from heats of binding, and the data were fit to a one-site binding model.

IC_{50} Measurement with WH-15.

Micelle buffer was prepared with HEPES (50 mM, pH 7.4), KCl (70 mM), $CaCl_2$ (3 mM), EGTA (3 mM), 0.5% (w/v) sodium cholate, and DTT (2 mM). Inhibitor dilutions containing 5% (v/v) DMSO were prepared as described above using micelle buffer and added to a microplate in quadruplicate aliquots of 2 μL. Solutions of purified PLC-γ1 (D1165H) were diluted to the appropriate concentration with micelle buffer containing fatty acid-free BSA (1 mg/mL). Four microliters of a PLC solution was added, and the microplate was incubated at room temperature for 15 min. Subsequently, 4 μL of micelle buffer containing 25 μM WH-15 was added to initiate the reaction. The microplate was immediately placed in the plate reader, and the fluorescence intensity (λ_{ex} = 355 nm, and λ_{em} = 530 nm) was recorded every minute. The volumes of all final assay mixtures were 10 μL, and the mixtures contained PLC-γ1 (D1165H) (0.5 nM), WH-15 (10 μM), 1% (v/v) DMSO, HEPES (50 mM, pH 7.4), KCl (70 mM), $CaCl_2$ (3 mM), EGTA (3 mM), DTT (2 mM), 0.5% (w/v) sodium cholate, and fatty acid-free BSA (0.4 mg/mL).

Inhibition of the Phospholipase Activity in Live Cells.

HEK293A cells were plated at a density of ~75000 cells/well in 12-well tissue culture dishes in growth medium [DMEM containing 10% (v/v) FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B]. Twenty-four hours later, cells were transiently transfected with 100 ng of a plasmid encoding HA-tagged wild-type PLC-γ1 or PLC-γ1 (D1165H) using Continuum transfection reagent according to the manufacturer's protocol (GeminiBio). Twenty-four hours post-transfection, growth medium was removed and cells were metabolically labeled overnight with 1 μCi of [^{3}H]-*myo*-inositol in serum-free, inositol-free DMEM. Radiolabeling medium was then removed and replaced with vehicle [serum-free, inositol-free DMEM containing 1 μCi of [^{3}H]-*myo*-inositol and 1% (v/v) DMSO] or a vehicle containing 30 μM Hit-1, and cells were incubated for 1 h. Cells were then treated with 10 mM LiCl (final concentration) for an additional 1 h, and accumulation of [^{3}H]inositol phosphates was quantified by Dowex column chromatography as described previously. Expression of each version of PLC-γ1 was confirmed by immunoblotting of cell lysates using a monoclonal antibody against the HA epitope (BioLegend, clone 16B12; RRID: AB_2565335). A monoclonal antibody against β-actin (SigmaAldrich, clone AC-15; RRID: AB_476692) was used as a loading control.

RESULTS AND DISCUSSION

Development of a High-Throughput Assay Based on XY-69 in Liposomes.

We have chosen to use oncogenic and constitutively active PLC-γ1 (D1165H) as the screening target. This choice is driven by both practical and theoretical considerations. First, the basal activity of wild-type PLC-γ1 with membrane-embedded XY-69 is very low, and it would be difficult to produce a reasonable signal-to-noise ratio with this form of the enzyme. Alternatively, phosphorylated PLC-γ1 could be used, but it is difficult to produce a homogeneous amount of this form sufficient for large scale screens. Instead, the enzymatic activity of PLC-γ1 (D1165H) with membrane-embedded XY-69 is high enough to produce a robust signal-to-noise ratio.[31] This is likely due to D1165H-induced shifts in the equilibrium population of PLC-γ1 toward a more open distribution predisposed to engage membranes. However, the equilibrium likely continues to favor the autoinhibited state because PLC-γ1 (D1165H) retains phosphorylation-induced activation[31] and its intrinsic activity remains slow enough that initial rates of hydrolysis of XY-69 in liposomes remain linear (Figure S1). Consequently, screened compounds are expected to have opportunities to interact with populations of PLC-γ1 (D1165H) in the autoinhibited and open states before addition of liposomes containing XY-69.

To optimize a high-throughput assay using membrane-embedded XY-69 to screen compounds for inhibitors of PLC-γ1, we systematically assessed (i) the concentration of XY-69, (ii) the production and composition of liposomes, (iii) the concentration of PLC-γ1 (D1165H), (iv) the amount and order of reagent addition, and (v) the quench conditions. For the sake of brevity, only studies assessing the concentrations of XY-69 and PLC-γ1 (D1165H) are discussed in detail.

In previous experiments,[31] 0.5 nM PLC-γ1 (D1165H) was used in liposomes incorporated with 5 μM XY-69. This concentration of XY-69 was deemed prohibitive for large screens and was decreased over a 10-fold range while the concentration of PLC-γ1 (D1165H) was maintained at 0.5 nM (Figure 3A). The hydrolysis of XY-69 was continuously monitored using fluorescence (λ_{ex} = 485 nm, and λ_{em} = 520 nm), and the extent of activation was calculated. XY-69 embedded in liposomes produced robust changes in fluorescence over a wide range of concentrations. To minimize XY-69 consumption while maintaining a robust signal-to-noise ratio, 0.5 μM XY-69 was used in all subsequent experiments.

With the concentration of XY-69 fixed, amounts of PLC-γ1 (D1165H) were varied (Figure 3B). The assay was linear over the course of 1 h for several concentrations of PLC-γ1 (D1165H). Consequently, 1 nM PLC-γ1 was chosen for subsequent experiments. This choice was designed to minimize the consumption of PLC-γ1 (D1165H) while maintaining a robust, linear signal that allowed sufficient time for the numerous plate manipulations required for high-throughput screens.

The final protocol consisted of incubation of 1 nM PLC-γ1 (D1165H) with 0.5 μM XY-69 embedded in liposomes composed of 220 μM liver phosphoethanolamine (PE) and 20 μM PIP_2. The final volumes were 10 μL per well, and reactions were monitored at room temperature (~23 °C) for 60 min prior to the addition of 50 mM EGTA to chelate the Ca^{2+} cofactor needed for phospholipase activity. Importantly, this final assay format readily reads out the preferential interfacial activation of PLC-γ1 (D1165H) relative to that of the wild type (Figure 3C). In addition, the assay is immune to concentrations of DMSO of ≤5% (v/v) (Figure 3D). The fluorescence values (λ_{ex} = 485 nm, and λ_{em} = 520 nm) for the final assay conditions were measured for 16 parallel reactions in a 384-well plate for three consecutive days. In parallel, experiments under identical conditions were performed but with PLC-γ1 (D1165H) (high control) substituted for BSA (low control). From these data, the average Z'-factor[33] was calculated to be 0.77 (Figure S2), indicating a highly robust high-throughput assay.

Proof-of-Concept High-Throughput Screen with the $LOPAC_{1280}$ Library.

Using essentially the same conditions as described above, we completed a pilot screen in duplicate with the 1280 compounds comprising the Library of Pharmacologically Active Compounds 1280 ($LOPAC_{1280}$) (Figure 4). A correlation coefficient of approximately 0.86 for the two runs attests to the reproducibility of the format, and Z' factors were maintained above 0.7 for both runs. The screen produced 12 hits (Figure 5) flagged as having >50% inhibition in both runs for an estimated hit rate of 0.94%.

U73122 is widely accepted as an inhibitor of PLCs and also included in $LOPAC_{1280}$. However, it was not flagged as an inhibitor of PLC-γ1 in this screen. This result is consistent with our published data showing that U73122 does not inhibit a panel of purified PLCs, including PLC-γ1.[7]

The 12 hit compounds (Figure 5) were categorized into five groups (A–E) based on structural similarities and possible mechanisms of action. We previously used WH-15 to screen $LOPAC_{1280}$ for PLC inhibitors,[24] and under these conditions, only hit compounds in

groups D and E, including ATA (Hit-7), were identified. Clearly, the new assay format based on XY-69 can identify new classes of inhibitors, possibly allosteric, that are invisible to assays using WH-15.

Inhibitory Profiles of Two Hit Compounds from the Pilot Screen.

To further validate the hits and establish secondary assays, we measured the concentration-dependent inhibition of PLC-γ1 (D1165H) by Hit-1 in group A and Hit-3 in group B. The IC_{50} value for Hit-1 was 13.9 μM when XY-69 was used in the assay but increased to 36.0 μM when WH-15 was used (Figure 6A). Similarly, Hit-3 inhibited PLC-γ1 with differing IC_{50} values (0.26 μM vs 7.3 μM) when XY-69 and WH-15 were used (Figure 6A). When the substrate PIP_2 concentration was varied in the liposome (Figure 6B), Hit-3 showed a constant IC_{50} (~0.25 μM) indicative of a noncompetitive and likely allosteric mode of action.[34-36] Another cancer-associated mutant, PLC-γ1 (P867R), can also be inhibited by Hit-3 with an IC_{50} of 0.85 μM (Figure S3). Furthermore, Hit-3 demonstrated important isoform selectivity, showing strong inhibition of the wild-type PLC-γ isozymes but no significant inhibition of either PLC-β (β2 or β3) or PLC-δ1 isozymes (Figure 6C).

Hit-3 is an ATP analogue and is negatively charged. There are eight other ribonucleotides or derivatives (Figure S4) in $LOPAC_{1280}$. Despite the entire set being negatively charged, only the two ATP analogues significantly inhibited the phospholipase C activity of PLC-γ1 (D1165H), suggesting that the inhibition by Hit-3 is likely specific. This intriguing inhibitory profile prompted us to investigate whether ATP also inhibits the phospholipase activity of PLC-γ1 (D1165H). As shown in Figure 6D, ATP indeed inhibited with a potency (IC_{50} = 0.19 μM) similar to that of Hit-3. In contrast, ADP and GTP showed much weaker potency, with IC_{50} values of 4.3 and 12.7 μM, respectively.

To further understand the mechanism of inhibition by ATP, we carried out kinetic analysis of XY-69 hydrolysis by wild-type PLC-γ1 (Figure 7). The initial velocities at various substrate concentrations with or without ATP (1.5 and 5 μM) were measured, and the data were analyzed using a double-reciprocal plot (Lineweaver–Burk plot). The V_{max} of the enzymatic reaction was reduced by 1.9- and 4.1-fold when 1.5 and 5 μM ATP was present, respectively, while the K_M remained essentially unchanged. This result is consistent with noncompetitive inhibition. This outcome is also consistent with the unchanged IC_{50} when the PIP_2 concentration is varied in the presence of ATP analogue Hit-3 (Figure 6C). Given these results, isothermal titration calorimetry (ITC) was used to measure the affinity of ATP and wild-type PLC-γ1 (Figure 8). The titration showed unequivocal interaction (K_D of 2.6 μM) of ATP and wild-type PLC-γ1, suggesting the intriguing possibility of endogenous regulation of PLC-γ1 by ATP. In summary, the pilot screen and follow-up studies highlight the potential value of XY-69 for the identification of novel, allosteric inhibitors of PLCs.

However, neither Hit-3 nor ATP is cell permeable, and neither can be profiled in cell-based experiments. To further demonstrate that the primary hits identified in the pilot screen can be confirmed in secondary assays, we investigated the effect of the less potent but cell permeable Hit-1 in intact cells. Briefly, HEK293A cells were transfected with PLC-γ1 (D1165H) and then treated with Hit-1. Indeed, Hit-1 (30 μM) inhibited the phospholipase activity of PLC-γ1 (D1165H) by approximately 80% in intact cells (Figure 9).

CONCLUSIONS

We have developed a liposome-based, high-throughput assay to identify selective inhibitors of the PLC-γ isozymes. The assay is based on the fluorogenic, membrane-associated PLC reporter, XY-69, and is highly reproducible. The new format is further validated by a pilot screen of $LOPAC_{1280}$ in 384-well format with a Z'-factor of >0.7. The screen results demonstrate that the liposome-based assay can identify novel types of inhibitors, presumably allosteric inhibitors, that assays based on a soluble PIP_2 analogue will miss. These results also highlight the importance of liposomes in measuring the activity of the PLC-γ isozymes, consistent with the mechanism of PLC-γ activation as illustrated by structural studies. Liposome-based high-throughput screens have been underexplored, and examples in the literature are very limited,[37,38] possibly because of the concern about nonspecific disruption of liposomes by different compounds. The moderate hit rate (0.94%) of the pilot screen suggests that such a concern does not prevent practical use of our assay format.

Ribonucleotides including ATP have been shown to inhibit PLC-β4 at a high concentration (1 mM),[39] but their effects on the PLC-γ isozymes are not known. We have demonstrated that ATP, but not ADP or GTP, potently and selectively inhibits the phospholipase activity of the PLC-γ isozymes. The cellular concentration of ATP is typically in the range of 1–10 mM, raising the question of how the PLC-γ isozymes can be activated in cells. We have suggested several possibilities. For example, receptor tyrosine kinases such as Src have been shown to activate ATPases.[40] Because ADP and AMP have less or no inhibition of PLC-γ1, it is possible that ATP inhibition could be released through ATP hydrolysis in response to activated receptor tyrosine kinases. Alternatively, cellular cations such as Mg^{2+} and Ca^{2+} bind to ATP to form different complexes.[41-43] It is possible those complexes, as well as free ATP, have a heterogeneous distribution inside cells and differential capacities to inhibit PLC-γ1, especially at the membranes where PLC-γ1 is activated. The negative charges on membranes could also facilitate the dissociation of bound ATP from the enzyme. Although a detailed mechanism of action needs more extensive studies and is beyond the scope of this work, the discovery of ATP's inhibition of wild-type PLC-γ1 suggests that the assay platform might be suitable for discovering additional endogenous regulators of PLCs.

Hit-1 and Hit-3 were identified from a LOPAC that contains only 1280 compounds, including many newly appreciated pan assay interference compounds (PAINS).[44-46] Aggregator Advisor[47] is a widely used database for assessing whether screen hits are similar to known aggregators. Indeed, among the 12 hit compounds in Figure 5, five (including Hit-1) are considered as aggregators or similar to a known aggregator in the database. Some other considerations also preclude Hit-1 and Hit-3 from being good candidate inhibitors worthy of further development. For example, Hit-1 [6-bromoindirubin-3′-oxime (BIO)] has multiple other pharmacological activities, including inhibition of GSK-3β,[48] and also has poor solubility in aqueous buffer, while both Hit-3 and ATP are not cell permeable. Nonetheless, the pilot screen with this library is intended solely for the validation of the assay format for subsequent high-throughput screens. Our results suggest that the novel liposome-based assay platform developed in this work provides a unique opportunity to potentially identify allosteric modulators of the PLC-γ isozymes as chemical probes and for drug discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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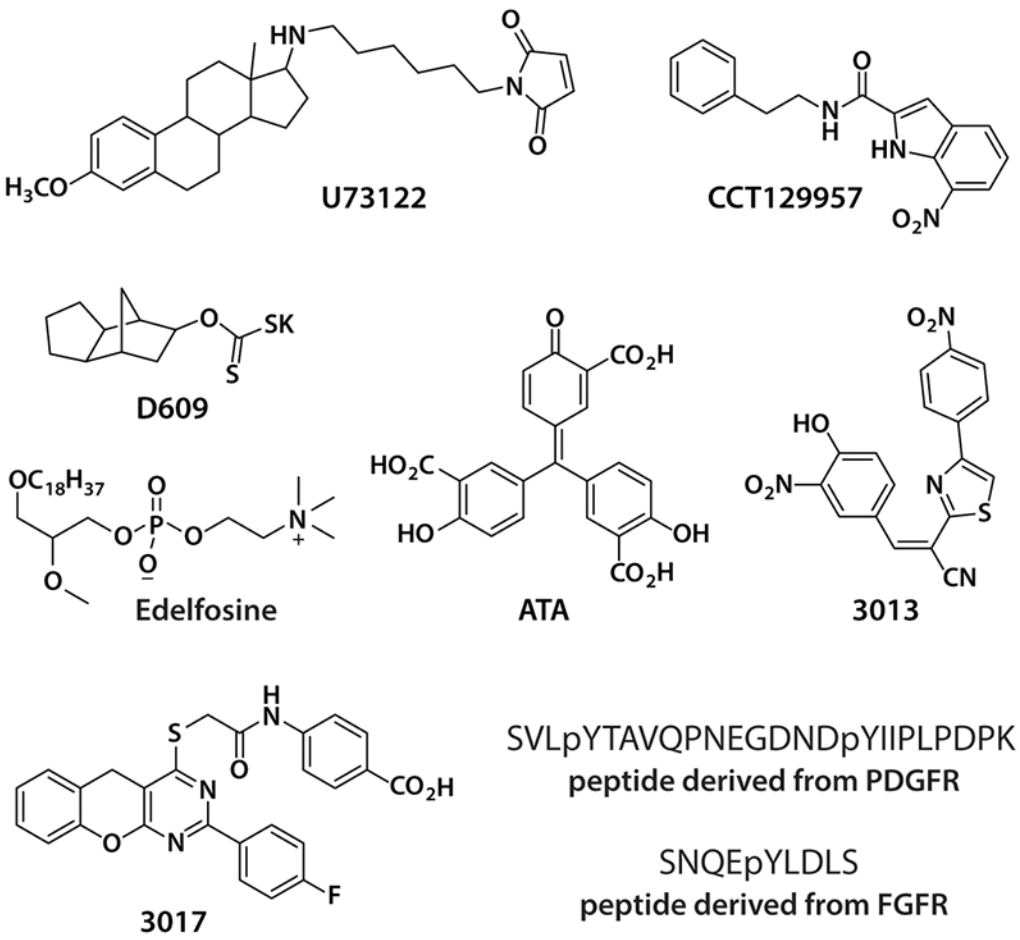

Figure 1.

Reported small molecule and peptide inhibitors of PLCs. These inhibitors either do not directly inhibit the PLC-γ isozymes or lack isoform selectivity and sufficient potency to be useful.

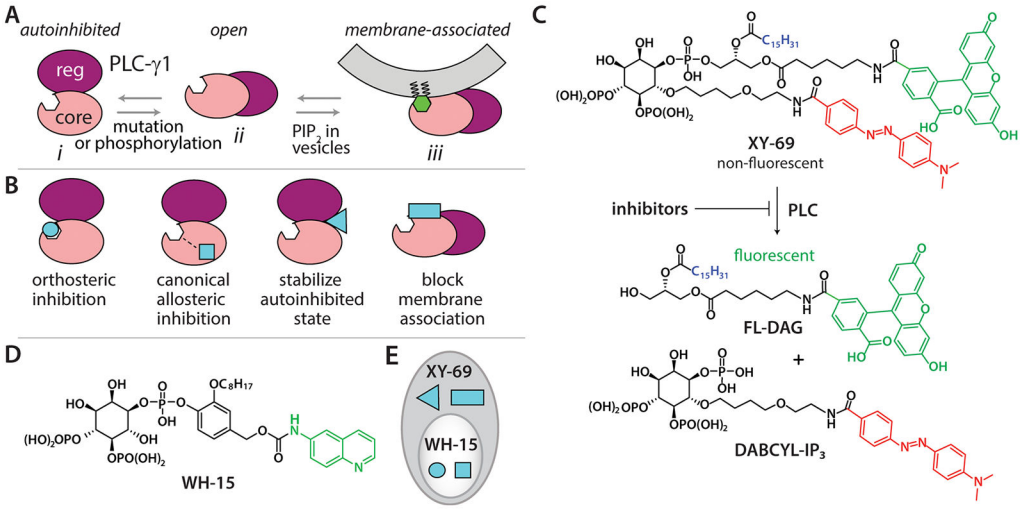

Figure 2.

Design of a high-throughput assay for selective, allosteric inhibitors of PLC-γ1. (A) Model for the activation of PLC-γ1. There are three major states that are in equilibrium. The autoinhibited state cannot bind PIP_2 in membranes and is greatly favored. Phosphorylation of Tyr783 or oncogenic mutations shift the equilibrium toward the open state to varying degrees, and only the membrane-associated active state can access PIP_2 or XY-69 in vesicles. (B) Four major classes of inhibitors are expected from high-throughput screens of PLC-γ isozymes. (C) XY-69 is a membrane-associated, fluorogenic reporter for PLC isozymes. Inhibitors are expected to block hydrolysis of XY-69 catalyzed by PLC-γ1. (D) Chemical structure of a soluble PIP_2 analogue, WH-15, that functions as a PLC substrate. (E) WH-15 and XY-69 can discern among the classes of inhibitors indicated in panel B.

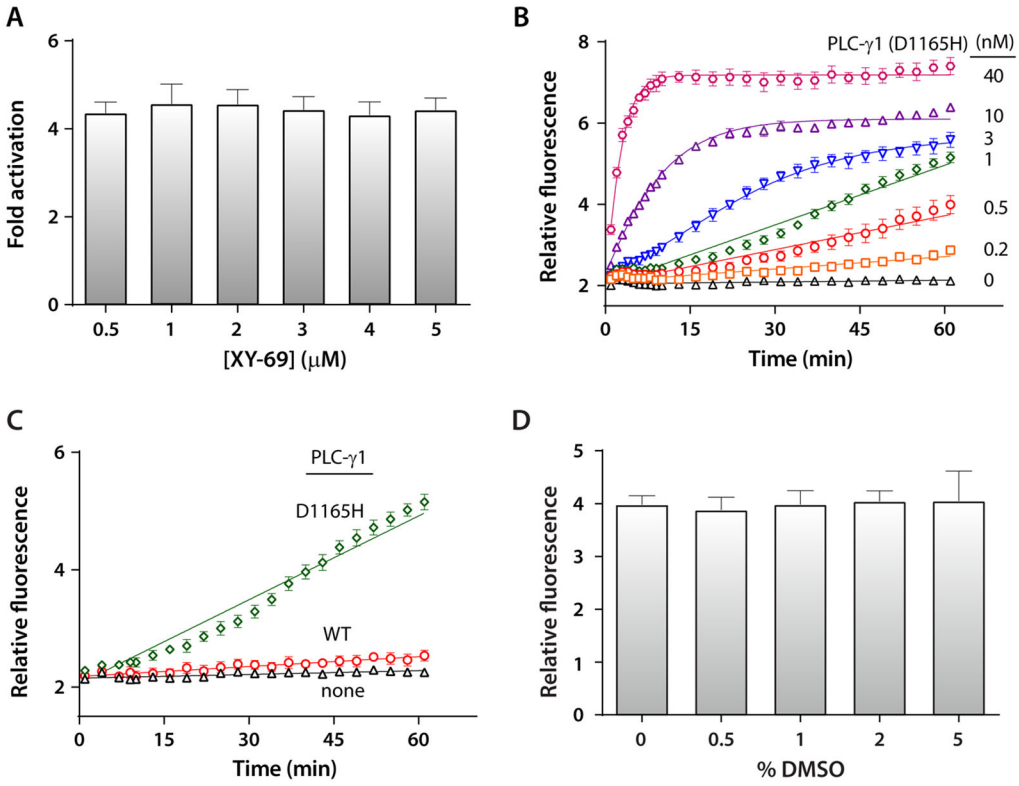

Figure 3.

Assay optimization using XY-69 embedded in liposomes. (A) Assay window assessed as a function of XY-69 concentration. The concentration of PLC-γ1 (D1165H) was 0.5 nM. (B) The level of hydrolysis of XY-69 increases with an increase in the concentration of PLC-γ1 (D1165H). The concentration of XY-69 was 0.5 μM. (C) Membrane-embedded XY-69 highlights the preferential interfacial activation of constitutively active PLC-γ1 (D1165H). PLCs at 1 nM; XY-69 at 0.5 μM. (D) Hydrolysis of XY-69 by PLC-γ1 (D1165H) tolerates up to 5% DMSO with no degradation of the signal.

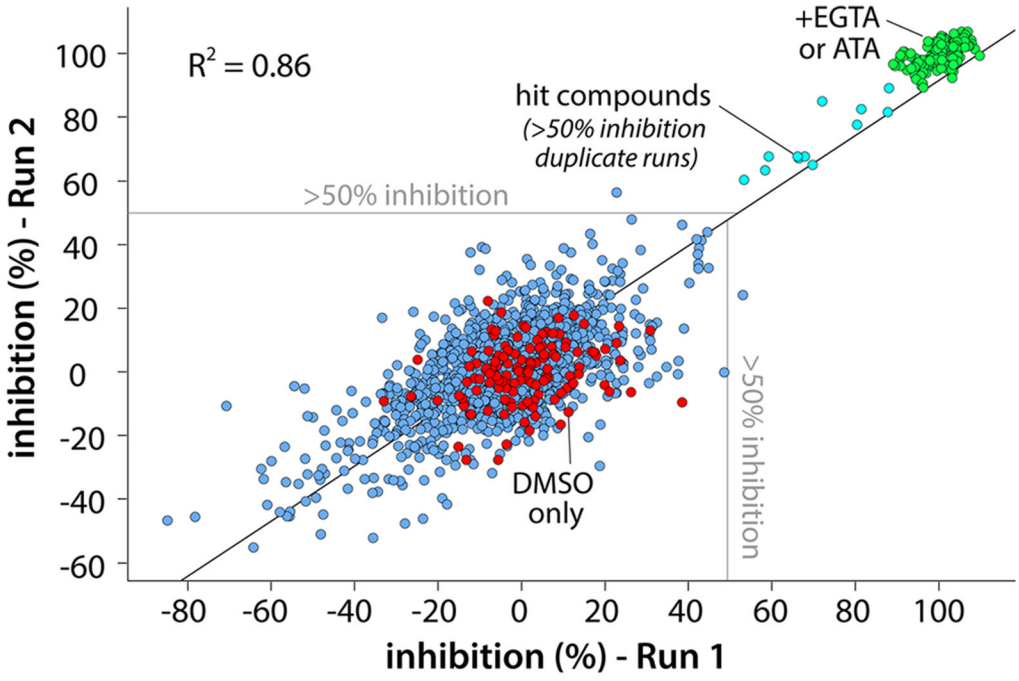

Figure 4.

Pilot screen of the $LOPAC_{1280}$ library. The high-throughput assay based on XY-69 was used to screen 1280 compounds in the Library of Pharmacologically Active Compounds 1280 ($LOPAC_{1280}$, 10 μM) for inhibitors of PLC-γ1 (D1165H). The screen was run in duplicate, and the correlation between runs was plotted. Full inhibition was obtained using either the promiscuous inhibitor aurintricarboxylic acid (ATA, 50 μM) or EGTA (10 mM) to chelate the Ca^{2+} cofactor. Twelve compounds produced >50% inhibition in both runs for an estimated hit rate of approximately 1%; the Z'-factor > 0.75 for both runs.

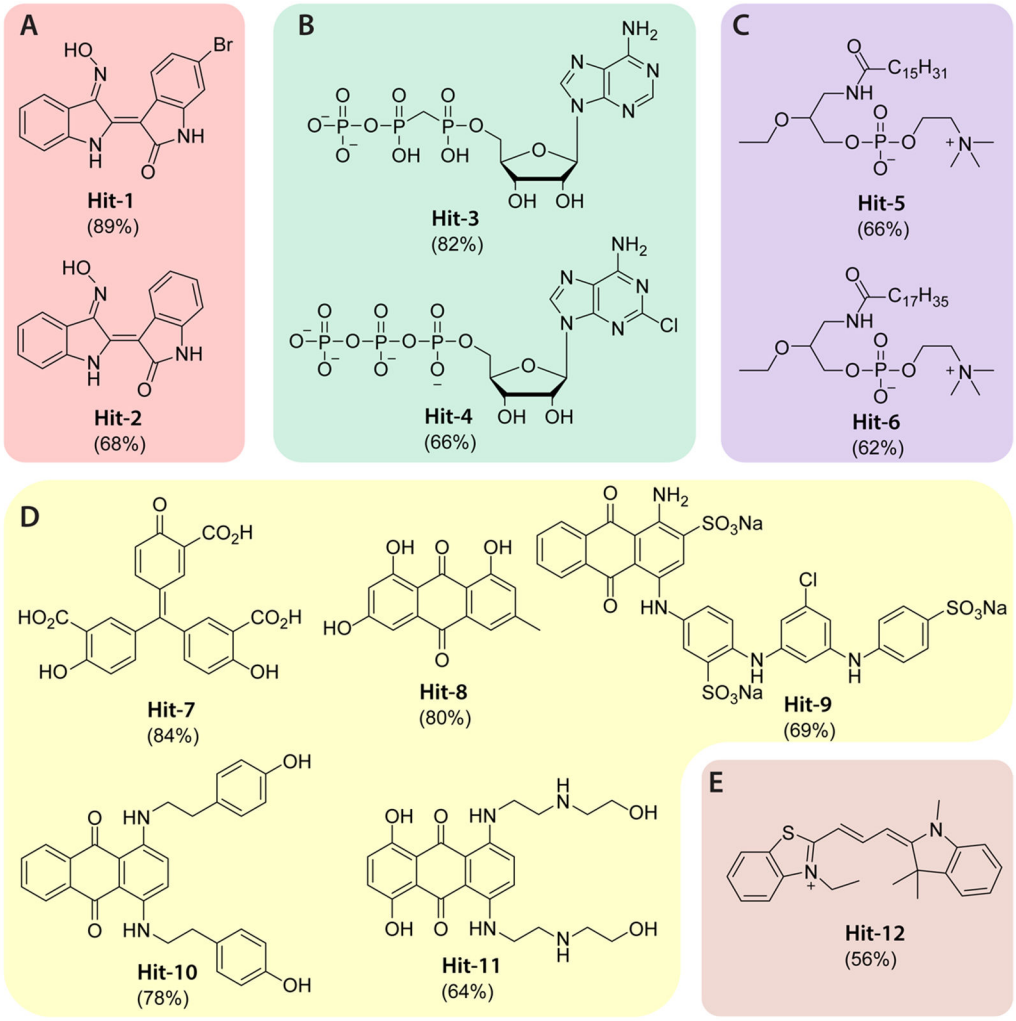

Figure 5.

Chemical structures of the hit compounds from the pilot screen. The 12 hits are categorized into five groups (A–E) based on structural features and possible mechanisms of action. Although hits in groups D and E were also identified from a previous screen based on the soluble PIP_2 analogue, WH-15, the other hits are unique to the screen based on XY-69. The number in parentheses represents the percentage inhibition of PLC-γ1 (D1165H) by the corresponding hit compound at 10 μM.

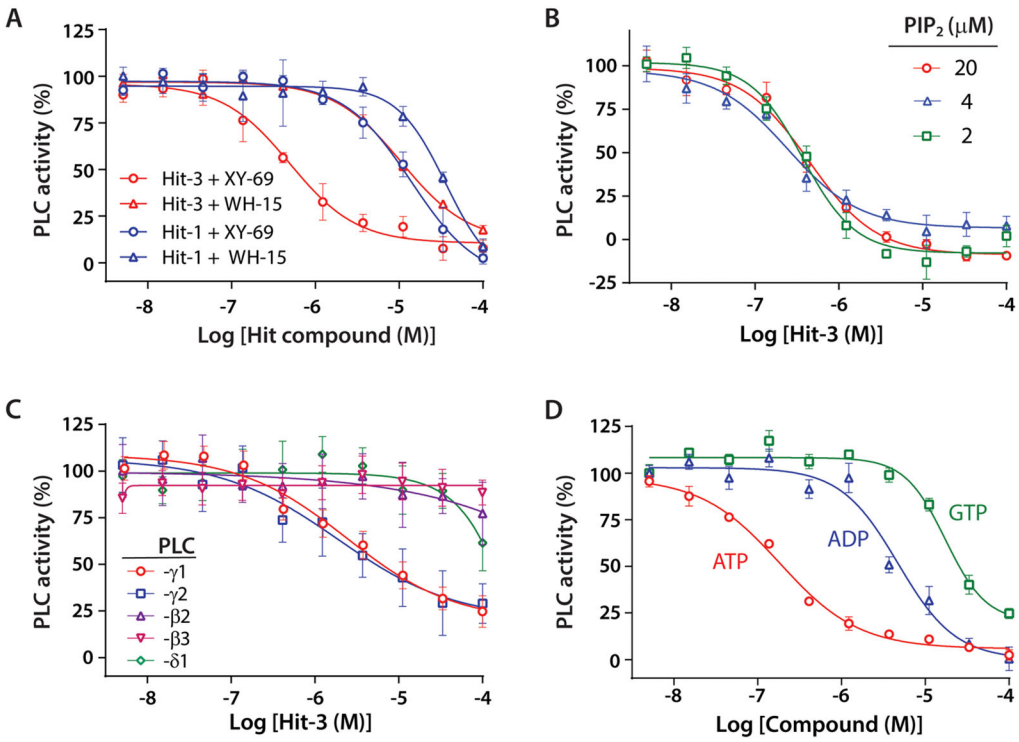

Figure 6.

Validation of two hit compounds in secondary assays. (A) The concentration-dependent inhibition of phospholipase activity of PLC-γ1 (D1165H) by Hit-1 or Hit-3 was quantified using either XY-69 or WH-15. (B) The inhibition of PLC-γ1 (D1165H) by Hit-3 is independent of the concentration of the substrate PIP_2 in liposomes, indicative of noncompetitive inhibition. (C) Isoform selectivity. The inhibition of the indicated wild-type PLC isozymes by Hit-3 was measured. (D) Inhibition of PLC-γ1 (D1165H) by ATP, ADP, and GTP.

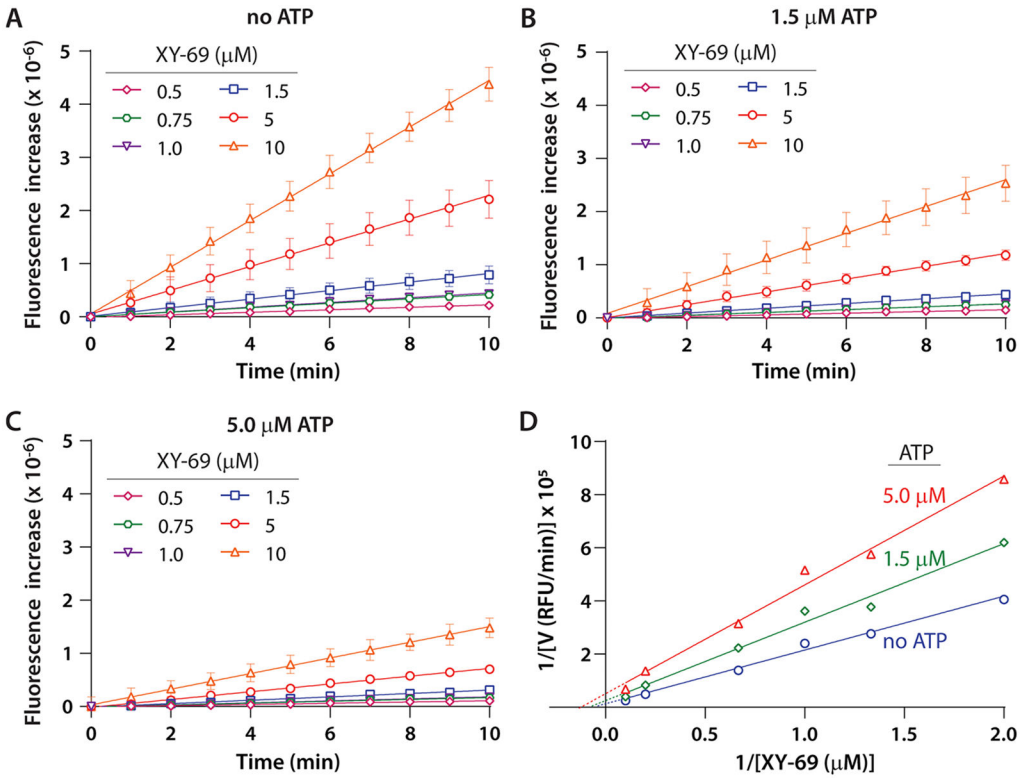

Figure 7.

Kinetic analysis of inhibition of wild-type PLC-γ1 by ATP. The concentration of wild-type PLC-γ1 was 0.8 nM. (A) Hydrolysis of XY-69 at the indicated concentrations was monitored by fluorescence. (B) Hydrolysis of XY-69 at the indicated concentrations in the presence of 1.5 μM ATP. (C) Hydrolysis of XY-69 at the indicated concentrations in the presence of 5 μM ATP. (D) Double-reciprocal plot. The initial velocities were calculated as fluorescence per minute, and its reciprocal was plotted against the reciprocal of XY-69 concentration. The data were fitted to linear regression in Prism 8.

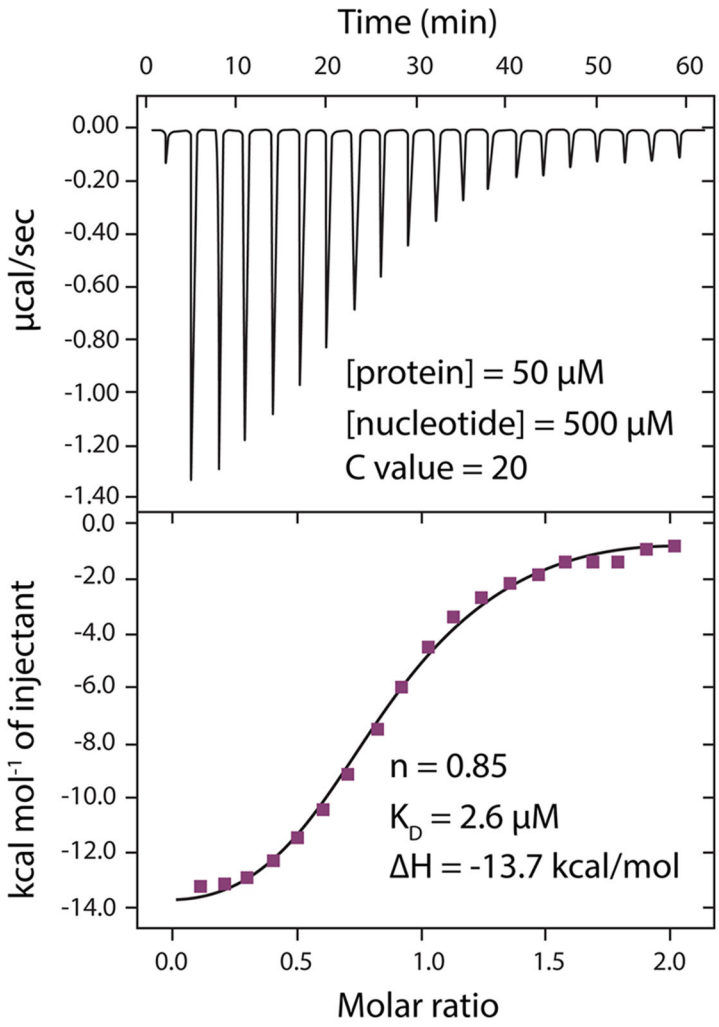

Figure 8.

ATP binds wild-type PLC-γ1 as measured by isothermal titration calorimetry. Thermogram (top) and binding isotherm (bottom) for ATP titrated into wild-type PLC-γ1 in a buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 μM $CaCl_2$, and 2 mM TCEP.

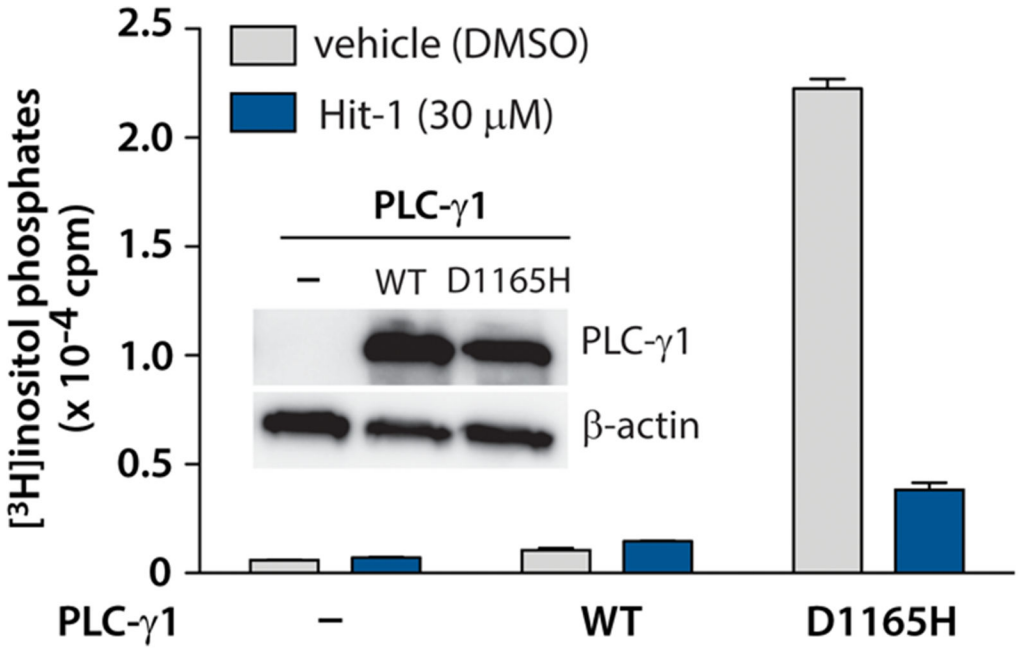

Figure 9.

Hit-1 inhibits PLC-γ1 (D1165H) in intact cells. HEK293 cells were transfected with PLC-γ1 (D1165H), wild-type PLC-γ1, or an empty vector control prior to metabolic labeling with [^{3}H]inositol and incubation with either DMSO or 30 μM Hit-1 for 1 h. Cells were subsequently lysed, and the radioactivity of the lysate was quantified. The expression of PLC-γ1 was confirmed by a Western blot (inset).