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A Fluorogenic, Small Molecule Reporter for Mammalian Phospholipase C Isozymes

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Abstract

Phospholipase C isozymes (PLCs) catalyze the conversion of the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol. This family of enzymes are key signaling proteins that regulate the physiological responses of many extracellular stimuli such as hormones, neurotransmitters, and growth factors. Aberrant regulation of PLCs has been implicated in various diseases including cancer and Alzheimer's disease. How, when, and where PLCs are activated under different cellular contexts are still largely unknown. We have developed a fluorogenic PLC reporter, **WH-15**, that can be cleaved in a cascade reaction to generate fluorescent 6-aminoquinoline. When applied in enzymatic assays with either pure PLCs or cell lysates, this reporter displays more than a 20-fold fluorescence enhancement in response to PLC activity. Under assay conditions, **WH-15** has comparable K_m and V_{max} with the endogenous PIP_2. This novel reporter will likely find broad applications that vary from imaging PLC activity in live cells to high throughput screening of PLC inhibitors.

Phospholipase C isozymes (PLCs) catalyze the conversion of the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers (1), inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Figure 1A). IP_3 mobilizes intracellular stores of Ca^{2+} while DAG activates protein kinase C (2). Furthermore, depletion of PIP_2 alters the membrane association and/or activity of many proteins that harbor phosphoinositide binding domains (3). Consequently, PLC isozymes are key signaling proteins that regulate the physiological responses of many extracellular stimuli such as hormones, neurotransmitters, and growth factors (4). Aberrant regulation of PLCs has been implicated in various diseases including cancer, Alzheimer's disease, and neuropathic pain (5–11). For example, activation of PLC-γ1 is required for the migration of breast cancer cells in response to epidermal growth factor. Similarly, PLC-γ1 is highly expressed in numerous breast carcinomas and colorectal tumor cell lines; down-regulation of PLC-γ1 expression in MD-MBA-231 breast cancer cells prevents the capacity of these cells to metastasize when injected into nude mice (7). Thus, PLCs are extensively studied and long considered as potential targets for drug development. Unfortunately, two major limitations continue to hinder studies of PLCs.

First, it is impossible to continuously monitor mammalian PLC activity, either in living cells or in enzymatic assays. Typically, cellular PLC activity is measured from the production of radiolabeled inositides after biosynthetic incorporation of radioactivity into phosphoinositide

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

pools (12). These assays are subject to cell-dependent differences in steady-state phosphoinositide metabolism and variable expression of PLCs. Alternatively, cell-permeable dyes that increase in fluorescence upon binding Ca^{2+} are also routinely used to monitor PLC activity (13). However, these dyes do not directly measure PLC activity and often generate confounding data due to diverse factors known to affect intracellular Ca^{2+} concentrations. For purified PLCs, radioactive PIP_2 is used as the substrate and the enzymatic activity is measured through quantifying radiolabeled IP_3. Unfortunately, none of the above assay formats allow for the continuous and direct monitoring of PLC activity.

Second, there are no direct, specific inhibitors of PLCs and this situation cannot easily be addressed with current assays of phospholipase activity since they are not amenable to high throughput screening. Several small molecule PLC inhibitors are indeed reported in the literature, among which U73122 is the most widely used (14). However, U73122 contains an electrophilic maleimide moiety that reacts with diverse nucleophiles including glutathione and various uncharacterized lipid components (15). More importantly, the direct inhibition of phospholipase activity by U73122 has not been demonstrated with purified PLCs. Instead, U73122 inhibits several other proteins including various calcium pumps (16), phosphatidylinositol-4-phosphate kinase (17), and 5-lipoxygenase (18).

Several fluorogenic reporters have been tested to monitor continuously the phospholipase activity of mammalian PLCs. None of them have been widely exploited likely due to limited applicability or ready availability. For instance, PLC-δ1 will efficiently hydrolyze phosphorothiolate analogs of PIP_2 (19). However, the resulting thiol products must be coupled to a secondary reaction in order to monitor absorbance changes; such coupled systems increase the potential for artifacts during high-throughput screens or detailed enzymological analyses. Similarly, the fluorescein derivative of phosphoatidylinositol-4-phosphate has been reported to be a substrate of PLC-δ1 with hydrolysis leading to increased fluorescence (20). Unfortunately, it is unclear how efficiently PLC-δ1 hydrolyzes this fluorescein derivative relative to its endogenous phosphatidylinositol substrates. Indeed, PLC-δ1 very poorly hydrolyzed the dibutyl derivative of phosphatidylinositol relative to longer acyl chain derivatives (21) suggesting that the fluorescein derivative might also be a poor substrate since it lacks acyl chains. Regardless, this fluorescein substrate is not commercially available and to the best of our knowledge there have been no subsequent reports of its use to monitor mammalian PLCs. Mammalian PLCs require a phosphate at the 4-position of the inositol ring for efficient hydrolysis of phosphatidylinositols (22) and fluorescent derivatives that do not preserve this phosphate are either inert (23) or not expected to be efficient substrates (24–27).

Here we report a rationally designed small molecule PLC reporter **WH-15** (Figure 1B) that features a 4-hydroxybenzyl alcohol linker to bridge the inositol phosphate head group and the fluorophore 6-aminoquinoline. The alkyl group C_8H_{17} is introduced into **WH-15** to retain the hydrophobic character of PIP_2 while still ensuring it is water soluble. Upon PLC action, the reporter **WH-15** is expected to be cleaved in a cascade reaction to generate inositol trisphosphate IP_3, quinomethide **1**, and 6-aminoquinoline **2** (Figure 1B). The carbamate derivative of the aminoquinoline in **WH-15** is predicted to have an emission maximum at 380 nm when excited at 344 nm. In contrast, 6-aminoquinoline **2** has emission maxima at 450 and 530 nm when similarly excited (28–29). Therefore, the enzymatic hydrolysis of **WH-15** by a PLC should be readily monitored by fluorescence at ~530 nm.

The synthesis of **WH-15** began with compound **3**, which was prepared in two steps from commercially available 4-(benzyloxy)-3-hydroxybenzaldehyde (Figure 1C). Phosphorylation of **3** with 1-benzyloxy-*N*,*N*,*N*′,*N*′-tetraisopropylphosphinediamine generated **4** quantitatively. The benzaldehyde **4** also served as the phosphorylation reagent

for enantiomerically and diastereomerically pure inositol phosphate derivative **5** (30), which was converted to **6** after reaction with **4** and oxidation with *t*-butylperoxide. Reduction of the aldehyde group in **6** to the corresponding benzylalcohol with sodium borohydride ($NaBH_4$) led to **7** in 95% yield. Coupling of **7** with N-(quinolin-6-yl)-1*H*-imidazole-1-carboxamide **8**, which was synthesized from 6-aminoquinoline and 1,1′-carbonyldiimidazole, followed by removal of the protective groups with trimethylsilyl bromide (TMSBr) and MeOH then produced the free phosphatidylinositide **WH-15**. The reporter is stable for 2 months with storage at −20 °C as judged by analyses with liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR).

To demonstrate the application of this reporter, **WH-15** was incubated with wild type, purified PLC-δ1 at 37 °C for 30 min and the emission spectrum of the reaction mixture was recorded (Figure 2A). Purified PLC-δ1 harboring a single substitution (E341A) within its active site has immeasurable lipase activity and was used in a parallel reaction (31). In addition, the reaction without PLC was also monitored. As shown in Figure 2A, wild type PLC-δ1 generates new emission peaks at 450 nm and 530 nm (green line), consistent with the formation of 6-aminoquinoline and confirmed by LC-MS analyses (Supporting Information, Figure S1). In contrast, the reaction mixture with either PLC-δ1(E341A) or no added PLC showed minimum emission at 530 nm and maximum emission at 380 nm (red and black lines), indicating that **WH-15** was not cleaved by either PLC-δ1(E341A) or other components in the assay buffer. These results were further confirmed by LC-MS since no 6-aminoquinoline **2** was detected from the reaction mixture (Supporting Information, Figure S1). To demonstrate that **WH-15** can be used to monitor PLC activity, the real-time fluorescence of the reaction mixture was recorded for purified PLC-δ1 (Figure 2B). **WH-15** generated approximately a 30-fold increase in fluorescence with PLC-δ1 relative to an identically treated sample containing either catalytically inactive PLC-δ1 (E341A) or BSA. These results suggest that **WH-15** specifically reports the enzymatic activity of PLC and the fluorescence-based assay is more sensitive and convenient than the traditional method.

The human genome encodes at least 13 distinct PLCs (1). To test whether **WH-15** functions as a substrate for other PLC isoforms, the reporter was incubated with either purified PLC-β2 or PLC-γ1 in reactions analogous to that described for PLC-δ1. As shown in Figure 2B, the fluorescence intensity of the reaction mixture with each of the three isoforms increases as reaction proceeds suggesting that **WH-15** is a substrate for all three isoforms of PLC tested. Although the kinetics profiles for the three isoforms are different, all reactions reach the same plateau in relative fluorescence intensity after incubation overnight. These results suggest that **WH-15** is a general substrate for different PLC isozymes.

To further characterize **WH-15**, we measured the kinetic properties of **WH-15** with PLC-γ1 (Figure 3). The K_m of PLC-γ1 for **WH-15** was 49 ± 7.2 μM with a V_{max} at 4.2 ± 0.26 pmol/min/ng. For comparison, the endogenous substrate, PIP_2, was also applied in the enzymatic reaction under similar conditions. The *Km* was measured as 28± 2.6 μM with a V_{max} of 2.7 ± 0.07 pmol/min/ng. Thus, despite the obvious structural differences between **WH-15** and endogenous PIP_2, both molecules serve as essentially equivalent substrates for PLC-γ1 under the assay conditions. Furthermore, PLC-δ1 (K_m = 30 ± 8.1 μM; V_{max} = 1.2 ± 0.11 pmol/min/ng) and -β2 (K_m = 86.1 ± 16.9 μM; V_{max} = 1.2 ± 0.10 pmol/min/ng) hydrolyze **WH-15** with similar kinetics suggesting that all PLC isozymes will cleave **WH-15** and PIP_2 with similar efficiencies (Supporting Information, Figure S2).

The reactive quinomethide intermediate formed upon the hydrolysis of **WH-15** is unlikely to modify covalently PLC-γ1 to alter its phospholipase activity. Otherwise, we would expect non-linear rates of **WH-15** consumption and this outcome is not observed. Instead, LC-MS analysis of the reaction mixture detected the formation of the products from reaction of

quinomethide with dithiothreitol (DTT) and water, suggesting that quinomethide was most likely quenched by nucleophiles in the assay buffer. We also tested substrate specificity of **WH-15** for other related lipases. Two other phospholipases, phospholipase A2 (PLA2) and phospholipase D (PLD), did not generate fluorescence enhancement from **WH-15** (Supporting Information, Figure S3), indicating that **WH-15** is a PLC-selective fluorogenic reporter.

To assess potential non-specific cleavage of **WH-15** by the entire repertoire of cellular phosphodiesterases, the stability of **WH-15** was evaluated in lysates derived from Human Embryonic Kidney HEK-293T cells (Figure 4). Cells transfected with plasmid encoding either PLC-δ1, PLC-δ1 (E341A), PLC-β2, PLC-γ1, or the parent vector were lysed 24 h after transfection; the resulting lysates were normalized for total protein and tested for capacity to hydrolyze **WH-15** (Figure 4A).

Importantly, lysates from cells transfected with either the parent vector or vector encoding catalytically dead PLC-δ1 (E341A) exhibited minimal capacity to hydrolyze **WH-15** as evidenced by minimal increases in fluorescence. These results confirm: i) the expected low basal activities of PLCs prior to upstream stimulation (32) and ii) that the cellular milieu does not contain substantial amounts of non-specific phosphodiesterases capable of hydrolyzing **WH-15**. Also importantly, cells that were transfected with either PLC-δ1, PLC-β2, or PLC-γ1 show a 10–20 fold increase in fluorescence (Figure 4A) consistent with the overexpression of these isozymes as determined by western blotting (Figure 4B). Taken together, these results unequivocally demonstrate that **WH-15** is a sensitive and selective reporter for PLC activities in complex cell lysates. The fact that the fluorescence window is as large as 20-fold highlights the potential application of **WH-15** in quantifying PLC activity in different cell lines.

In conclusion, we have developed a fluorogenic reporter **WH-15** for mammalian PLCs. This novel reporter functions in both enzymatic assays and cell lysates with high sensitivity, and represents a robust assay that is not based on radioactivity. Given the key roles that PLCs play in cell signaling and diseases, this new PLC reporter will likely find broad applications in profiling different cell types and disease states. Furthermore, the large signal-to-noise ratio of the assay with **WH-15** should enable its use in high throughput screens to identify small molecule PLC inhibitors. **WH-15** also provides a starting point for developing fluorescent reporters to monitor PLC activities in real-time in cells. Finally, the 4-hydroxybenzyl alcohol linker used in **WH-15** should also be applicable to the development of reporters for other enzymes that cleaves a P-O or C-O bond, such as esterases and tyrosine phosphatases.

Methods

Synthesis of the fluorogenic WH-15

The syntheses of **WH-15** and intermediates are described in detail in the Supporting Information. The identity and purity of compounds were assessed by LC-MS and nuclear magnetic resonance (NMR) spectroscopy.

Assay with Mammalian PLC Isoforms

All fluorescence assays were performed in Perkin Elmer 384-well Plates and the fluorescence was recorded on a Perkin Elmer Wallac Envision™ 2103 Multilabel Reader with the excitation wavelength of 355 nm (ex filter: 355 nm, 10 nm) and the emission wavelength of 535 nm (em filter: 535 nm, 10 nm). To carry out the assay, the reporter **WH-15** (44 μM, final concentration) was dissolved in the assay buffer (15 μL) that contains 133 μg/mL fatty-acid free BSA, 50 mM HEPES (pH 7.2), 70 mM KCl, 3 mM $CaCl_2$, 3 mM

EGTA, and 2 mM DTT at 37 °C. Assays were initiated upon addition of 20 ng of purified PLC protein and data were recorded every 2 min. Experiments were repeated at least three times. The excitation and emission spectra were recorded on a QM-4 PTI Spectral Fluorometer. Equivalent amounts of purified PLCs (3 μg, inset) were verified by SDS-PAGE followed by staining with Coomassie Brilliant Blue.

Kinetic Studies of WH-15 and Endogenous PIP_2

The reporter **WH-15** was dried under a stream of argon and resuspended in 20 mM HEPES which contains 0.5% cholate. This solution was diluted to obtain final assay conditions with 175, 117, 47, 35, 29, 23, 12, or 6 μM **WH-15** in the assay buffer (10 mM HEPES, pH 7.4, 120 mM KCl, 10 mM NaCl, 2 mM EGTA, 5.8 mM $MgSO_4$, 0.5% cholate, 160 μg/μL fatty acid-free BSA, and 100 μM free Ca^{2+}) in a final volume of 12 μL. The assays were started by the addition of 4 ng of purified full-length wild type PLC-γ1. The reaction mixtures were then incubated at 30 °C and fluorescence was measured continuously as is described above.

For kinetic studies with the endogenous PIP_2, a mixture of PtdIns(4,5)P_2 (300 μM, Avanti Polar Lipids) and ~10,000 cpm of [^{3}H]PtdIns(4,5)P_2 was dried under a stream of nitrogen and resuspended in 0.5% cholate. The resulting lipid stock was diluted to obtain final assay conditions with either 280, 160, 80, 24, or 8 μM PtdIns(4,5)P_2 in the same buffer as described above in a final volume of 60 μL. Assays were initiated by the addition of 17 ng of purified full-length wild-type PLC-γ1. After incubation at 30 °C at time intervals between 0–8 min, reactions were stopped by the addition of 200 μL of 10% (*v/v*) trichloroacetic acid (TCA) and 100 μL of 10 mg/ml BSA to precipitate uncleaved lipids and protein. Centrifugation of the reaction mixture isolated soluble [^{3}H]Ins(1,4,5)P_3, which was quantified using liquid scintillation counting.

Transfected Cell Lysate Assay

Cell lysates were prepared from transiently transfected HEK-293T cells plated in 12-well dishes at a cell density of 65,000 cells/well in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10,000 units/mL penicillin, 10,000 units/mL streptomycin, and 25 units/mL amphotericin B. Following incubation for 24 h at 37 °C in an atmosphere of 95% air/5% CO_2, cells were transfected with 600 ng of the indicated DNA and 100 ng of empty vector, for a total of 700 ng of DNA per well. DNA was complexed with FuGENE 6 transfection reagent (Roche Applied Sciences) in a 4:1 ratio of FuGENE 6 reagent to DNA prior to transfection. Twenty-four hours post transfection, media was aspirated and replaced with serum-free DMEM for 12–16 h. Subsequently, cells were lysed in 200 μL of RIPA buffer (Sigma) as per manufacturer's protocol. Lysates were normalized for total protein concentration using a Bio-Rad Protein assay (Bio-Rad Dye Reagent) prior to use in the reporter assay. Reporter assays were initiated by the addition of 50 μL of normalized cell lysate to the mixture (final volume 120 μL) that contains 50 μM reporter **WH-15**, 83 μg/mL fatty-acid free BSA, 50 mM HEPES (pH 7.2), 70 mM KCl, 3 mM $CaCl_2$, 3 mM EGTA and 2 mM DTT at 37 °C. Data was collected every 3 min using a Wallac Victor^{2}™ 1420 Multilabel Counter (Model: 1420-011, Perkin Elmer Life Sciences) with an excitation wavelength of 340 nm and an emission wavelength of 535 nm. Western blotting was performed on normalized cell lysates to confirm the expression of PLC-β2, -δ1, and -γ1 using monoclonal antibodies (Santa Cruz).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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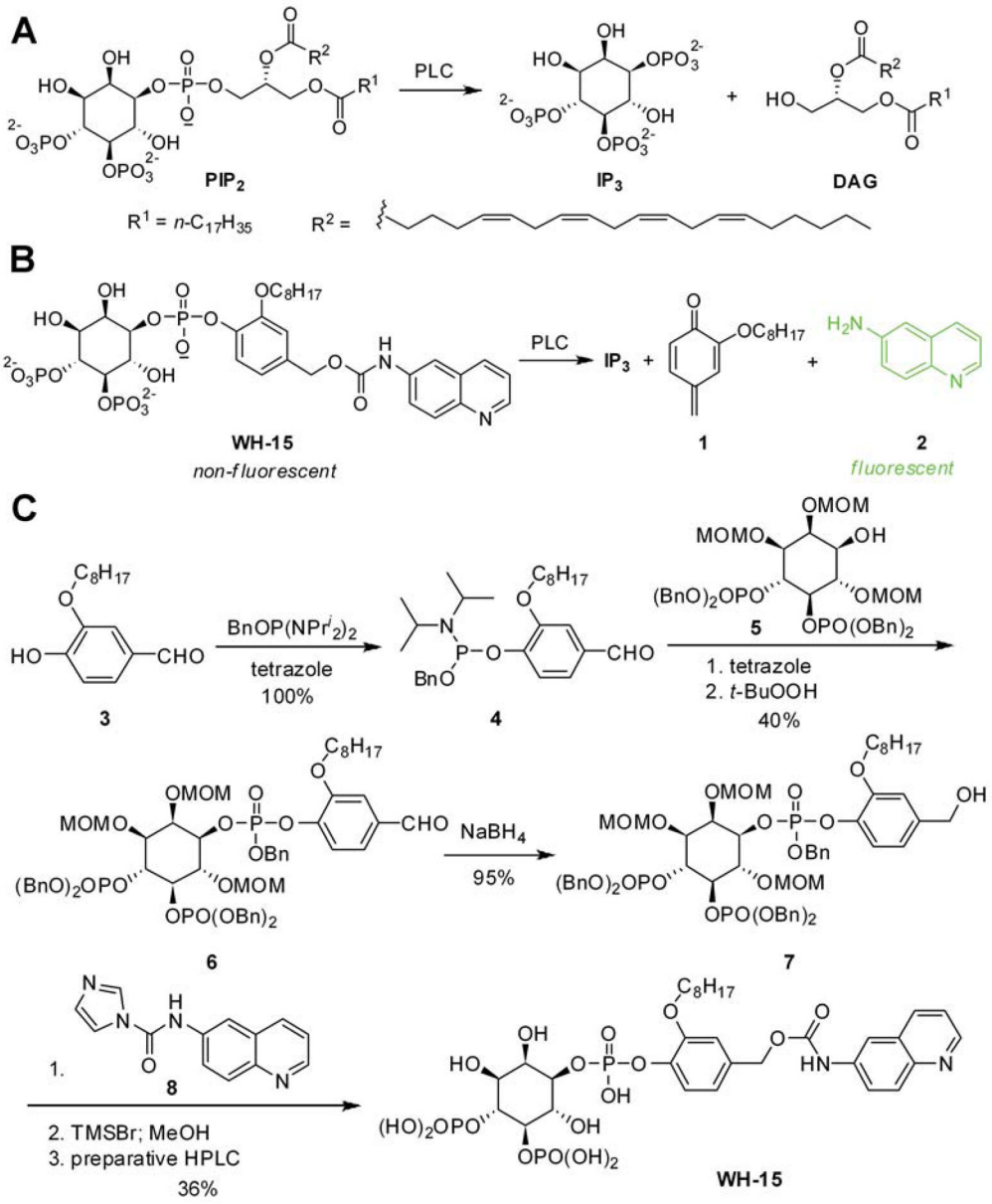

Figure 1.

Fluorogenic reporter design for mammalian PLCs. (A) PIP_2 is cleaved to IP_3 and DAG by PLC; (B) **WH-15** is cleaved by PLC to form IP_3, quinomethide **1**, and 6-aminoquinoline **2**. When excited at 344 nm and monitored the emission at 535 nm, **WH-15** is essentially non-fluorescent while 6-aminoquinoline is highly fluorescent. (C) Chemical synthesis of **WH-15**.

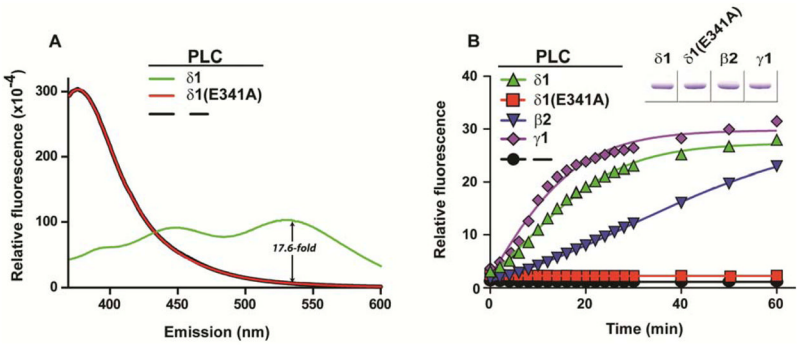

Figure 2.

WH-15 is a reporter for PLC isozymes. (A) **WH-15** was incubated with either wild-type (green) or catalytically-inactive (red) PLC-δ1 or no added PLC (black) for 60 min prior to recording emission spectra (λex = 344 nm). The ~17.6-fold increase in emission at 535 nm between reactions with wild-type PLC-δ1 and no added PLC is also shown. (B) Real-time fluorescence of **WH-15** cleavage catalyzed by different PLC isoforms and normalized to the initial fluorescence of the reaction without PLC. Equivalent amounts of purified PLCs (inset) were verified by SDS-PAGE followed by staining with Coomassie Brilliant Blue. Initial concentration of **WH-15** (44 μM) and PLC isozymes (20 ng/15 μL) are the same in both panels.

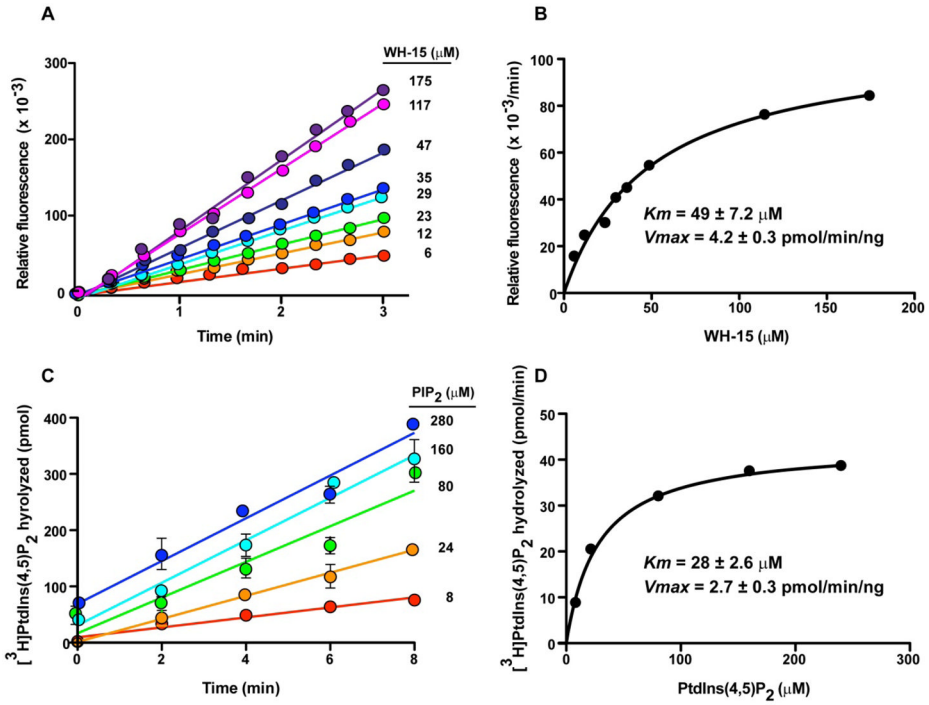

Figure 3.
Kinetics studies of **WH-15** (A and B) and endogenous PIP_2 (C and D) with PLC-γ1.

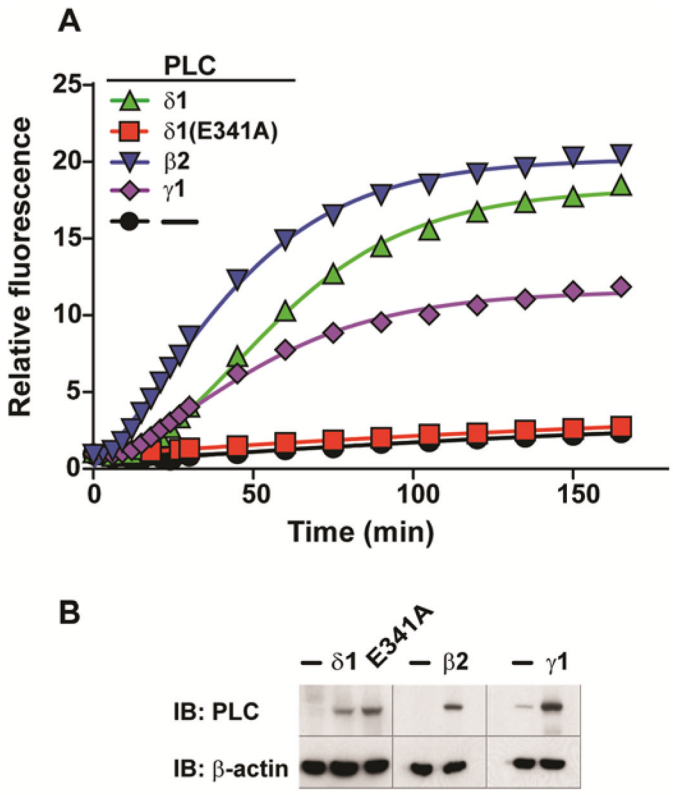

Figure 4.

WH-15 reports PLC enzymatic activity in cell lysates. (A) Real-time fluorescence of **WH-15** (50 μM) cleavage catalyzed by PLC isozymes in cell lysates and normalized to the initial fluorescence of the reaction with lysates derived from cells transfected with empty vector. (B) HEK-293T cells were lysed and western blotting was performed for the indicated PLC isozymes.