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Small Molecule Inhibitors of Phospholipase C from a Novel High-throughput Screen*

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Background: Phospholipase C (PLC) isozymes are increasingly attractive therapeutic targets; however, pharmacological modulators are lacking.
Results: A facile fluorescent high-throughput screen was developed and used to identify small molecule inhibitors of PLC activity.
Conclusion: The new assay is robust and suitable for the rapid discovery of novel PLC modulators.
Significance: This new methodology eliminates the major roadblock hampering the discovery of small molecule PLC inhibitors.

Phospholipase C (PLC) isozymes are important signaling molecules, but few small molecule modulators are available to pharmacologically regulate their function. With the goal of developing a general approach for identification of novel PLC inhibitors, we developed a high-throughput assay based on the fluorogenic substrate reporter WH-15. The assay is highly sensitive and reproducible: screening a chemical library of 6280 compounds identified three novel PLC inhibitors that exhibited potent activities in two separate assay formats with purified PLC isozymes *in vitro*. Two of the three inhibitors also inhibited G protein-coupled receptor-stimulated PLC activity in intact cell systems. These results demonstrate the power of the high-throughput assay for screening large collections of small molecules to identify novel PLC modulators. Potent and selective modulators of PLCs will ultimately be useful for dissecting the roles of PLCs in cellular processes, as well as provide lead compounds for the development of drugs to treat diseases arising from aberrant phospholipase activity.

Extracellular stimuli, including hormones, growth factors, and neurotransmitters, promote activation of phospholipase C (PLC)[2] isozymes and cleavage of the membrane lipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) into the classical second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (1). These second messengers coordinately control numerous signaling cascades through the mobilization of intracellular Ca^{2+} stores and the activation of protein kinase C. Aberrant regulation of PLCs contributes to diverse human diseases such as cancer (2–4), cardiovascular diseases (5, 6), and neuropathic pain (7). Consequently, small molecule PLC inhibitors will be valuable pharmacological tools to dissect the roles of PLCs in development and disease and could potentially serve as candidates for drug development.

Few small molecule PLC inhibitors have been reported, among which U73122 is the most widely used agent (8, 9). However, U73122 has been reported to have pleiotropic effects on cellular processes. Because most experiments with U73122 monitored events secondary to activation of PLCs, *e.g.* intracellular calcium release, it seems increasingly likely that U73122 does not faithfully report direct inhibition of these isozymes. For example, it was shown that U73122 prevented calcium release by directly inhibiting various Ca^{2+} pumps (10–13). In addition, the maleimide group within U73122 makes it highly reactive such that most U73122 modifies membrane components and does not enter cells (14). Internalized U73122 reacts with and inhibits a variety of unrelated enzymes, including key enzymes regulating lipid metabolism: phosphatidylinositol-4-phosphate kinase and 5-lipoxygenase (15, 16). U73122 has also been reported to sequester the PLC substrate PtdIns(4,5)P_2 (17) and even activate the phospholipase activity of purified PLCs (18). Thus, U73122 is a singularly poor reagent to probe signaling by PLC isozymes. Similarly, small peptides previously used to inhibit PLC enzymes also suffer from indirect effects, as well as from limited bioavailability. Thus, there is overwhelming evidence that the current repertoire of small molecules used to inhibit PLCs do so indirectly and can generate effects that are mistakenly attributed to PLCs. Clearly, a substantial need exists to develop small molecules that directly and selectively modulate PLC isozymes.

Current assays of the phospholipase activity of PLCs rely upon quantification of radioactive inositol phosphates derived from the hydrolysis of radiolabeled PtdIns(4,5)P_2. These assays are not readily amenable to high-throughput screens. Although several fluorogenic reporters have been tested to monitor continuously the phospholipase activity of PLCs, they have significant drawbacks, including limited applicability, availability, and reproducibility. For example, fluorescent substrates typically used to study bacterial PLCs are expected to be poorly hydrolyzed by mammalian PLCs (19–23), which have more

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[2] The abbreviations used are: PLC, phospholipase C; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; DMSO, dimethyl sulfoxide; ATA, aurintricarboxylic acid.

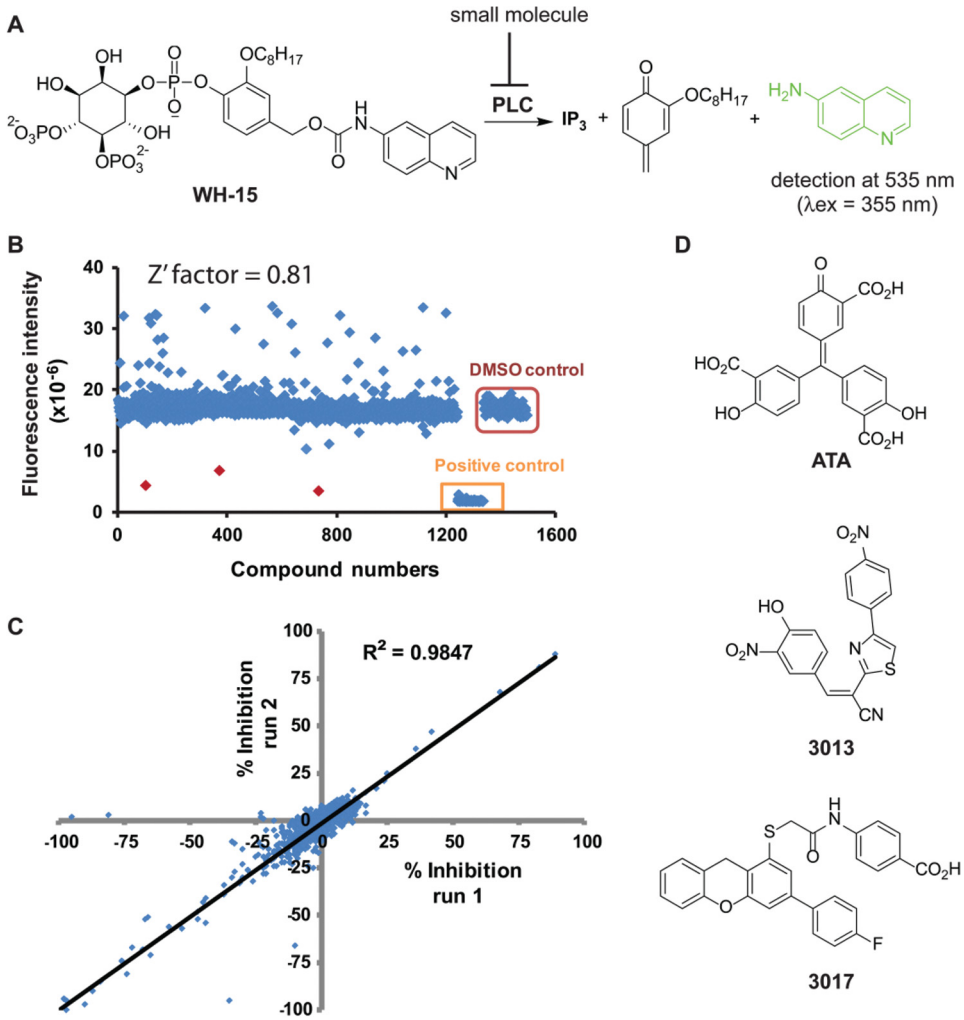


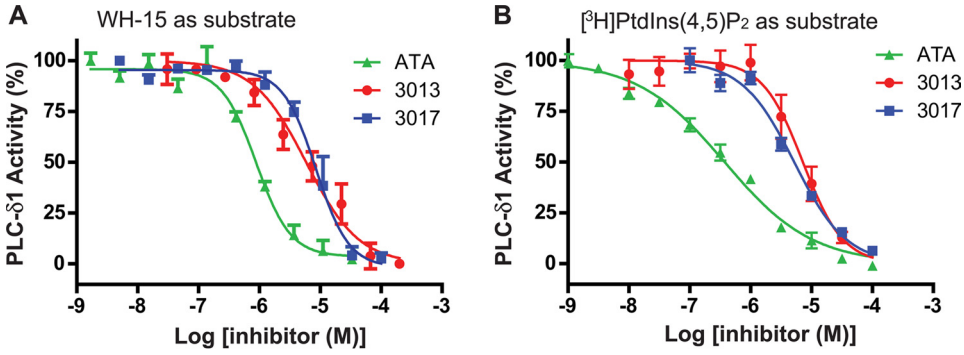
FIGURE 1. **High-throughput screen for small molecule PLC inhibitors.** *A*, fluorescence-based high-throughput assay of PLC activity. Upon PLC action, WH-15 is cleaved to inositol 1,4,5-trisphosphate (*IP*$_3$), a quinomethide derivative, and 6-aminoquinoline. When excited at 355 nm and detected at 535 nm, WH-15 is non-fluorescent, whereas 6-aminoquinoline is highly fluorescent. *B*, scatter plot of fluorescence changes after incubation of PLCδ1 and WH-15 with individual compounds of the LOPAC1280 collection. The *Z*′-factor for the screen is 0.81, with a hit rate of 0.23% for >50% inhibition relative to DMSO. *C*, screen reproducibility. The correlation coefficient for two parallel screens of the LOPAC1280 library is 0.98. *D*, chemical structures of representative hits.

stringent substrate requirements, including an absolute need for a 4′-phosphate on the inositol ring (24) that is absent from these compounds and some more recently described reporters (25). A second-generation fluorescein derivative of phosphatidylinositol 4-phosphate has been reported to be a fluorescent substrate of PLCδ1 (26); however, it is not commercially available and has not been used in subsequent reports to monitor mammalian PLC activity. Furthermore, this compound is likely to be a poor substrate for mammalian PLCs because it lacks an acyl chain shown to be necessary for efficient hydrolysis by these enzymes (27), a common flaw for most fluorescent substrates reported for mammalian PLCs. More recently, PLCδ1 was shown to efficiently hydrolyze phosphorothiolate analogues of PtdIns(4,5)P_2 (28). However, product detection requires a coupled secondary assay that would introduce unnecessary artifacts during high-throughput screens.

We recently developed WH-15, a robust fluorescent reporter useful for directly monitoring the phospholipase activity of mammalian PLCs (29). Here, we used WH-15 to develop a high-throughput PLC assay and verified its utility by identifying three new PLC inhibitors.

EXPERIMENTAL PROCEDURES

Screening of the LOPAC1280 Collection—Chemical compounds (1 mM in 1 μl of dimethyl sulfoxide (DMSO)) were added to assay buffer (19 μl) containing 50 mM HEPES (pH 7.2), 70 mM KCl, 3 mM $CaCl_2$, 3 mM EGTA, 2 mM DTT, and 0.04 mg/ml fatty acid-free BSA. The resulting stock solutions (2 μl) were then added to each well of a PerkinElmer ProxiPlate™-384 Plus F black plate that contained purified PLCδ1 (4 ng) in assay buffer (4 μl). The mixture was incubated at room temperature for 10 min, and the fluorogenic reporter WH-15 (30 μM) in assay buffer (4 μl) was added to initiate the reaction. After incubation at room temperature for 1 h, 5 μl of stop solution (0.2 M EGTA in H_2O (pH 10.2)) was added, and fluorescence was recorded on a PerkinElmer Wallac EnVision 2103 multilabel reader with an excitation wavelength of 355 nm (bandwidth



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		ATA	3013	3017
IC_{50} (μM)	fluorescence-based assay	0.53 ± 0.36	7.3 ± 2.1	8.0 ± 0.3
	radioactivity-based assay	0.67 ± 0.12	7.4 ± 1.1	5.0 ± 0.5

FIGURE 2. **Concentration-dependent inhibition of phospholipase activity of PLCδ1 by inhibitors.** *A*, plot of PLC activity in the presence of increasing concentrations of ATA, 3013, or 3017 in the fluorescence-based assays. Purified full-length PLCδ1 was incubated with inhibitors at the indicated concentrations for 15 min before WH-15 was added to initiate the enzymatic reactions. Phospholipase activity was calculated as described under "Experimental Procedures." The experiments were carried out in triplicate. *Error bars* are S.D. *B*, plot of PLC activity in the presence of increasing concentrations of ATA, 3013, or 3017 in the radioactivity-based assays. Purified full-length PLCδ1 was incubated with inhibitors at the indicated concentrations for 15 min before [^{3}H]PtdIns(4,5)P_2 was added to initiate the enzymatic reactions. Phospholipase activity was calculated as described under "Experimental Procedures." The experiments were carried out in triplicate. *Error bars* are S.D. *C*, The IC_{50} values for ATA, 3013, and 3017.

of 10 nm) and an emission wavelength of 535 nm (bandwidth of 10 nm).

Quantification of PLC Inhibition in the Fluorescence-based Assay—Similar to the procedure described above, 2 μl of small molecule inhibitors (10 mM) in DMSO were diluted with assay buffer (78 μl) to make 250 μM stock solutions, which were subsequently serially diluted at a 1:3 ratio with assay buffer containing 2.5% DMSO. Inhibitors (4 μl) at the indicated concentrations were incubated with PLCδ1 (0.5 ng) in assay buffer (2 μl) in a PerkinElmer ProxiPlate™-384 Plus F black plate at room temperature for 15 min before WH-15 (30 μM, 4 μl) was added to initiate the reaction. 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), PLCδ1 (0.5 ng), WH-15 (12 μM), 1% DMSO, HEPES (50 mM, pH 7.2), KCl (70 mM), $CaCl_2$ (3 mM), EGTA (3 mM), DTT (2 mM), cholate (0.5%), and fatty acid-free BSA (0.1 mg/ml). DMSO was used instead of inhibitors as a control. Fluorescence was recorded every 5 min as described above, and phospholipase activity was quantified as the ratio of fluorescence intensity in the presence and absence (DMSO only) of inhibitor.

Quantification of Phospholipase Activity of Purified PLC Enzymes—Detergent mixed micelles containing 50 μM PtdIns(4,5)P_2 and ~10,000 cpm of [^{3}H]PtdIns(4,5)P_2 per assay were generated by combining lipids in a borosilicate glass tube and drying under a stream of nitrogen. The dried lipids were resuspended by sonication in assay buffer, and assays were initiated by the addition of PLC dissolved in assay buffer. Activity assays were run for 10 min in 60 μl and stopped with trichloroacetic acid (200 μl) and 10 mg/ml BSA (100 μl). The reactions were centrifuged at 5000 × *g* for 10 min, and the supernatant containing soluble [^{3}H]inositol 1,4,5-trisphosphate was measured by scintillation counting.

Quantification of [^{3}H]Inositol Phosphate Accumulation in Cells—HEK293A cells were maintained in high-glucose DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere of 95% air and 5% CO_2. Forty-eight hours prior to measurements, cells were seeded at a density of ~20,000 cells/well into a 96-well culture dish coated with 0.01% poly-L-lysine. The culture medium was changed to inositol-free DMEM containing 1 μCi/well *myo*-[2-^{3}H]inositol (American Radiolabeled Chemicals) ~24 h after plating, and metabolic labeling was allowed to proceed for 12–18 h. One hour prior to measurements, the medium was changed to 20 mM HEPES-buffered Hanks' balanced salt solution with various concentrations of the PLC inhibitors. After 1 h, cells were challenged with 25 μl of a 5-fold solution of carbachol in 20 mM HEPES (pH 7.2) containing LiCl (50 mM) at 37 °C for 30 min. Incubations were terminated by aspiration of the medium and addition of ice-cold formic acid (50 mM), followed by neutralization with NH_4OH (150 mM) after cell lysis. [^{3}H]Inositol phosphates were isolated and quantified using Dowex chromatography. Experiments using 1321N1 human astrocytoma cells stably expressing the human $P2Y_6$ receptor were carried out similarly, except the cells were maintained with 5% fetal bovine serum, and uridine diphosphate (100 μM) was used as the agonist.

cAMP Accumulation and Quantification of ATP Levels—cAMP accumulation in $P2Y_6$-1321N1 cells was quantified as described previously (30). Briefly, cells in 24-well plates were preincubated with [^{3}H]adenine (1 μCi) for 2 h in serum-free DMEM. This medium was then aspirated, and various concentrations of aurintricarboxylic acid (ATA), 3013, or 3017 were added in 25 mM HEPES-buffered Hanks' balanced salt solution. After a 30-min incubation at 37 °C, a solution of 200 μM 3-isobutyl-1-methylxanthine, 10 μM forskolin, and 10 μM iso-

proterenol was added, and the incubation was continued for an additional 15 min. Incubations were terminated by aspiration of the medium and addition of 5% trichloroacetic acid. [^{3}H]cAMP was isolated by sequential Dowex and alumina chromatography and quantified by liquid scintillation counting as described previously (31). [^{3}H]ATP was isolated by Dowex chromatography and quantified by liquid scintillation counting as described previously (32).

Thermal Shift Assay—Melting temperatures were determined by monitoring binding of the dye SYPRO Orange (Invitrogen) to versions of PLCδ1 during thermal denaturation. PLCδ1 (1 μM) or PLCδ1(E341A) (1 μM) was incubated with ATA in buffer containing 100 mM HEPES (pH 7.5), 70 mM KCl, 3 mM EGTA, 10 mM $CaCl_2$, 2 mM DTT, 1% (v/v) DMSO, and 1:1000 SYPRO Orange. To determine melting temperatures in the absence of Ca^{2+}, the buffer consisted of 100 mM HEPES (pH 7.5), 70 mM KCl, 10 mM EGTA, 2 mM DTT, 1% (v/v) DMSO, and 1:1000 SYPRO Orange. All reactions were performed in triplicate in a volume of 20 μl in 384-well PCR plates (Genesee Scientific). Fluorescence was monitored with an Applied Biosystems 7900HT fast real-time PCR system, using the filter sets for NED™, as the temperature was increased from 25 to 95 °C at a ramp rate of 3%.

RESULTS AND DISCUSSION

Assay Development and Pilot Screen—The hydrolysis of WH-15 by PLC isozymes generates up to a 20-fold increase in fluorescence. Thus, we determined whether WH-15 and PLCδ1 could be used to implement a prototype assay suitable for high-throughput screens using 384-well microtiter plates (Fig. 1*A*). Several parameters were assessed, including (i) order of reagent addition; (ii) concentrations of WH-15, DMSO, and PLCδ1; (iii) reaction time; and (iv) potential stop conditions.

For instance, the capacity of PLCδ1 to hydrolyze WH-15 was unaffected by up to 5% DMSO (data not shown), indicating that residual DMSO in the final assay conditions and carried over from the compound stocks will not affect assay performance. Furthermore, the hydrolysis of 10 μM WH-15 by as little as 4 ng of wild-type PLCδ1 was sufficient to generate an ~15-fold increase in fluorescence over the course of 1 h (data not shown). In contrast, in the absence of PLCδ1 or in the presence of a catalytically inactive form (E341A) of the isozyme, no increase in fluorescence occurred over the same time. WH-15 is stable for up to 24 h at room temperature over a pH range of 7–10 (data not shown). Differences in fluorescence upon incubation of WH-15 with either wild-type or catalytically inactive PLCδ1 in multiple wells of a 384-well microtiter plate were used to calculate an initial *Z′*-factor of 0.9, indicating that this format is highly reproducible for high-throughput screening (data not shown). Because PLCδ1, PLCβ2, and PLCγ1 hydrolyze WH-15 with similar rates (29), we expect this assay format to be applicable to all PLC isozymes with only minor variations. Finally, the addition of EGTA at a final concentration of 60 mM completely terminated the phospholipase activity of PLCδ1, presumably through chelation with Ca^{2+} normally required with the catalytic site. Efficient termination of reaction conditions provides for flexibility in plate handling.

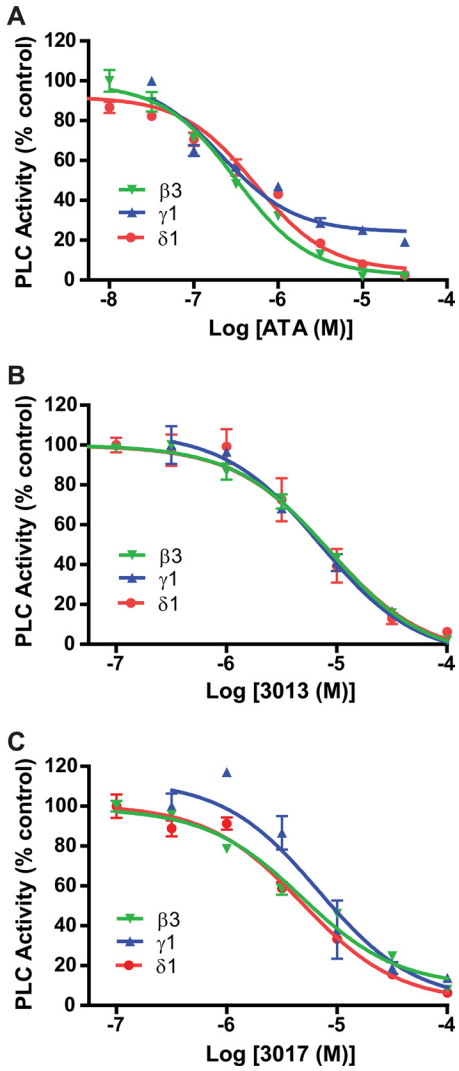


FIGURE 3. **Inhibitory effects of ATA, 3013, and 3017 on different PLC isoforms.** The phospholipase activities of purified PLCδ1, PLCγ1, and PLCβ3 in the presence of increasing concentrations of ATA (*A*), 3013 (*B*), or 3017 (*C*) were measured and plotted as described in the legend to Fig. 2.

Using the optimized assay conditions, we screened in duplicate the 1280 compounds composing the Library of Pharmacologically Active Compounds LOPAC1280 (Fig. 1*B*). Two microliters of a 50 μM stock of each compound was incubated with 4 ng of PLC (4 μl) for 10 min prior to incubation with 10 μM WH-15 in a final volume of 10 μl. The correlation coefficient for the parallel runs was 0.98, with an average *Z′*-factor of 0.81 for all plates (DMSO *versus* EGTA) (Fig. 1*C*). The hit rate was 0.23% for compounds (10 μM) exhibiting >50% inhibition relative to identical reactions containing DMSO alone. Although this format exhibits excellent characteristics for a high-throughput screen, we noted identification of a disproportionate number of putative activators relative to inhibitors of PLCδ1 (Fig. 1*B*). The majority of these putative activators most likely represent false hits due to high intrinsic fluorescence. This contention was confirmed by incorporating a secondary counterscreen using catalytically inactive PLCδ1. Overall, the high signal-to-noise ratio and reproducibility of the assay should ensure the successful identification of both activators and inhibitors of PLCs.

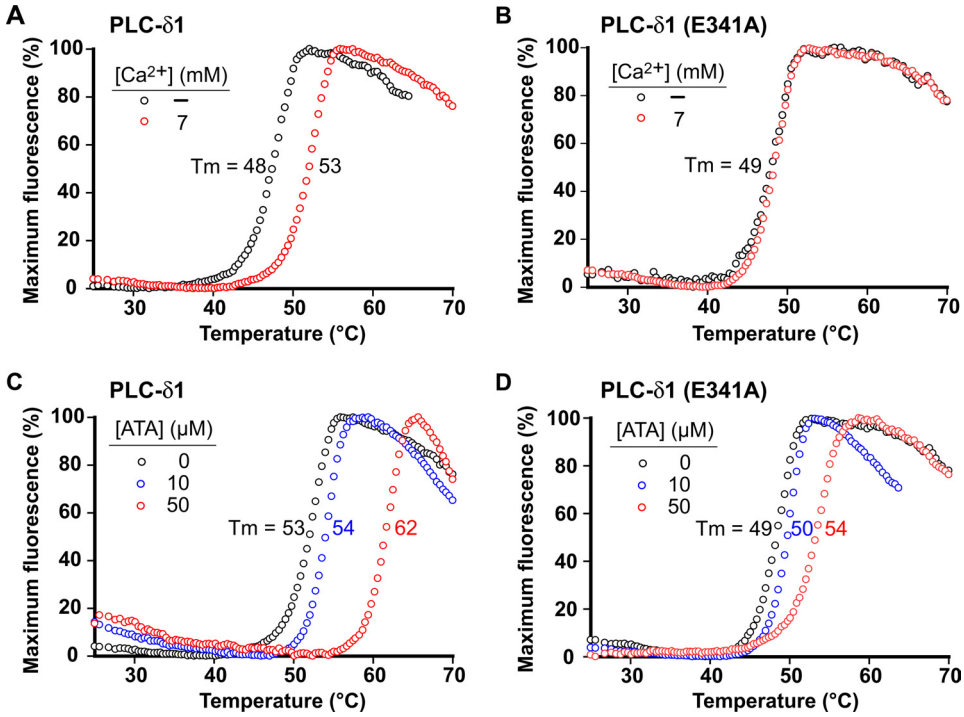


FIGURE 4. **PLCδ1 is stabilized by ATA in thermal shift assay.** Shown are plots of the fluorescence changes of PLCδ1 (*A*) and PLCδ1(E341A) (*B*) as the temperature was increased in the presence or absence of Ca^{2+} (7 mM). With the concentration of Ca^{2+} at 7 mM, the fluorescence changes of PLCδ1 (*C*) and PLCδ1(E341A) (*D*) as the temperature was increased in the presence of increasing concentrations of ATA were then recorded. The mean T_m was calculated from a single experiment in which each condition was assayed in triplicate as described under "Experimental Procedures."

As an interesting aside, U73122, which is included in LOPAC1280 and is widely accepted as an inhibitor of PLCs, was not flagged as an inhibitor of PLCδ1 in this screen. This result is consistent with literature reports showing that U73122 does not inhibit a panel of PLCs, including PLCδ1 (18). The success of the pilot screen prompted us also to screen an in-house collection of 5000 compounds with diverse scaffolds. The three most promising hit compounds from both screens (Fig. 1*D*) inhibited >80% of the phospholipase activity of PLCδ1 at 10 μM and were further assessed for inhibitory potential in additional complementary assays.

Inhibitory Profiles of Hit Compounds with Purified Enzymes—An unknown fraction of the hits identified from high-throughput screens are expected to consist of nonspecific modulators. For instance, a ubiquitous class of "promiscuous" inhibitors tends to self-aggregate, leading to nonspecific adsorption of proteins and concomitant inhibition (33–35). The addition of low amounts (0.01–0.1%, w/v) of various detergents tends to disrupt these aggregates and prevent nonspecific inhibition (36). Unfortunately, the addition of detergents also tends to increase artifacts in high-throughput screens due to increased difficulties in reproducible liquid handling, such as bubbles and varying menisci arising from lowered surface tension. In an effort to minimize potential difficulties in handling the low volumes required for 384-well plates, the high-throughput assays described above were designed without detergents.

We thus retested the three identified hits (ATA, 3013, and 3017) to generate concentration effect curves for each molecule in the presence of 0.5% cholate. This concentration of cholate is traditionally used to solubilize PtdIns(4,5)P_2 in detergent mixed micelles for presentation to PLCs and does not diminish phospholipase activity relative to the presentation of PtdIns(4,5)P_2 in lipid vesicles (37). Accordingly, the inhibitors at various concentrations were incubated with PLCδ1 in the presence of 0.5% cholate at room temperature for 15 min before WH-15 was added to initiate the reactions. A plot of the percentage modulation of activity as a function of compound concentration was used to calculate the potency of each inhibitor (Fig. 2*A*). The half-maximal inhibitory concentrations (IC_{50}) for ATA, 3013, and 3017 were 0.53 ± 0.36, 7.3 ± 2.1, and 8.0 ± 0.3 μM, respectively (Fig. 2*C*).

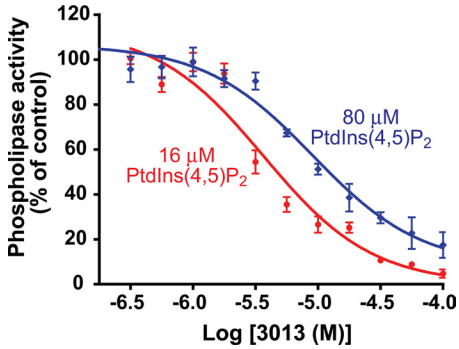
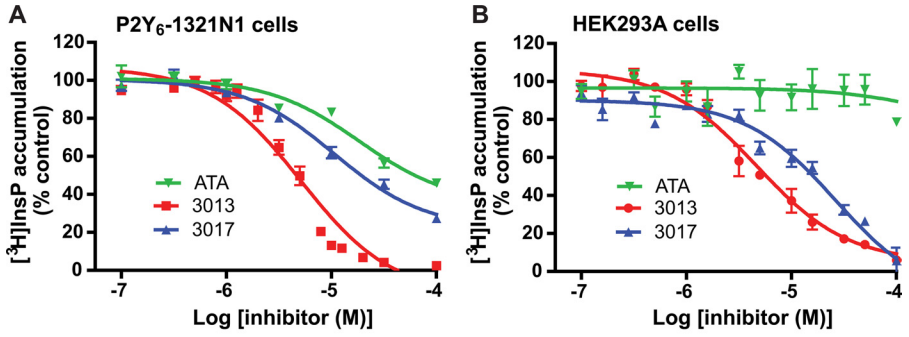


FIGURE 5. **Concentration-dependent inhibition of PLCδ1 by 3013 at two different concentrations of PtdIns(4,5)P_2.** Purified PLCδ1 was incubated with the indicated concentrations of 3013 in the presence of two different concentrations (16 and 80 μM) of the PtdIns(4,5)P_2 substrate. Phospholipase activity was calculated as described under "Experimental Procedures."

The above analyses monitored fluorescence change derived from hydrolysis of WH-15 by PLCs. However, compounds that either are intrinsically fluorescent or affect the fluorescent properties of WH-15 will likely produce confounding results. To prevent mistaken interpretations based on monitoring the



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		ATA	3013	3017
IC_{50} (μM)	$P2Y_6$-1321N1 cells	19 ± 4	5.6 ± 0.6	12 ± 2.5
	HEK293A cells	not determined	7.1 ± 1.8	30 ± 13

FIGURE 6. **Concentration-dependent inhibition of receptor-promoted inositol lipid signaling by ATA, 3013, or 3017.** *A*, $P2Y_6$-1321N1 cells metabolically prelabeled with [^{3}H]inositol were incubated with increasing concentrations of ATA, 3013, or 3017 for 1 h before stimulation with UDP (100 μM). The cells were lysed, and the radioactivity of the lysate was quantified as described under "Experimental Procedures." The experiment was repeated three times. *B*, as described for *A*, HEK293A cells were metabolically prelabeled with [^{3}H]inositol and treated with increasing concentrations of the inhibitors before incubation with carbachol (10 μM) and quantification of radioactivity. *C*, the IC_{50} values for ATA, 3013, and 3017.

fluorescence of WH-15, we also measured the effects of hit compounds on the enzymatic activity of purified PLC proteins using [^{3}H]PtdIns(4,5)P_2 solubilized in cholate as described in the literature (Fig. 2*B*). In this traditional assay, the IC_{50} values for ATA, 3013, and 3017 were 0.67 ± 0.12, 7.4 ± 1.1, and 5.0 ± 0.5 μM, respectively (Fig. 2*C*). Although the slopes of the IC_{50} plots are slightly different (possibly due to different forms of presentation of WH-15 and PtdIns(4,5)P_2 in mixed micelles), these values are essentially the same as those obtained from the fluorescence-based assay, indicating that ATA, 3013, and 3017 are direct inhibitors of PLC isozymes.

Next, we tested whether the inhibitors are selective among PLC isoforms. Under initial velocity conditions, the concentration-dependent inhibition of the phospholipase activity of PLCδ1, PLCγ1, and PLCβ3 by ATA (Fig. 3*A*), 3013 (Fig. 3*B*), or 3017 (Fig. 3*C*) was measured. Each of the three inhibitors exhibited similar effects on the different PLC isoforms, suggesting that they are general inhibitors of PLCs. These results also are consistent with the idea (but do not prove) that the inhibitors bind to the active site because PLC isozymes share a conserved binding pocket and mechanism of catalysis.

A fluorescence-based thermal shift assay was applied to confirm that the inhibitors directly bind to PLCδ1. Complex formation of a small molecule with PLCδ1 should stabilize the native state of the protein, leading to enhanced thermal stability. In the active site of PLCs, Ca^{2+} binds to both the enzyme and the substrate to facilitate catalysis. Consequently, we used Ca^{2+} as a positive control for the assay. Indeed, the melting temperature (T_m) (27) of wild-type PLCδ1 was 5 °C higher in the presence of a high concentration of Ca^{2+} than when Ca^{2+} was depleted (Fig. 4*A*). In contrast, the T_m of PLCδ1(E341A), a mutant form that is unable to properly ligate Ca^{2+} in its active site due to a single mutation, did not change irrespective of the Ca^{2+} concentrations (Fig. 4*B*). Increasing concentrations of ATA were added, and T_m values were quantified similarly. In the presence of 50 μM ATA, the T_m of wild-type PLCδ1 was increased by ~9 °C (Fig. 4*C*), whereas that of PLCδ1(E341A) was increased by only ~5 °C (Fig. 4*D*). These results suggest that ATA directly binds within the active site of PLCδ1. The other two hits, 3013 and 3017, were not sufficiently soluble under the required solution conditions for the assay.

Finally, concentration-dependent inhibition of phospholipase activity of PLCδ1 by 3013 was quantified in experiments in which different concentrations of PtdIns(4,5)P_2 were used (Fig. 5). The IC_{50} values of 3013 were 3.7 and 9.1 μM in the presence of PtdIns(4,5)P_2 at 16 and 80 μM, respectively. These data also are consistent with the idea that 3013 binds in the lipase active site and inhibits activity at least in part by a competitive mechanism.

Inhibitory Potential of Hit Compounds in Cellular Assays—One critical feature of a useful chemical probe is its cell permeability and interaction with its molecular target in intact cells. Consequently, we also assessed the three inhibitors in two cell-based assays. First, we investigated whether cellular PLCs were inhibited by ATA, 3013, or 3017 using previously published protocols to measure phospholipase activity (38, 39). Briefly, 1321N1 human astrocytoma cells stably expressing the UDP-activated $P2Y_6$ receptor were metabolically prelabeled with *myo*-[2-^{3}H]inositol, and UDP-stimulated [^{3}H]inositol phosphate accumulation was quantified in the presence of various concentrations of ATA, 3013, or 3017 (Fig. 6*A*). Similarly, carbachol-stimulated [^{3}H]inositol phosphate accumulation was quantified in HEK293A cells in the presence of various concentrations of inhibitors (Fig. 6*B*). Both 3013 and 3017 caused concentration-dependent inhibition of agonist-stimulated responses in the 1321N1 and HEK293A cell test systems. The potency of ATA in these assays was lower than that of 3013 or 3017, likely due to low cell permeability.

To rule out the possibility that these molecules cause a non-specific loss of cell viability, their effect on ATP levels was determined. Thus, $P2Y_6$-1321N1 cells were preincubated with [^{3}H]adenine for 2 h, and these metabolically labeled cells were then incubated with 100 μM ATA, 100 μM 3013, or 100 μM 3017

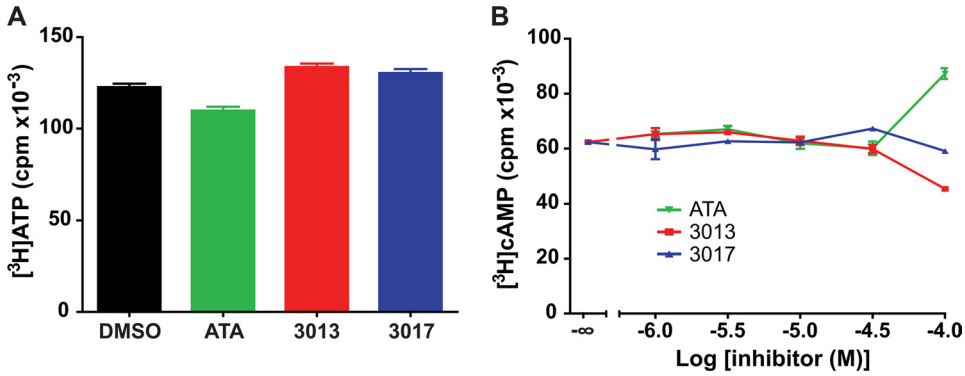

FIGURE 7. **ATA, 3013, and 3017 do not change cellular levels of basal ATP or (forskolin + isoproterenol)-stimulated cAMP levels.** *A*, ATP levels in the presence of ATA, 3013, and 3017. ATP pools of $P2Y_6$-1321N1 cells were metabolically labeled by preincubation with [^{3}H]adenine for 3 h. ATA (100 μM), 3013 (100 μM), or 3017 (100 μM) was then added, the incubation was terminated after 30 min, and [^{3}H]ATP levels were quantified as described under "Experimental Procedures." The results are means ± S.E. and are representative of results obtained in two independent experiments. *B*, (forskolin + isoproterenol)-stimulated cAMP accumulation in the presence of ATA, 3013, or 3017. [^{3}H]Adenine-prelabeled $P2Y_6$-1321N1 cells were incubated with the indicated concentrations of ATA, 3013, or 3017 for 30 min prior to a 10-min challenge with 10 μM forskolin and 10 μM isoproterenol. Accumulation of [^{3}H]cAMP was quantified as described under "Experimental Procedures." The results are means ± S.E. and are representative of results obtained in two independent experiments. Basal cpm accumulation (data not shown) was ~400 cpm for each condition and was not changed by the addition of ATA, 3013, or 3017 at concentrations up to 100 μM.

for an additional 30 min. [^{3}H]ATP levels were quantified as described under "Experimental Procedures," and as illustrated in Fig. 7*A*, no effect of ATA, 3013, or 3017 on ATP concentration was observed. We also examined whether these phospholipase inhibitors exhibited inhibitory effects on another G protein-regulated effector enzyme. Preincubation of $P2Y_6$-1321N1 cells with a broad range of concentrations of ATA, 3013, or 3017 had no effect on adenylyl cyclase-dependent cAMP accumulation in the presence of forskolin plus the β-adrenergic receptor agonist isoproterenol (Fig. 7*B*). We conclude from these studies that the inhibitory effects of ATA, 3013, and 3017 on receptor-stimulated inositol lipid signaling are not due to nonspecific effects that diminish cell viability or generally interfere with G protein-mediated signaling cascades.

In summary, we have developed a robust fluorogenic high-throughput assay of PLC activity and used it to screen a collection of 6280 compounds. Three new PLC inhibitors were identified and verified in assays with purified enzymes or in cells. Given the small set of compounds that have been screened and the preliminary nature of the studies on the hit compounds, the current three inhibitors might not be optimal to inhibit cellular PLC activity due to poor cell permeability, insufficient potency, or unaccounted nonspecific effects. Indeed, ATA has been shown to inhibit platelet aggregation (40), modulate glucocorticoid receptor-mediated signaling (41), and promote survival and regeneration of retinal ganglion cells (42). In addition, ATA inhibits the activity of multiple enzymes such as DNA topoisomerase II (43), the cytosine deaminase APOBEC3G (44), and the kinase c-Met (45). Thus, it is unlikely that ATA could serve as an effective chemical probe to study PLC-regulated cellular processes. Although there are no reports of the biological activity of 3013 and 3017 in the literature, their moderate potency and solubility should be reasons for caution when using these two inhibitors to modulate the phospholipase activity of PLCs. Despite these limitations, this work provides a complete set of screening protocols that are suitable for high-throughput screening to identify PLC-selective modulators. These modulators would then be exceptionally useful for dissecting signaling cascades controlled by PLCs. Abnormal signaling through PLC isozymes is implicated in a multitude of diseases, including numerous cancers (2, 3, 46–51), atherosclerosis and cardiac failure (5, 52), and schizophrenia and epilepsy (7, 53). Thus, this work also has the potential to provide new and exciting avenues to understand and treat human diseases.

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