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5-Stabilized Phosphatidylinositol 3,4,5-Trisphosphate Analogues Bind Grp1 PH, Inhibit Phosphoinositide Phosphatases, and Block Neutrophil Migration

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Abstract

Metabolically-stabilized analogues of PtdIns(3,4,5)P_3 have shown long-lived agonist activity for cellular events and selective inhibition of lipid phosphatase activity. We describe an efficient asymmetric synthesis of two 5-phosphatase resistant analogues of PtdIns(3,4,5)P_3, the 5-methylene phosphonate (MP) and 5-phosphorothioate (PT). Furthermore, we illustrate the biochemical and biological activities of a total of five stabilized PtdIns(3,4,5)P_3 analogues in four contexts. First, the relative binding affinities of the 3-MP, 3-PT, 5-MP, 5-PT, and 3,4,5-PT_3 analogues to the Grp1 PH domain are shown, as determined by NMR. Second, the enzymology of the five analogues is explored, showing the relative efficiency of inhibition of SHIP1, SHIP2, and PTEN, as well as the greatly reduced ability of these phosphatases to process these analogues as substrates as compared to PtdIns(3,4,5)P_3. Third, exogenously-delivered analogues severely impair complement factor C5a-mediated polarization and migration of murine neutrophils. Finally, the new analogues show long-lived agonist activity in mimicking insulin action in sodium transport in A6 cells.

Introduction

The phosphoinositide 3-kinase (PI 3-K) signaling pathway contains important therapeutic targets in human pathophysiology (1,2). Phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3) is a ubiquitous signaling lipid found in higher eukaryotic cells (3) and activates a plethora of downstream cellular processes (4). These signaling events include cell proliferation and transformation (5), cell shape and motility (6), and insulin action and alteration of glucose transport (7). PtdIns(3,4,5)P_3-regulated signaling can be modulated by the lipid 3-phosphatase PTEN (8) and SH2 domain-containing inositol 5-phosphatases

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Supporting Information Available: Complete experimental details for synthesis and characterization of new compounds with chemical structures; inhibition of PTEN by metabolically stabilized analogues; Akt activation by analogues. This material is available free of charge via the internet.

SHIP1 and SHIP2 (9). Indeed, PTEN and SHIP1/2 are crucial regulators of PI 3-kinase signaling in T-lymphocytes (10), drug discovery targets in type 2 diabetes (11) and intimately involved in signaling networks for a myriad of other disease states (12).

A metabolically-stabilized (ms) analogue of PtdIns(3,4,5)P_3 that resists lipid 3- and 5-phosphatases would have numerous applications in understanding the role of PtdIns(3,4,5)P_3 in cell physiology. The ms-PtdIns(3,4,5)P_3 analogues could separate the activation of signal transduction from the degradation of the signal by phosphatase action in cells. This chemical biology approach to the dissection of the PI 3-K pathway would be complementary to the use of siRNA knockdowns or genetic knockouts for PTEN and SHIP.

We recently described the preparation and activity of the 3-phosphatase-resistant, metabolically-stabilized (ms) analogues **1** and **2** (Figure 1) of PtdIns(3,4,5)P_3 that were both stable to degradation by PTEN and acted as inhibitors of PTEN (13). One of these analogues incorporated a single phosphorothioate (PT) substituent, and an additional study examined the chemistry and biological activity of the 3,4,5-trisphosphorothioate analogue **3** of PtdIns(3,4,5)P_3 (14). Phosphorothioates are important phosphomimetics that show greatly reduced rates of enzyme-mediated hydrolysis (15). However, the replacement of P=O by P=S also affects the pKa of the phosphate and removes a H-bond acceptor (16,17). Indeed, we demonstrated that PtdIns(3)PT had reduced binding activity for cognate PtdIns(3)P-selective FYVE and PX domain binding proteins, which was attributable to reduced H-bonding (18). We also observed that a PT analogue of PtdIns(5)P was a long-lived agonist for chromatin remodeling (19), and a tris(PT) cyclopentyl analogue (20) of Ins(1,4,5)P_3 was a long-lived agonist activities for the IP_3 receptor. We hypothesized that a 5-PT and 5-MP analogues of PtdIns(3,4,5)P_3 could be either antagonists or long-lived PtdIns(3,4,5)P_3 agonists, as they would be more slowly dephosphorylated by the 5-phosphatase SHIP and could potentially block the normal receptor-mediated signaling involving PtdIns(3,4,5)P_3. We also wished to test whether the 5-PT and 5-MP modifications could alter processing of PtdIns(3,4,5)P_3 by the 3-phosphatase PTEN.

Herein, we report the total asymmetric synthesis and characterization of the two 5-stabilized PtdIns(3,4,5)P_3 analogues **4** and **5** (Figure 1). First, we investigated the relative binding affinities of the 3-, 5-, and 3,4,5-stabilized PtdIns(3,4,5)P_3 analogues to Grp1 PH domain using NMR titration methods. Second, we examined the relative efficiency of inhibition of SHIP1, SHIP2, and PTEN by these analogues, often with unexpected results. The reduced processing of the analogues as substrates, in an enzyme and modification-selective fashion, was also studied. Third, we explored the effects of the exogenously-delivered the 3-, 5-, and 3,4,5-stabilized PtdIns(3,4,5)P_3 analogues on complement factor C5a-mediated polarization and migration of wild-type murine neutrophils. Finally, the long-lived agonist activities of the 5-PT (**4**) and 5-MP (**5**) analogues in mimicking insulin action in sodium transport in A6 cells were compared to previous data for the 3-PT (**1**), 3-MP (**2**), and 3,4,5-PT_3 (**3**) analogues (13,14).

Results and Discussion

Chemistry and Synthesis

The PTEN resistant analogues, 3-PT- PtdIns(3,4,5)P_3 (**1**) and 3-MP-PtdIns(3,4,5)P_3 (**2**),(13) and 3,4,5-PT- PtdIns(3,4,5)P_3 (**3**) (14) were synthesized previously. The synthesis of 5-ms-PtdIns(3,4,5)P_3 required selective protection of the 5-OH and the use of enantiomerically pure *myo*-inositol derivatives. A number of strategies have been developed for the synthesis of optical pure phosphoinositides, either using the enantiopure natural precursors, such as D-glucose (21) or quinic acid (22). Kinetic resolution or desymmetrization via enantioselective enzymatic acylation, nonenzymatic phosphorylation of *myo*-inositol derivatives (23–25),

and the separation of diastereomeric derivatives of *myo*-inositol with chiral auxiliaries are also commonly used (13,26–28). The syntheses of the 5-PT and 5-MP PtdIns(3,4,5)P_3 analogues are illustrated in Figure 2; complete step-by-step synthetic details and structures may be found in the Supporting Information. As with our previous syntheses of stabilized PtdIns(3,4,5)P_3 analogues (13,14), we first prepared the 3,4,5-unprotected inositol using methods developed by Bruzik (27,28): (a) acetalization of inositol with D-camphor dimethyl acetal, (b) selective silylation of 1-OH (c) removal of the camphor ketal, (d) selective benzoylation of 3,4,5-OH, (e) complete protection of the hydroxyl groups; (f) and debenzoylation. Next, the appropriately protected 5-PT intermediate **10** bearing a free 1-hydroxyl group for introduction of the diacylglyceryl-bearing phosphodiester was prepared through 14 steps after TIPDS-bis-silylation, benzoylation, desilylation, and phosphorylation as depicted in Figure 2 (see Supporting Information for complete experimental details). Thus, the appropriately protected 5-MP intermediate **15** equipped with a free 1-hydroxyl group was synthesized from *myo*-inositol through eleven steps (Figure 2; see Supporting Information for complete experimental details) in close analogy to the synthesis (13) of 3-MP-PtdIns(3,4,5)P_3 (**2**).

The phosphoroamidates **11** were prepared from 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite with diacylglycerols bearing dibutanoyl, dioctanoyl, or dihexadecanoyl chains (13,18), and coupled with 5-PT intermediate **10** or with 5-MP intermediate **15**, in the presence of 1*H*-tetrazole. Phosphodiesters were produced by *t*-BuOOH oxidation, and no overoxidation of the 5-PT group was observed. The cyanoethyl groups were removed using triethylamine plus bis(trimethylsilyl)trifluoroacetamide (BSTFA) in anhydrous acetonitrile (13,14). Addition of BSTFA prevents the phosphorothioate anion from undergoing re-alkylation. Protocols (see Supporting Information) analogous to those for the 3-MP and 3-PT-PtdIns(3,4,5)P_3 analogue syntheses (13) provided the desired analogues 5-PT-PtdIns(3,4,5)P_3 (**4**) and 5-MP-PtdIns(3,4,5)P_3 (**5**) (13,14) in the acid form. The final products **4** and **5** were converted to the sodium salts by ion exchange chromatography (Dowex 50@ X 8 200 Na^+ exchange resin) and stored lyophilized at −80 °C prior to use in biological experiments.

High-Affinity Binding to Grp1 PH

In mammalian cells, PtdIns(3,4,5)P_3 is transiently accumulated in plasma membranes following activation of PI 3-kinase (29). PtdIns(3,4,5)P_3 is specifically recognized by the pleckstrin homology (PH) domain-containing proteins such as general receptor for phosphoinositides 1 (GRP1) (30,31). To test the biological activity of the PtdIns(3,4,5)P_3 analogues, we investigated their interactions with GRP1 PH domain by NMR spectroscopy (Figure 3 and Supporting Figure 1). The GRP1 PH domain recognizes all five PtdIns(3,4,5)P_3 analogues. In Figure 3, the chemical shift changes observed in ^1H-^{15}N heteronuclear single quantum coherence (HSQC) spectra of the ^{15}N-labeled GRP1 PH domain indicate that the binding 3-PT-PtdIns(3,4,5)P_3 (**1**) and 5-PT-PtdIns(3,4,5)P_3 (**4**) is nearly equivalent to the binding of unmodified PtdIns(3,4,5)P_3. Supporting Figure 1 shows an overlay of the ^1H-^{15}N HSQC spectra of GRP1 PH collected as three dibutanoyl lipids were added (unmodified PtdIns(3,4,5)P_3, 3-PT-PtdIns(3,4,5)P_3 (**1**), and 3,4,5-PT_3-PtdIns(3,4,5)P_3 (**3**)). The slow exchange on the NMR time scale for all three experiments suggests a robust lipid-protein interaction. Taken together, these NMR data indicate that the binding affinity decreases in the order PtdIns(3,4,5)P_3 ≥ 3-PT-PtdIns(3,4,5)P_3 (**1**)≈ 5-PT-PtdIns(3,4,5)P_3 (**4**) > 3-MP-PtdIns(3,4,5)P_3 (**2**) ≈ 5-MP-PtdIns(3,4,5)P_3 (**5**) > 3,4,5-PT_3-PtdIns(3,4,5)P_3 (**3**). It is important to note that while the phosphorothioate is considered isosteric with the phosphate, the methylene phosphonate is a methylene-extended analogue of the phosphate, rather than an isostere. Thus, these synthetic analogues can effectively

substitute for PtdIns(3,4,5)P_3 in biochemical and biological assays while retaining affinity for PtdIns(3,4,5)P_3 binding sites.

Stabilized Analogues are Poor Substrates for SHIP2 and PTEN

Incubation of 100 μM of diC_8-5-MP (**5**), diC_8-5-PT (**4**), or diC_8-3,4,5-PT_3 (**3**) with SHIP2 for 10 min resulted in no phosphate release, confirming the metabolic stability of these 5-stabilized analogues to the 5-phosphatase activity (Figure 4). In contrast, both the 3-MP (**2**) and 3-PT (**1**) substrates were at least partially dephosphorylated by SHIP2, showing that the 5-phosphatase activity was capable of removing a non-stabilized phosphomonoester in the analogues containing a 3-stabilized group.

In contrast, none of the five analogues were processed by PTEN during the 10 min incubation period (Figure 5). The absence of phosphate release confirms the metabolic stability of these analogues to the 3-phosphatase activity of PTEN, but is somewhat unexpected. One would have expected that 5-stabilization should not completely abrogate 3-phosphate removal from a non-stabilized position. An alternative explanation is that the chemical modifications in 5-position of the 5-ms analogues are not well tolerated in the enzyme active site, and simply result in poor enzyme-substrate binding rather than binding with reduced turnover.

Competitive Inhibition of SHIP1, SHIP2 and PTEN

We chose to work with Ins(1,3,4,5)P_4 as substrate in order to use relatively low concentrations of substrate, taking advantage of the available tritium labeled tracer and a well characterized assay (32)(see Methods). The alternative malachite green phosphate release assay used to examine substrate ability of the analogues required much higher substrate concentrations (50–100 μM).

The new 5-stabilized PtdIns(3,4,5)P_3 analogues as well as the previously described 3-stabilized PtdIns(3,4,5)P_3 analogues (13) and 3,4,5-tris(phosphorothioate) analogue (14) of PtdIns(3,4,5)P_3 were examined as potential inhibitors of the 5-phosphatase and 3-phosphatase activities. Figure 6A summarizes the comparative inhibition of the 5-phosphatase activity of recombinant SHIP1, using 0.3 μM tritium-labeled Ins(1,3,4,5)P_4 as the substrate and each of the diC_8 analogues at 10 μM. DiC_8-PtdIns(3,4,5)P_3 itself did not show any competitive inhibition of SHIP1 activity under these conditions, but each of the analogues significantly reduced SHIP1 activity. Interestingly, the most potent analogues in this assay were diC_8-5-MP (**5**), diC_8-3-PT (**1**) and diC_8-3,4,5-PT_3 (**3**). In contrast to the situation with SHIP1, little or no inhibition of SHIP2 activity was observed for any of the analogues under the same incubation conditions (Figure 6B). This most likely reflects the difference in affinity of the substrate Ins(1,3,4,5)P_4 for SHIP1 and SHIP2 (32).

Evaluation of the five stabilized analogues for inhibition of PTEN 3-phosphatase activity is shown in Supporting Figure 2, for which 0.3 μM tritium-labeled Ins(1,3,4,5)P_4 as the substrate and each of the diC_8 analogues was employed at 10 μM. Only the 3,4,5-PT_3 analogue (**3**) appeared to inhibit PTEN 3-phosphatase activity with this substrate.

Neutrophil Polarity and Motility

Chemotaxis is a crucially important cellular response implicated in physiological activities (33–36). Chemotatic stimulation of cells results in a complex sequence of events: increased actin organization, cell-shape changes and the polarity developing (37,38). The selective translocation of PtdIns(3,4,5)P_3 to the plasma membrane at the leading edge leads to the cytoskeleton reorganization and pseudopod formation (39), so the localized accumulation of PtdIns(3,4,5)P_3 was deemed to be the key event directing the recruitment and activation of

signaling components required for cell polarization and chemotaxis (40,41). The amoeba *Dictyostelium discoideum* and mammalian neutrophils are commonly used as representative chemotactic cells. In *D. discoideum,* PI(3)Ks and PTEN control cell polarization and chemotaxis by regulating spatially localized PtdIns(3,4,5)P_3 accumulation (42,43). In neutrophils, both PI3Kγ and SHIP1 play critical roles in polarization and motility (36,44); SHIP1 restricts the localization of PtdIns(3,4,5)P_3 accumulation, and thereby governs the cell polarization required for proper motility and chemotaxis (36). Thus, we examined the effects of metabolically-stablized PtdIns(3,4,5)P_3 analogues in wild-type neutrophils using C5a as chemoattractant, which could in principle occur by a possible interaction with SHIP1 or with Akt or both. As shown in Figure 7, the polarization of wild-type neutrophils was greatly impaired by exogenous addition of each of the five PtdIns(3,4,5)P_3 analogues. The 3- and 5-ms-PtdIns(3,4,5)P_3 analogues may bind the SHIP1 as an inhibitor or antagonist, thus the ability of SHIP1 in governing the PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 accumulation at the leading edge of chemotaxing neutrophils is decreased, the formation of the pseudopod is retarded.

To determine if the metabolically stabilized analogues might be acting at the level of Akt activation, mouse peritoneal neutrophils were purified, stimulated and lysed as described (36). Lysates were separated by 10% SDS-PAGE and immunoblotted with phospho-specific antibodies against Akt pS473 and pan-Akt. As shown in Supporting Figure 3, none of the five analogues activated Akt phosphorylation and the two analogues tested, 3-PT and 3,4,5-PT3, failed to inhibit C5a-stimulated Akt phosphorylation.

We had hypothesized that the 5-stabilized analogs would be able to prevent the physiological production of PtdIns(3,4)P_2 that is, at least in part, produced by the action of SHIP1 on PtdIns(3,4,5)P_3. In other words, by interfering with SHIP1 activity, we had envisaged that the exogenous addition of an analogue such as diC_8-3,4,5-PT_3-PtdIns(3,4,5)P_3 could qualitatively phenocopy the effect of the absence of SHIP1 in altering neutrophil polarization (36) (see Figure 7). In the end, however, this desired result was not achieved.

Sodium Transport

To test the function of these analogues, we used A6 cell monolayers, a renal epithelium model that expresses epithelial sodium channels (ENaC), in which carrier-mediated intracellular delivery (45) of PtdIns(3,4,5)P_3 activates sodium transport (46). ENaC activity is the rate-limiting step of the sodium transport and is stimulated by insulin (47). DiC_{16}-PtdIns(3,4,5)P_3 is an early mediator of the insulin-stimulated sodium transport in A6 cells. Thus, we compared the effect of the unmodified diC_{16}-PtdIns(3,4,5)P_3 with diC_{16}-5-PT-PtdIns(3,4,5)P_3 **4** and diC_{16}-5-MP-PtdIns(3,4,5)P_3 **5** on sodium transport across confluent monolayers of A6 cells. As shown in Supporting Figure 4 apical addition of analogues **4** and **5** increased sodium transport. The 5-ms analogue **4** and **5** also mimicked the activity unstabilized PtdIns(3,4,5)P_3. It is worth noting that there was no significant variation of the resistance of the monolayers of A6 cells upon addition of the PtdIns(3,4,5)P_3 analogs tested in this study up to 60 min. Moreover, addition of amiloride to the apical bathing medium completely inhibited the current stimulated by either insulin or PtdIns(3,4,5)P_3 (46). We propose that these analogues may have other applications as pharmacological tools to probe role of PtdIns(3,4,5)P_3 in a cellular context.

Conclusions

Two 5-phosphatase resistant analogues of PtdIns(3,4,5)P_3 were synthesized and characterized in biochemical and biological contexts. First, the modification of a single phosphate of PtdIns(3,4,5)P_3 resulted in retention of binding to the PtdIns(3,4,5)P_3-specific

GRP1 PH domain, with varying relative affinities. Second, modest inhibition of the action of SHIP1 phosphatase activity on Ins(1,3,4,5)P_4 was evident, and less marked inhibition of SHIP2 or PTEN dephosphorylation of Ins(1,3,4,5)P_4 by any of the analogues was observed. Third, while none of the five phosphatase-resistant analogues released phosphate when incubated with the 3-phosphatase PTEN, the 3-stabilized analogues **1** and **2** were partially dephosphorylated by the 5-phosphatase activity. Fourth, each of the analogues severely impaired complement factor C5a-mediated polarization and migration of murine neutrophils, with 3-MP (**2**) showing the greatest effect on polarization and 3,4,5-PT_3 (**3**) showing the greatest increase in eccentricity. Finally, the new 5-stabilized analogues **4** and **5**, similar to the previously tested analogues **1**, **2**, and **3**, both activated sodium transport in A6 cells.

We had originally envisaged the utility of these new analogues as phosphatase-resistant mimics of PtdIns(3,4,5)P_3 to selectively manipulate cell responses. Unfortunately, the complexity of differential interactions of the analogues with different PtdIns(3,4,5)P_3 targets, e.g., Akt, GRP1, PDK1, among others, has rendered this expectation unrealistic in a cellular context.

Experimental Section

Chemical Syntheses

The synthesis of the 3-metabolically stabilized PtdIns(3,4,5)P_3 analogues **1** and **2**, and 3,4,5-PT-PtdIns(3,4,5)P_3 **3** have been previously published. The 5-stabilized PtdIns(3,4,5)P_3 analogues **4** and **5** were synthesized in a similar way, and full details for their preparation and characterization can be found in the supporting information.

Protein Expression and Purification

The DNA fragment encoding residues 261–385 of the PH domain of human GRP1 was cloned in pRSET A vector (Invitrogen). The ^{15}N-labeled protein was expressed in *E. coli* Rosetta in minimal media supplemented with $^{15}NH_4Cl$ (Cambridge Isotope). Bacteria were harvested by centrifugation after induction with IPTG (0.1mM) and lysed by French Press. The 6x His fusion protein was purified on a Talon-resin column (Clontech Laboratories, Inc.). The His tag was cleaved with EKMax (Invitrogen). The protein was further purified by ion exchange chromatography on HiTrap SP HP column (Amersham) in Bis-Tris buffer pH 6.5, and concentrated in Millipore concentrators.

NMR Spectroscopy and Titration of PtdIns(3,4,5)P_3 Analogues

NMR spectra were recorded at 25°C on Varian INOVA 500 MHz spectrometer. The ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra of 0.2 mM uniformly ^{15}N-labeled PH domain were collected while dibutanoyl (C_4)-PtdIns(3,4,5)P_3 analogues were added stepwise. (See also Supporting Figure 1).

Expression of Human SHIP1 and SHIP2 Enzymes

The SHIP2 catalytic domain was expressed as GST fusion protein and expressed in bacteria as reported (49). The cDNA encoding human SHIP1 was subcloned in pTrHis bacterial expression vector (this construct was provided by Dr Len Stephens, Babraham, UK) and expressed as His-tagged fusion protein (50). Briefly, 0.4 L of an overnight culture of bacteria was twofold diluted the next day to reach an OD_{600} of 0.6 and induced by IPTG for 5 h at 20°C. The crude lysate was taken up in 50 mM Tris-HCl, pH 7.5 containing 0.1% Triton X-100, 300 mM NaCl, 10% glycerol, 5 mM $MgCl_2$ and complete protease inhibitors (Roche). The enzyme was applied to a Ni-NTA agarose column (Quiagen), washed, and

eluted with 100 mM imidazole in the same buffer. GST-PTEN was expressed and purified as reported (49).

Determination of SHIP1, SHIP2, and PTEN Phosphatase Activities

SHIP1 and SHIP2 phosphatase activities were determined in the presence of 0.3 μM [^{3}H]-Ins(1,3,4,5)P_4 and the use of Dowex columns to isolate the dephosphorylated product (51). When the substrate specificity of SHIP2 and PTEN was addressed with the various analogues, the malachite phosphate release assay was used as described (49). The enzyme activity data are shown as means of triplicates ± standard deviation. (See Supporting Figure 2 for PTEN inhibition.)

Western Blot for Akt Activation

Mouse peritoneal neutrophils were purified, stimulated and lysed as described (36). Lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and followed by immunoblotting with phospho-specific antibodies against Akt pS473 and pan-Akt (Cell Signaling Technology). (See Supporting Figure 3.)

Neutrophil Morphology

Mouse neutrophils (4×10^5) were seeded onto glass coverslips. Cells were allowed to attach for 10 min at room temperature, incubated at 37°C with or without 10 μM diC_{16}-PtdIns(3,4,5)P_3 analogues and carrier (1.5 μM histone H1) for 30 min, and stimulated with 5 nM complement factor 5a (C5a). The reaction was stopped by fixing the cells in 4% paraformaldehyde. Fixed cells were washed and incubated for 1 hr in PBS containing 0.2U/ml Alexa Fluor 488 phalloidin (Molecular Probes, Eugene, OR), 0.1% lysophosphatidylcholine (Nacalai, Kyoto, Japan), and 1% BSA. Cell shape was determined using confocal images of fluorescently-labeled cells. (A) Cells with distinctive, F-actin-rich leading edge were qualified as polarized (n>300 cells). A cell's perimeter, area, eccentricity and radial standard deviation (radial S.D.), were determined for each cell (n>43) using the Analysis Functions of IPLab software (Scanalytics, Fairfax, VA). The formula used to determine roundness (R) was $R = 1 - 4\pi y\, x^{-2}$, where x is a cell's perimeter and y is its area. Low values for eccentricity, radial S.D. and roundness indicate a lack of polarization.

Experimental Determination and Evaluation of Sodium Transport (I_{Na}+, μA/cm^2)

Briefly, A6 cells were subcultured onto 24-mm Millicell inserts (Millipore, Bedford, MA) for 10 days and the day before the experiment, incubated overnight in a serum-free 260 mosmol/kg H_2O amphibian Ringer solution. diC_{16}-PtdIns(3,4,5)P_3, analogues **4** and **5** was complexed by histone H1 carrier (50 μM) and then added to the apical side of the monolayer. Results were compared with insulin basolateral stimulation (100 nM) and control (histone H1 alone). This experiment is representative of three independent experiments. (See supporting Figure 4.)

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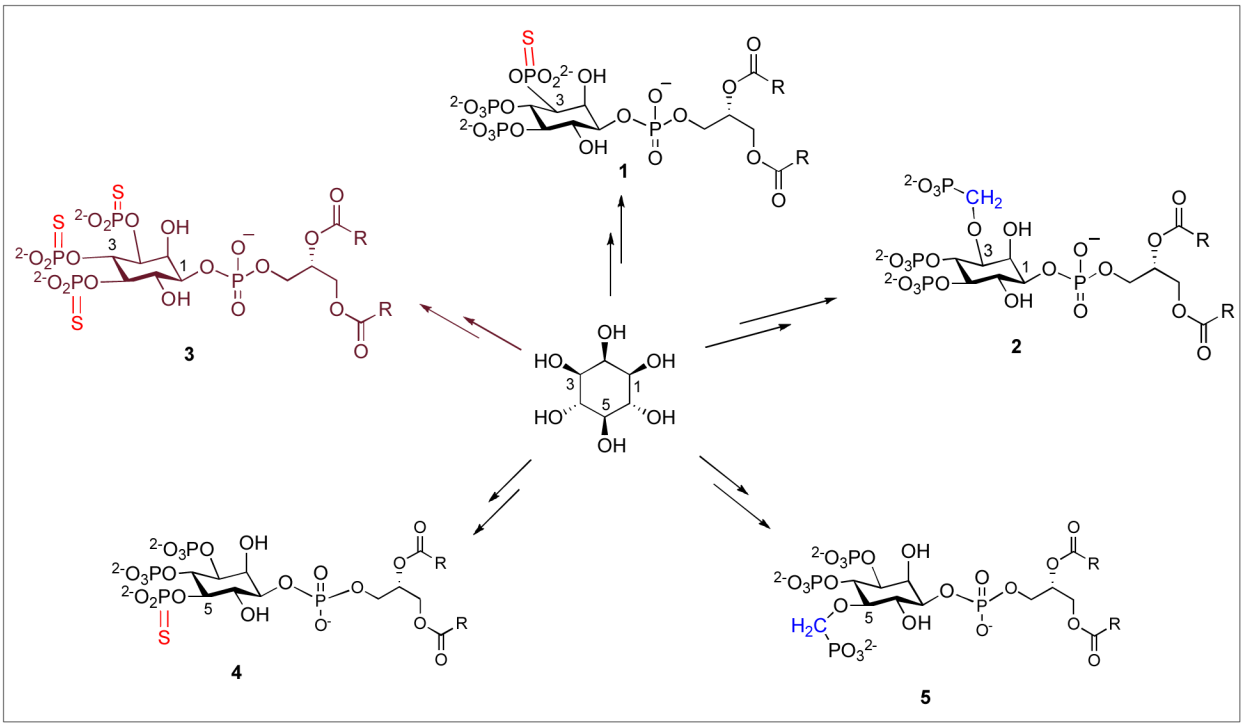

Figure 1.
Analogues of phosphatidylinositol 3,4,5-trisphosphate synthesized from *D-myo*-inositol.

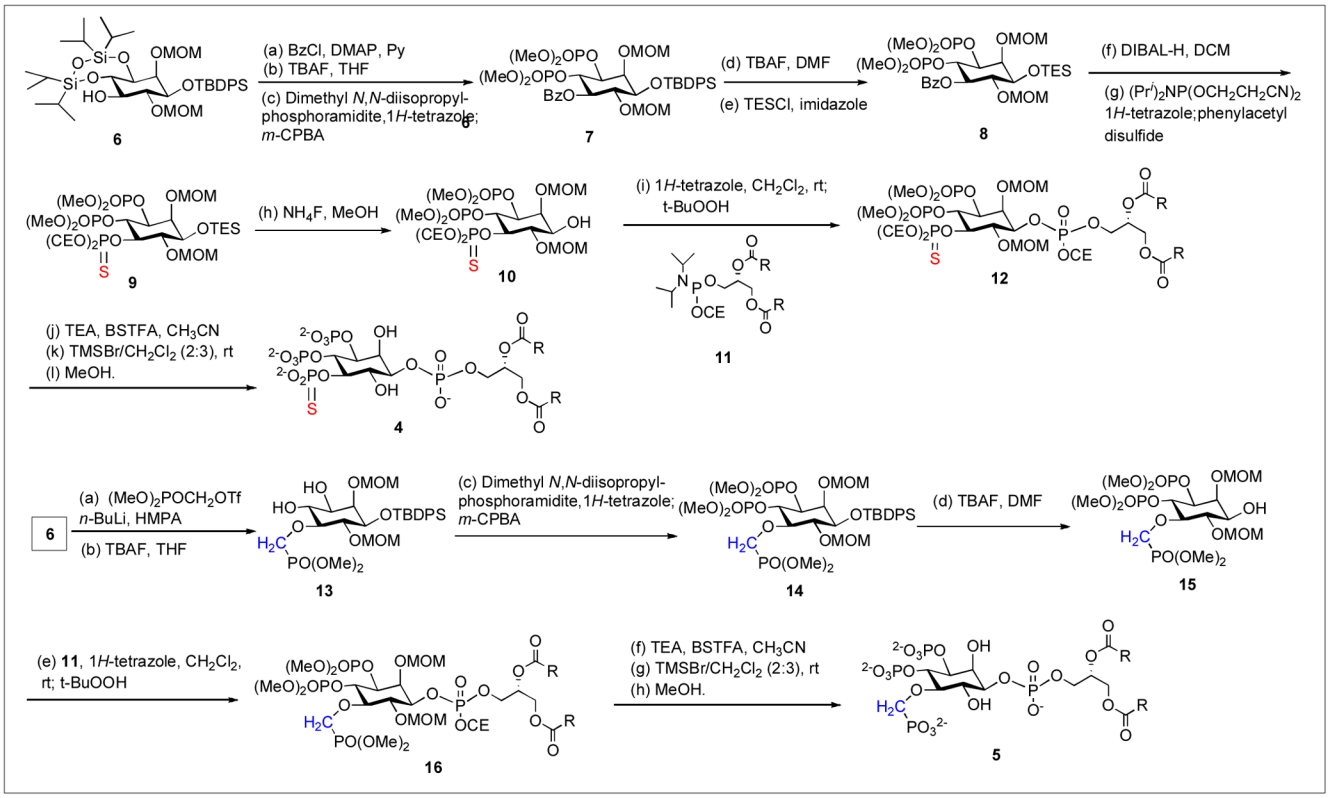

Figure 2.

Synthesis of 5-PT and 5-MP analogues. Key: CE = cyanoethyl; MOM = methoxymethyl ether; R = C_3H_7, C_7H_{15}, $C_{15}H_{31}$, $C_{17}H_{33}$.

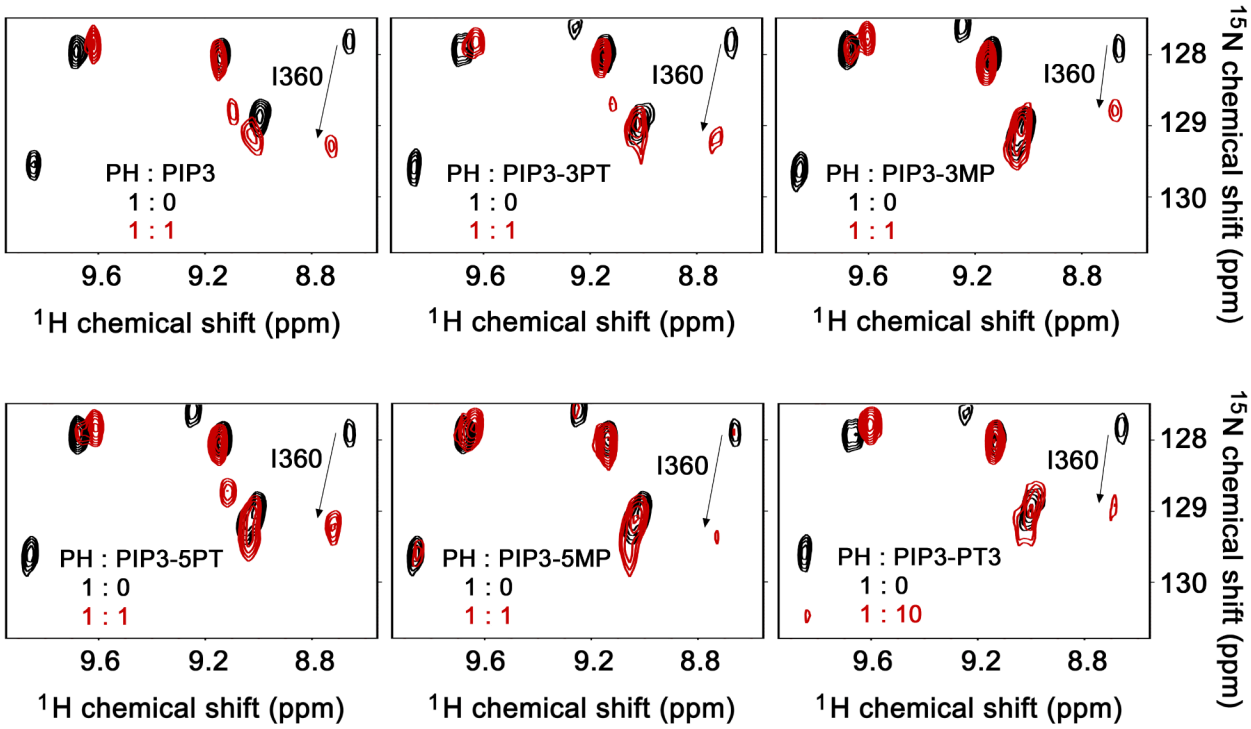

Figure 3.

PtdIns(3,4,5)P_3 analogues are recognized by the PH domain of GRP1.

Superimposed ^{1}H-^{15}N HSQC spectra of 0.2 mM ^{15}N-labeled GRP1 PH domain collected before (black) and after (red) addition of indicated diC_4-PtdIns(3,4,5)P_3 analogues. The chemical shift changes for the amide groups of Ile360 in the presence or absence of each analogue are indicated by arrows for comparison.

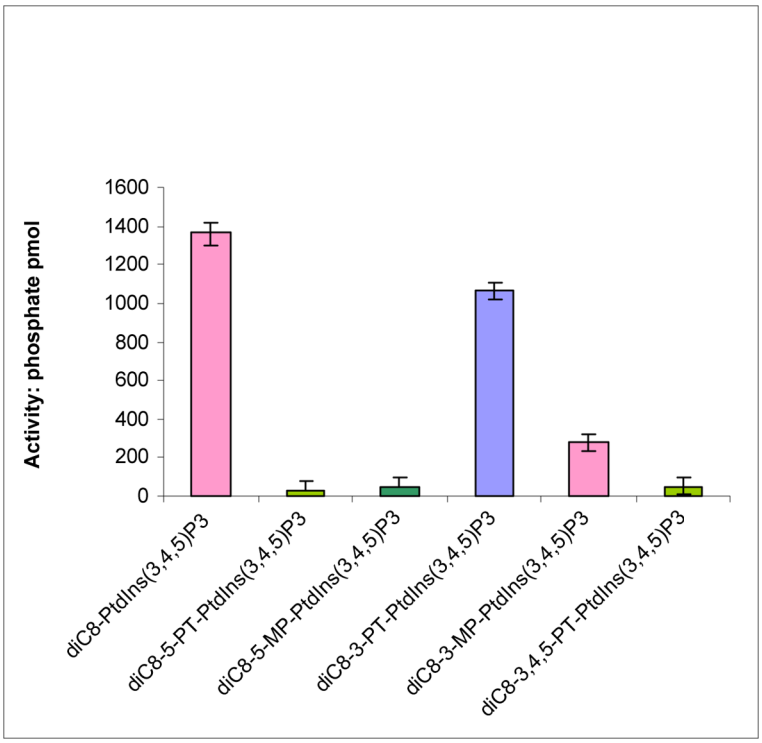

Figure 4.

Phosphate release from metabolically stabilized analogues by SHIP2 in the presence of 200 μM PtdSer (49), as detected by malachite green assay. Analogues were added at 100 μM. Analogues, including the positive control diC_8-PtdIns(3,4,5)P_3, were added at 100 μM. The final enzyme concentrations were 3.4 μg/ml and incubation time was 10 min at 37°C. Enzyme activity is expressed as pmol of phosphate released (means of triplicates ± SD).

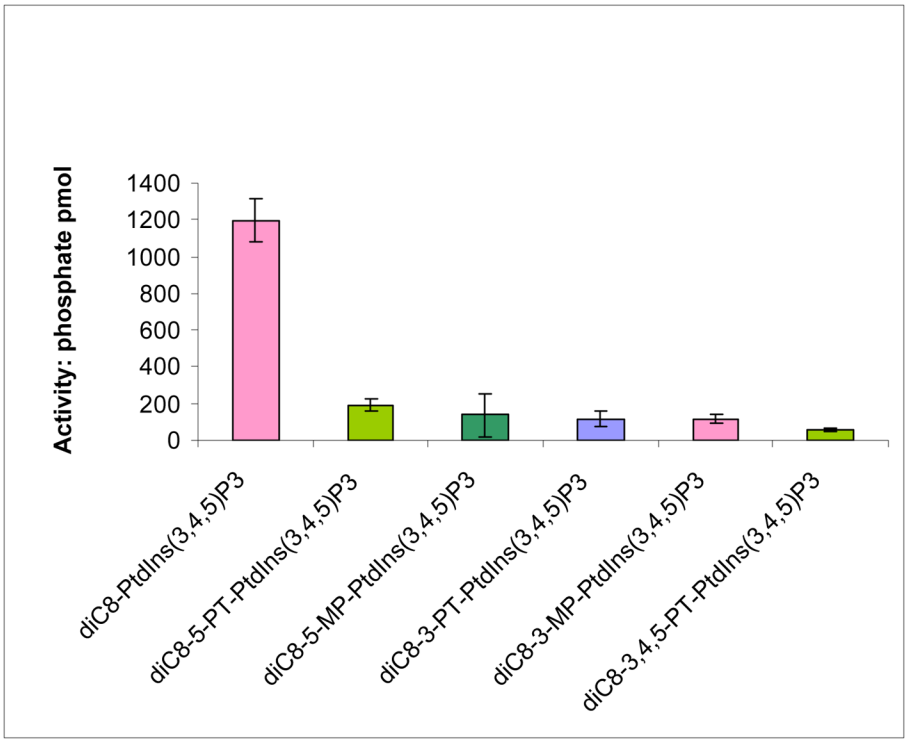

Figure 5.

Phosphate release from metabolically-stabilized analogues by PTEN in the presence of 200 μM PtdSer (49), as detected by malachite green assay. Analogues were added at 100 μM. Analogues, including the positive control diC_8-PtdIns(3,4,5)P_3, were added at 100 μM at 37°C. The final enzyme concentration was 0.125 mg/ml and incubation time was 10 min. Enzyme activity is expressed as described in the legend for Figure 4.

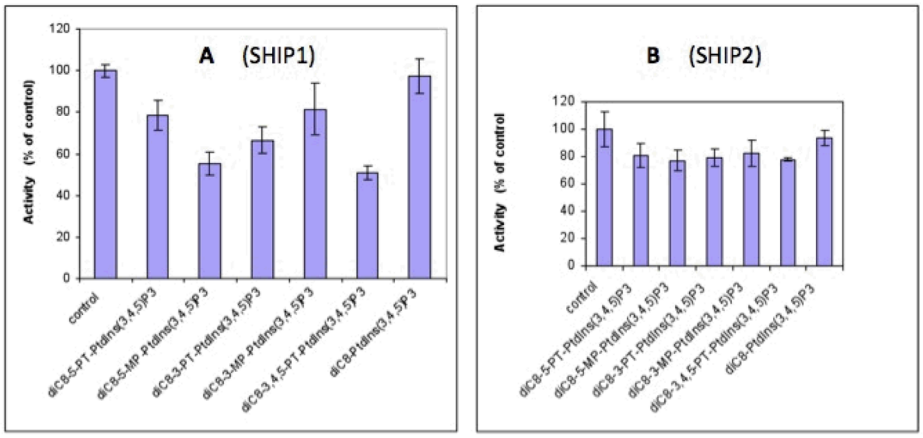

Figure 6.

Inhibition of phosphatase activity by stabilized analogues, using 0.3 μM tritium-labeled Ins(1,3,4,5)P_4 as the substrate and each of the diC_8 analogues at 10 μM. Panel A: inhibition of the 5-phosphatase activity of recombinant SHIP1. Panel B: inhibition of the 5-phosphatase activity of recombinant SHIP2. Panel A: inhibition of the 5-phosphatase activity of recombinant SHIP1 at 4.9 μg/ml. Panel B: inhibition of the 5-phosphatase activity of recombinant SHIP2 at 11.3 μg/ml. Analogues were preincubated for 5 min in the presence of enzyme at 4 °C. The mixture was then mixed with the labelled substrate and incubated for 10 min at 37 °C. The 100% control refers to the activity in the presence of water replacing analogue. Each value below 100% represents the activity that was reached in the presence of analogue (mean value ± SD).

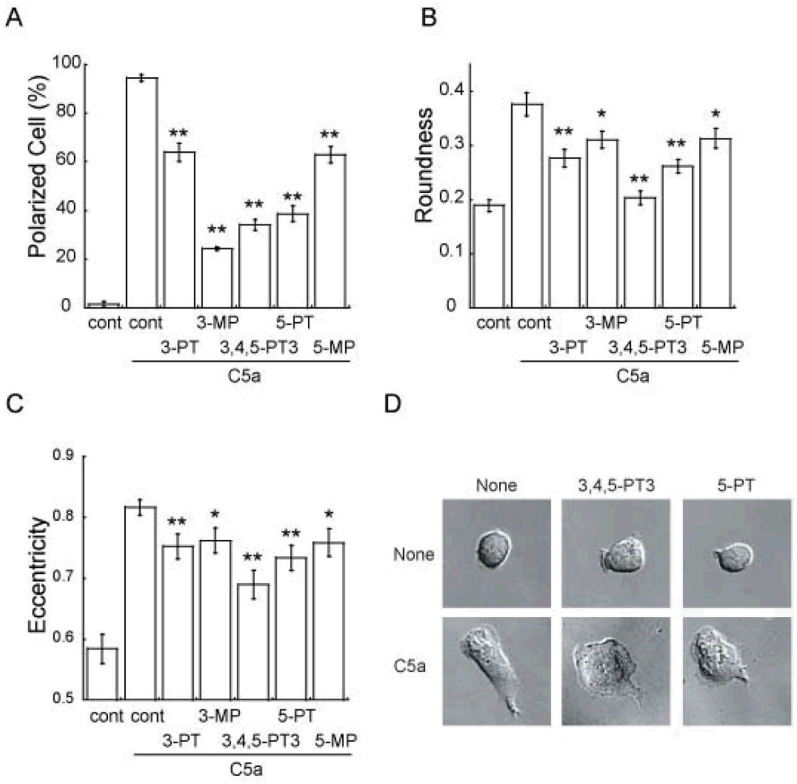

Figure 7.

Effects of metabolically-stabilized PtdIns(3,4,5)P_3 analogues on cell polarization. Mouse neutrophils (4×10^5) were preincubated with or without metabolically-stabilized PtdIns(3,4,5)P_3, followed by the addition of C5a and a further incubation for 15 min. Subsequently, cells were stained with fluorescent phalloidin; and the percentage of polarized cells (A), roundness (B) and eccentricity (C) was determined as described in the Methods. Higher values of roundness and eccentricity indicate that the cell shape is further from a circle and the cell is more polarized. All data are presented as the mean ± SD. Significant differences in each parameter from cells treated with C5a in the absence of a PtdIns(3,4,5)P_3 analogue are indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$ versus control (histone H1 carrier) (Student t test). Panel D illustrates selected micrographs of unactivated (top) or C5a-activiated (bottom) neutrophils in treated with no ligand or one of the two most potent ligands, 5-PT-PtdIns(3,4,5)P_3 or 3,4,5-PT_3-PtdIns(3,4,5)P_3.