Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 133–139

BIOORGANIC & MEDICINAL CHEMISTRY

A Phosphatidylinositol 3,4,5-Trisphosphate Analogue with Low Serum Protein-Binding Affinity

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Received 19 April 2000; accepted 15 August 2000

Abstract—Phosphatidylinositol 3,4,5-trisphosphate (PIP_3) plays an important role in the regulation of diverse physiological functions. Recent evidence indicates that PIP_3 is cell permeant, and can be added exogenously to modulate cellular responses. However, like many other phospholipids, PIP_3 binds serum proteins with high affinity, resulting in rapid deactivation of this lipid second messenger. Our study indicates that bovine serum albumin (BSA) at concentrations as low as 10 μg/mL abrogated the biological activity of dipalmitoyl-PIP_3. This nonspecific interaction with serum proteins hampers the use of PIP_3 in biological studies where serum is needed. We report here an ether-linked PIP_3 analogue, 1-*O*-(1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycero-3-phosphoryl)-*myo*-inositol 3,4,5-trisphosphate (C_{16}Me-PIP_3), which displays low serum protein-binding affinity while retaining the biological function of PIP_3. The affinity of C_{16}Me-PIP_3 with BSA was two orders of magnitude lower than that of its dipalmitoyl-counterpart. Biochemical data indicate that C_{16}Me-PIP_3 was able to stimulate Ca^{2+} influx in T cells in the presence of moderate levels (up to 1 mg/mL) of BSA. Thus, C_{16}Me-PIP_3 may provide a useful tool to study the physiological function of phosphoinositide (PI) 3-kinase in vivo.

Introduction

Phosphatidylinositol 3,4,5-trisphosphate (PIP_3) is a lipid second messenger generated by phosphoinositide (PI) 3-kinase in response to a wide array of external stimuli.[1–5] Substantial evidence indicates that PIP_3 plays a crucial role in diverse cellular processes including mitogenic signaling, cell survival, cytoskeletal remodeling, and vesicular trafficking. Putative downstream effectors of PIP_3 identified to date include Ca^{2+}-independent PKC isozymes,[6,7] Akt,[8,9] PDK1 (phosphoinositide-dependent kinase-1),[10–12] PLC-γ (phospholipase C-γ),[13] Btk (Bruton's tyrosine kinase),[14] GRP1 (general receptor for phosphoinositides 1),[15] α-centaurin,[16] and so forth. Many of these proteins harbor pleckstrin (PH) and/or Src homology-2 (SH2) domains that mediate the initiation of signaling cascades leading to an array of biochemical responses.[14,17] Furthermore, PIP_3 is a membrane-permeant molecule. Published data from this and other laboratories have shown that exogenous PIP_3 can readily fuse with cell membranes, and exert cellular and biochemical responses in different cell types, including platelets,[18] T cells,[19] NIH 3T3 cells,[20] and adipocytes.[21] This new approach allows a direct assessment of the physiological function of PI 3-kinase by supplementing PIP_3 to intact cells. However, this strategy is hampered by a potential drawback, i.e., PIP_3, like many other phospholipids, binds serum proteins with high affinity, resulting in rapid deactivation of this lipid second messenger. This nonspecific protein binding prevents the use of PIP_3 under conditions where serum is needed to sustain cell viability. To circumvent this problem, we have synthesized a series of PIP_3 analogues with modified side chains, aiming at minimizing the nonspecific interaction with serum proteins without disrupting their cellular function. Here, we report a unique ether-linked PIP_3 derivative that shows low binding affinity with bovine serum albumin (BSA), while retaining the physiological activity in stimulating T-cell Ca^{2+} signaling.

Results and Discussion

Deactivation of dipalmitoyl-PIP_3 by BSA

Two lines of evidence demonstrate that PIP_3 binds BSA with high affinity. First, exposure of tritium-labeled dipalmitoyl- or di-octanoyl-PIP_3 to BSA (100 μg/mL) resulted in the association of virtually all radioactivity with the protein (data not shown). Second, BSA at low concentrations inhibited PIP_3-induced cellular response. Previously, we raised evidence that PI 3-kinase was

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0968-0896/01/$ - see front matter
PII: S0968-0896(00)00227-3

involved in T-cell Ca^{2+} signaling via a PI(3,4,5)P_3-sensitive Ca^{2+} entry mechanism.[19] Dipalmitoyl-PIP_3 stimulated intracellular Ca^{2+} increase in Jurkat T cells in a dose-dependent manner. Nonetheless, BSA suppressed this PIP_3-induced Ca^{2+} entry at concentrations as low as 1 μg/mL (Fig. 1). The extent of inhibition reached 70% with 10 μg/mL of BSA, and 90% with 100 μg/mL. Conceivably, attenuation of the Ca^{2+} response was due to the sequestration of PIP_3 by protein binding.

Side-chain modifications of PIP_3

It is noteworthy that all PIP_3-interacting proteins examined so far exhibit a high degree of tolerance with respect to the length of PIP_3 side chains. For example, the dioctanoyl- and dibutyryl-analogues of PIP_3 retain the activity of their dipalmitoyl-counterpart, though with diminished potency.[22] In view of this relaxed specificity, we hypothesized that the side-chain of PIP_3 could be modified without abrogating its biological activity. With regard to the structural design, we turned our attention to the ether-linked phospholipid PAF (platelet-activating factor; 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine). Evidence shows that PAF was able to activate cellular responses in platelets and macrophages in the presence of BSA as high as 10 mg/mL.[23] Based on the PAF structure, we proposed two series of PIP_3 derivatives for biochemical testing (Fig. 2): 1-*O*-[1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphoryl]-*myo*-inositol 3,4,5-trisphosphate (**C_8Ac-**, **C_{16}Ac-** and **C_{18}Ac-PIP_3**) and 1-*O*-[1-*O*-alkyl-2-*O*-methyl-*sn*-glycero-3-phosphoryl]-*myo*-inositol 3,4,5-trisphosphate (**C_8Me-**, **C_{16}Me-** and **C_{18}Me-PIP_3**).

Figure 3 illustrates the synthesis of these compounds. The mixed 1,2-disubstituted glycerols (−)-**4a,b,** and **cAc** and **4a,b,** and **cMe** were prepared from (−)-3-*O*-(4-methoxybenzyl)-*sn*-glycerol [(−)-**1**] in three steps in 60% yield. These mixed glycerides were then converted to the corresponding phosphoramidites (+)-**5a,b,** and **cAc** and **5a,b,** and **cMe** in 90% yield. The key intermediate (−)-**6** was synthesized from (−)-1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol as previously described.[24,25] Coupling of the **5 Ac** and **Me** series with **6** in the presence of 1*H*-tetrazole, followed by *m*-CPBA oxidation, gave the perbenzylated derivative (−)-**7a,b,** and **cAc** and **7a,b,** and **cMe**, respectively, that underwent hydrogenolysis to

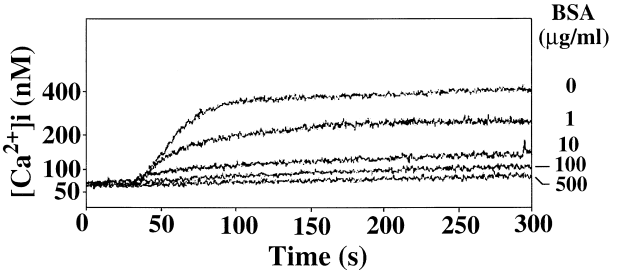
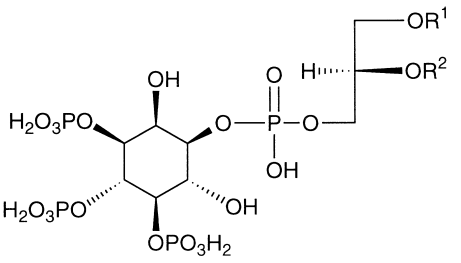


Figure 1. Inhibitory effect of BSA on dipalmitoyl-PIP_3-induced Ca^{2+} influx in Jurkat T cells. Fura-2-loaded Jurkat T cells were stimulated with 10 μM dipalmitoyl-PIP_3 in the presence of indicated levels (0–500 μg/mL) of BSA. Intracellular Ca^{2+} was analyzed by fluorescence spectrophotometry as described in the Experimental.



C_8Ac-PIP_3: $R^1 = C_8H_{17}$, $R^2 = COCH_3$
C_{16}Ac-PIP_3: $R^1 = C_{16}H_{33}$, $R^2 = COCH_3$
C_{18}Ac-PIP_3: $R^1 = C_{18}H_{37}$, $R^2 = COCH_3$
C_8Me-PIP_3: $R^1 = C_8H_{17}$, $R^2 = CH_3$
C_{16}Me-PIP_3: $R^1 = C_{16}H_{33}$, $R^2 = CH_3$
C_{18}Me-PIP_3: $R^1 = C_{18}H_{37}$, $R^2 = CH_3$

Figure 2. Structures of ether-linked PIP_3 derivatives.

afford the respective PIP_3 derivatives. The overall yield from (−)-**1** was approximately 30% for these analogues.

C_{16}Me-PIP_3 stimulates Ca^{2+} influx in Jurkat T cells in the presence of moderate levels of BSA

The aforementioned PIP_3 analogues were tested for the stimulation of Ca^{2+} entry in Jurkat T cells by using fura-2 fluorimetry.[19] Fura-2-loaded Jurkat T cells were exposed to individual derivatives in the presence of increasing concentrations of BSA, and $[Ca^{2+}]i$ was monitored by changes in the fluorescence intensity. Among the six compounds examined, only C_{16}Me-PIP_3 elicited substantial increase in $[Ca^{2+}]i$ even in the presence of BSA (Fig. 4). C_{18}Me-PIP_3 could also trigger $[Ca^{2+}]i$ increase, however, to a lesser extent than C_{16}Me-PIP_3. In addition, this C_{18} derivative caused instantaneous cell aggregation. This cell-aggregating activity might be attributable to the nonspecific interaction of the long acyl side chain with cell membranes. In contrast, C_8Me-PIP_3 and the 2-acetyl-PIP_3 derivatives lacked appreciable effect on $[Ca^{2+}]i$. C_8Me-PIP_3 displayed high solubility in water, which might hinder permeability across cell membranes. Nevertheless, it remains unclear why C_{16}Ac-PIP_3 and C_{18}Ac-PIP_3 were inactive in eliciting Ca^{2+} entry, since their physical properties were similar to those of the 2-methyl counterparts. One possible explanation was that these compounds were susceptible to rapid deactivation via deacetylation and/or dephosphorylation when entering the cells.

Although the potency of C_{16}Me-PIP_3 in inducing T-cell Ca^{2+} response was about one half of that of dipalmitoyl-PIP_3, it was able to withstand substantially higher concentrations of BSA. For example, the maximum $[Ca^{2+}]i$ attained at 500 and 1000 μg/mL of BSA was 93% and 55%, respectively, of that without BSA (Fig. 5). The activity, however, was completely abolished at 2.5 mg/mL of BSA. In contrast, dipalmitoyl-PIP_3 lost 70% of activity at 10 μg/mL of BSA (Fig. 1).

According to the dose–response relationship shown in Figure 5, the affinity of C_{16}Me-PIP_3 with BSA was at

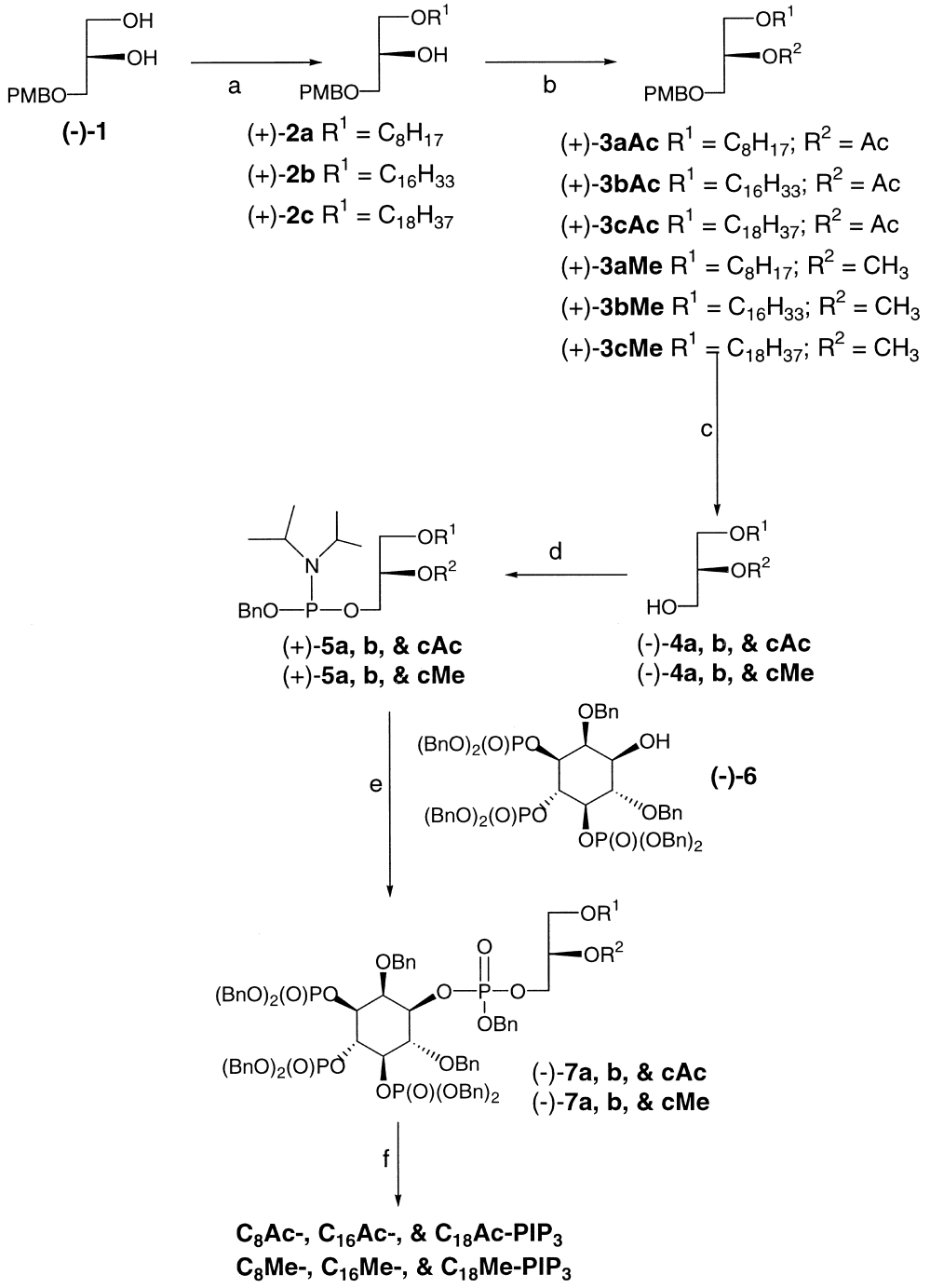


Figure 3. Synthesis of ether-linked PIP_3 derivatives. Key: (a) $Bu_2SnO/PhCH_3$, followed by R^1Br, CsF/DMF; (b) Ac_2O, Et_3N, DMAP/CH_2Cl_2 or CH_3I, NaH/DMF; (c) DDQ/CH_2Cl_2; (d) (BnO)P[N(*i*Pr)$_2$]$_2$, 1*H*-tetrazole, *m*-CPBA/CH_2Cl_2; (e) 1*H*-tetrazole, *m*-CPBA/CH_2Cl_2; (f) Pd black, H_2/85% EtOH.

least two orders of magnitude lower than that of its dipalmitoyl-counterpart. Especially noteworthy is that the range of BSA concentrations for complete inhibition of C_{16}Me-PIP_3-induced Ca^{2+} response was less than 1 log unit, which is a good indication of specific protein–lipid interactions.

Because serum albumin carries a number of binding sites for fatty acids, it nondiscriminatingly binds phospholipids through the fatty acyl moiety. However, due to lack of acyl functions, C_{16}Me-PIP_3 might interact with BSA through specific electrostatic binding between the phosphoinositol head group and the cationic domain on BSA, a phenomenon that warrants further investigation.

Conclusion

The role of PIP_3 as a second messenger in transmembrane signal transduction is well documented. Although PIP_3 is a cell-permeant molecule, its use in biochemical

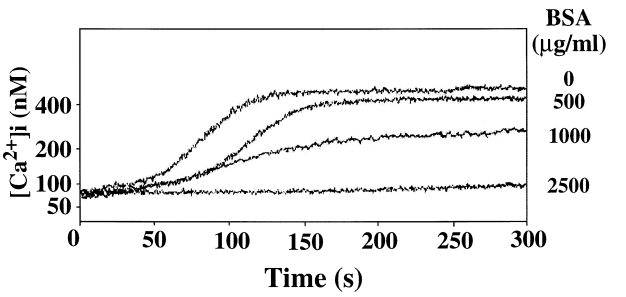


Figure 4. Inhibitory effect of BSA on C_{16}Me-PIP_3-induced Ca^{2+} influx in Jurkat T cells. Fura-2-loaded Jurkat T cells were stimulated with 20 μM C_{16}Me-PIP_3 in the presence of indicated levels (0–2500 μg/mL) of BSA. Intracellular Ca^{2+} was analyzed by fluorescence spectrophotometry as described in the Experimental.

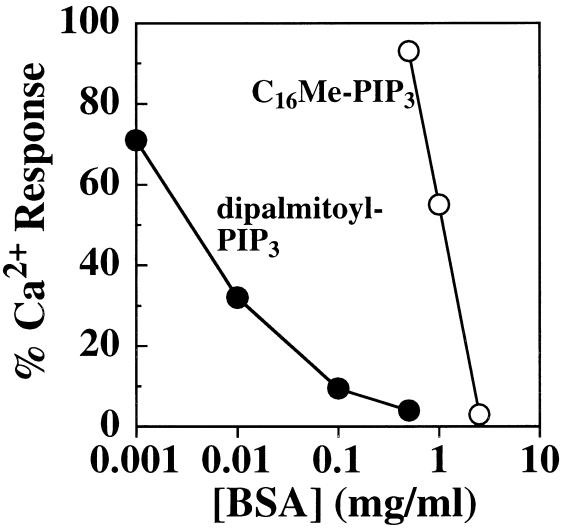


Figure 5. Dose-dependent inhibitory effect of BSA on T-cell Ca^{2+} response induced by dipalmitoyl-PIP_3 (10 μM) and C_{16}Me-PIP_3 (20 μM).

studies is hampered by high affinity with serum proteins. Based on the structure of PAF, we designed an ether-linked PIP_3 derivative that displays reduced affinity with BSA, while retaining activity in eliciting cellular response. Application of this analogue in studying the in vivo role of PI 3-kinase in T-cell and platelet activation is currently under way in this laboratory.

Experimental

General procedure for the regioselective preparation of (+)-2a, (+)-2b, and (+)-2c. A solution of 3-(4-methoxybenzyl)-*sn*-glycerol **1** (2.2 mmol) and Bu_2SnO (2.4 mmol) in toluene was stirred under reflux with azeotropic removal of water for 2 h, and then concentrated to dryness. To the residue were added DMF (2 mL), CsF (5.6 mmol), and the respective alkyl bromide (2.4 mmol) at −15 °C. After being stirred at −15 °C for 2 h, the mixture was allowed to warm up to rt and stirred overnight. The resulting reaction mixture was concentrated. The residue was diluted with CH_2Cl_2, washed with brine, dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography (silica gel; hexane:ether, 3:1) to give the respective 1-*O*-alkyl ether. (+)-1-*O*-Octyl-3-*O*-(4-methoxybenzyl)-*sn*-glycerol [(+)-**2a**]: colorless oil (602 mg, 84%); $[\alpha]_D^{20}$ +3.0° (*c* 4, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.27 (br s, 10H), 1.50–1.60 (m, 2H), 2.56 (br s, 1H), 3.40–3.54 (m, 6H), 3.79 (s, 3H), 3.92–3.98 (m, 1H), 4.47 (s, 2H), 6.88 (d, *J* = 8.7 Hz, 2H), 7.26 (d, *J* = 8.7 Hz, 2H). (+)-1-*O*-Hexadecyl-3-*O*-(4-methoxybenzyl)-*sn*-glycerol [(+)-**2b**]: amorphous solid (834 mg, 86%); $[\alpha]_D^{20}$ +1.7° (*c* 1.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 26H), 1.50–1.60 (m, 2H), 2.48 (d, *J* = 4.2 Hz, 1H), 3.40–3.54 (m, 6H), 3.80 (s, 3H), 3.92–3.99 (m, 1H), 4.48 (s, 2H), 6.89 (d, *J* = 8.7 Hz, 2H), 7.26 (d, *J* = 8.7 Hz, 2H). HRMS: Calcd *m*/*z* for $C_{27}H_{48}O_4$: 436.355. Found: 459.350 (M + Na). (+)-1-*O*-Octadecyl-3-*O*-(4-methoxybenzyl)-*sn*-glycerol [(+)-**2c**]: amorphous solid (920 mg, 89%); $[\alpha]_D^{20}$ +1.5° (*c* 0.8, $CHCl_3$); ^{1}H NMR (CDCl3) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.25 (br. s, 30H), 1.48–1.58 (m, 2H), 2.47 (d, *J* = 4.2 Hz 1H), 3.41–3.52 (m, 6H), 3.80 (s, 3H), 3.92–3.99 (m, 1H), 4.48 (s, 2H), 6.89 (d, *J* = 8.7 Hz, 2H), 7.26 (d, *J* = 8.7 Hz, 2H).

General procedure for the preparation of (+)-3aAc, (+)-3bAc, and (+)-3cAc. To a solution of **2a**, **2b**, or **2c** (0.67 mmol), triethylamine (2.1 mL) and DMAP (89 mg, 0.73 mmol) in CH_2Cl_2 was added Ac_2O (0.21 mL, 2.22 mmol). The resulting mixture was stirred at rt for 3 h, concentrated, and the residue was purified by column chromatography (silica gel; hexane:ether, 5:1) to yield the 2-acetylated product. (+)-1-*O*-Octyl-2-*O*-acetyl-3-(4-methoxybenzyl)-*sn*-glycerol [(+)-**3aAc**]: colorless syrup (230 mg, 94%); $[\alpha]_D^{20}$ +1.8° (*c* 1.4, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 10H), 1.45–1.60 (m, 2H), 2.07 (s, 3H), 3.37—3.46 (m, 2H), 3.57 (t, *J* = 6.3 Hz, 4H), 3.80 (s, 3H), 4.47 (dd, *J* = 11.7, 17.1 Hz, 2H), 5.10–5.16 (m, 1H), 6.88 (d, *J* = 8.7 Hz, 2 H), 7.26 (d, *J* = 8.7 Hz, 2 H). (+)-1-*O*-Hexadecyl-2-*O*-acetyl-3-(4-methoxybenzyl)-*sn*-glycerol [(+)-**3bAc**]: amorphous solid (297 mg, 93%); $[\alpha]_D^{20}$ +1.2° (*c* 0.6, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 26H), 1.46–1.55 (m, 2H), 2.08 (s, 3H), 3.34–3.48 (m, 2H), 3.57 (t, *J* = 6.3 Hz, 4H), 3.80 (s, 3H), 4.47 (dd, *J* = 11.7, 17.1 Hz, 2H), 5.10–5.15 (m, 1H), 6.88 (d, *J* = 8.7 Hz, 2H), 7.26 (d, *J* = 8.7 Hz, 2H). (+)-1-*O*-Octadecyl-2-*O*-acetyl-3-(4-methoxybenzyl)-*sn*-glycerol [(+)-**3cAc**]: amorphous solid (320 mg, 96%); $[\alpha]_D^{20}$ +0.9° (*c* 0.6, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 30H), 1.45–1.59 (m, 2H), 2.08 (s, 3H), 3.37–3.46 (m, 2H), 3.57 (t, *J* = 6.3 Hz, 4H), 3.80 (s, 3H), 4.48 (dd, *J* = 11.7, 17.1 Hz, 2H), 5.12–5.15 (m, 1H), 6.88 (d, *J* = 8.7 Hz, 2H), 7.26 (d, *J* = 8.7 Hz, 2H).

General procedure for the preparation of (+)-3aMe, (+)-3bMe, and (+)-3cMe. To a solution of (+)-**2a**, (+)-**2b**, or (+)-**2c** (0.67 mmol) in DMF (2 mL) was added NaH (32.2 mg, 1.34 mmol) and stirred at 0 °C for 30 min, then CH_3I (83 μL, 1.34 mmol) was added. The resulting mixture was stirred at rt overnight, and concentrated. The residue was diluted with ethyl acetate, washed with brine, dried over Na_2SO_4, and concentrated. Purification by column chromatography (silica gel; hexane:ether, 5:1) afforded the 2-methylation product. (+)-1-*O*-Octyl-2-*O*-methyl-3-(4-methoxybenzyl)-*sn*-glycerol

[(+)-**3aMe**]: colorless oil (216 mg, 96%); $[\alpha]_D^{20}$ +2.9° (*c* 1.5, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.27 (br s, 10H), 1.49–1.60 (m, 2H), 3.41 (t, *J* = 6.6 Hz, 2H), 3.45 (s, 3H), 3.48–3.58 (m, 5H), 3.80 (s, 3H), 4.48 (s, 2H), 6.88 (d, *J* = 8.7 Hz, 2H), 7.26 (d, *J* = 8.7 Hz, 2H). (+)-1-*O*-Hexadecyl-2-*O*-methyl-3-(4-methoxybenzyl)-*sn*-glycerol [(+)-**3bMe**]: amorphous solid (273 mg, 91%); $[\alpha]_D^{20}$ +2.4° (*c* 1.2, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 26H), 1.48–1.58 (m, 2H), 3.41 (t, *J* = 6.6 Hz, 2H), 3.45 (s, 3H), 3.48–3.58 (m, 5H), 3.80 (s, 3H), 4.48 (s, 2H), 6.88 (d, *J* = 8.7 Hz, 2H), 7.25 (d, *J* = 8.7 Hz, 2H). HRMS: Calcd *m*/*z* for $C_{28}H_{50}O_4$: 450.371. Found: 473.366 (M + Na). (+)-1-*O*-Octadecyl-2-*O*-methyl-3-(4-methoxybenzyl)-*sn*-glycerol [(+)-**3cMe**]: amorphous solid (302 mg, 95%); $[\alpha]_D^{20}$ +2.2° (*c* 0.8, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 30H), 1.47–1.60 (m, 2H), 3.41 (t, *J* = 6.6 Hz, 2H), 3.45 (s, 3H), 3.49–3.57 (m, 5H), 3.80 (s, 3H), 4.48 (s, 2H), 6.88 (d, *J* = 8.7 Hz, 2H), 7.25 (d, *J* = 8.7 Hz, 2H).

General procedure for the preparation of (−)-4aAc, (−)-4bAc, (−)-4cAc, (−)-4aMe, (−)-4bMe, and (−)-4cMe. To a solution of the aforementioned 3-PMB ether (0.57 mmol) in wet CH_2Cl_2 (20 mL), DDQ (270 mg, 1.19 mmol) was added in portions. The resulting mixture was stirred at rt for 4 h, diluted with CH_2Cl_2 (20 mL), washed with 10% aq $NaHCO_3$, and brine, dried over Na_2SO_4, and concd. The residue was purified by column chromatography (silica gel; hexane:ether, 1:2) to give the 3-hydroxy derivative. (−)-1-*O*-Octyl-2-*O*-acetyl-*sn*-glycerol [(−)-**4aAc**]: colorless oil (132 mg, 93%); $[\alpha]_D^{20}$ −18.9° (*c* 0.8, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.27 (br s, 10H), 1.50–1.62 (m, 2H), 2.10 (s, 3H), 2.31 (br s, 1H), 3.41–3.50 (m, 2H), 3.60–3.64 (m, 2H), 3.81 (d, *J* = 4.2 Hz, 2H), 4.95–5.01 (m, 1H). (−)-1-*O*-Hexadecyl-2-*O*-acetyl-*sn*-glycerol [(−)-**4bAc**]: amorphous solid (191 mg, 91%); $[\alpha]_D^{20}$ −11.1° (*c* 0.4, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 26H), 1.55–1.59 (m, 2H), 2.11 (br s, 3H), 2.21 (br s, 1H), 3.42–3.48 (m, 2H), 3.61–3.64 (m, 2H), 3.82 (d, *J* = 3.6 Hz, 2H), 4.96–5.02 (m, 1H). (−)-1-*O*-Octadecyl-2-*O*-acetyl-*sn*-glycerol [(−)-**4cAc**]: amorphous solid (192 mg, 85%); $[\alpha]_D^{20}$ −11° (*c* 1.3, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 30H), 1.51–1.61 (m, 2H), 2.11 (br s, 3H), 2.22 (br s, 1H), 3.38–3.51 (m, 2H), 3.57–3.67 (m, 2H), 3.82 (d, *J* = 3.6 Hz, 2H), 4.95–5.02 (m, 1H). (−)-1-*O*-Octyl-2-*O*-methyl-*sn*-glycerol [(−)-**4aMe**]: colorless oil (110 mg, 86%); $[\alpha]_D^{20}$ −7.8° (*c* 1.8, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.26 (br s, 10H), 1.52–1.60 (m, 2H), 2.18 (br s, 1H), 3.38–3.45 (m, 3H), 3.46 (s, 3H), 3.49–3.58 (m, 2H), 3.65 (dd, *J* = 5.4, 11.4 Hz, 1H), 3.74 (dd, *J* = 4.2, 11.1 Hz, 1H). (−)-1-*O*-Hexadecyl-2-*O*-methyl-*sn*-glycerol [(−)-**4bMe**]: amorphous solid (148 mg, 78%); $[\alpha]_D^{20}$ −6.0° (*c* 1.3, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 26 H), 1.51–1.61 (m, 2 H), 2.11 (br s, 1 H), 3.39–3.46 (m, 3 H), 3.46 (s, 3 H), 3.53 (t, *J* = 4.5 Hz, 2 H), 3.67 (dd, J = 5.1, 11.4 Hz, 1 H), 3.75 (dd, *J* = 4.2, 11.1 Hz, 1 H). HRMS: Calcd *m*/*z* for $C_{20}H_{42}O_3$: 330.313. Found: 353.307 (M + Na). (−)-1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycerol [(−)-**4cMe**]: amorphous solid (160 mg, 74%); $[\alpha]_D^{20}$ −5.7° (*c* 0.9, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 30H), 1.52–1.61 (m, 2H), 2.16 (br s, 1H), 3.39–3.46 (m, 3H), 3.46 (s, 3H), 3.52 (t, *J* = 4.5 Hz, 2H), 3.65 (dd, *J* = 5.1, 11.4 Hz, 1H), 3.75 (dd, *J* = 4.2, 11.1 Hz, 1H).

General procedure for the preparation of (+)-5aAc, (+)-5bAc, (+)-5cAc, (+)-5aMe, (+)-5bMe, and (+)-5cMe. To a solution of the aforementioned alcohol (0.49 mmol) in anhyd CH_2Cl_2 (3 mL) was added a solution of benzyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (197 mg, 0.58 mmol) in CH_2Cl_2 (1 mL), followed by 1*H*-tetrazole (42 mg, 0.58 mmol) under an argon atmosphere. After 3 h, the volatiles were removed in vacuo, and the residue was purified by column chromatography (silica gel; hexane:triethylamine, 10:1) to produce the phosphoramidite derivative. (+)-1-*O*-Octyl-2-*O*-acetyl-*sn*-glycerol benzyl-(*N*,*N*-diisopropylamino)phosphoramidite [(+)-**5aAc**]: colorless oil (187 mg, 96%); $[\alpha]_D^{20}$ +6.2° (*c* 1.6, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.18 (d, *J* = 3 Hz, 6H), 1.20 (d, *J* = 3 Hz, 6H), 1.26 (br s, 10H), 1.48–1.58 (m, 2H), 2.06 (s, 3H), 3.35–3.88 (m, 8H), 4.62–4.77 (m, 2H), 4.90–5.16 (m, 1H), 7.24–7.34 (m, 5H); ^{31}P NMR ($CDCl_3$, H_3PO_4 as external reference) δ 150.90 and 151.05 (1:1). (+)-1-*O*-Hexadecyl-2-*O*-acetyl-*sn*-glycerol benzyl-(*N*,*N*-diisopropylamino)phosphoramidite [(+)-**5bAc**]: colorless oil (289 mg, 95%); $[\alpha]_D^{20}$ +4.8° (*c* 1.5, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.18 (d, *J* = 3 Hz, 6H), 1.22 (d, *J* = 3 Hz, 6H), 1.25 (br s, 26H), 1.48–1.58 (m, 2H), 2.05 (s, 3H), 3.34–3.86 (m, 8H), 4.62–4.77 (m, 2H), 5.08–5.16 (m, 1H), 7.20–7.34 (m, 5H); ^{31}P NMR ($CDCl_3$, H_3PO_4 as external reference) δ 150.89 and 151.04 (1:1). (+)-1-*O*-Octadecyl-2-*O*-acetyl-*sn*-glycerol benzyl-(*N*,*N*-diisopropylamino)phosphoramidite [(+)-**5cAc**]: colorless oil (298 mg, 98%); $[\alpha]_D^{20}$ +4.1° (*c* 2.1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.18 (d, *J* = 3 Hz, 6H), 1.20 (d, *J* = 3 Hz, 6H), 1.25 (br s, 30H), 1.48–1.58 (m, 2H), 2.06 (s, 3H), 3.38–3.87 (m, 8H), 4.50–4.60 (m, 2H), 5.08–5.16 (m, 1H), 7.20–7.40 (m, 5H); ^{31}P NMR ($CDCl_3$, H_3PO_4 as external reference) δ 150.25 and 151.42 (1:1). (+)-1-*O*-Octyl-2-*O*-methyl-*sn*-glycerol benzyl-(*N*,*N*-diisopropylamino)phosphoramidite [(+)-**5aMe**]: colorless oil (202 mg, 91%); $[\alpha]_D^{20}$ +4.5° (*c* 3, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.18 (d, *J* = 3 Hz, 6H), 1.20 (d, *J* = 3 Hz, 6H), 1.26 (br s, 10H), 1.51–1.61 (m, 2H), 3.40–3.80 (m, 10H), 4.63–4.79 (m, 2H), 7.22–7.41 (m, 5H); ^{31}P NMR ($CDCl_3$, H_3PO_4 as external reference) δ 150.25 and 151.41 (1:1). (+)-1-*O*-Hexadecyl-2-*O*-methyl-*sn*-glycerol benzyl-(*N*,*N*-diisopropylamino)phosphoramidite [(+)-**5bMe**]: colorless oil (262 mg, 93%); $[\alpha]_D^{20}$ +3.7° (*c* 1.4, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.19 (d, *J* = 3 Hz, 6H), 1.21 (d, *J* = 3 Hz, 6H), 1.25 (br s, 26H), 1.51–1.60 (m, 2H), 3.40–3.80 (m, 10H), 4.62–4.80 (m, 2H), 7.20–7.38 (m, 5H); ^{31}P NMR (CD Cl_3, H_3PO_4 as external reference) δ 150.28 and 151.45 (1:1). (+)-1-*O*-Octadeyl-2-*O*-methyl-*sn*-glycerol benzyl-(*N*,*N*-diisopropylamino)phosphoramidite [(+)-**5cMe**]: colorless oil (281 mg, 97%); $[\alpha]_D^{20}$ +4.2° (*c* 2.9, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.19 (d, *J* = 3 Hz, 6H), 1.21 (d, *J* = 3 Hz, 6H), 1.25 (br s, 30H), 1.52–1.60 (m, 2H), 3.36–3.80 (m, 10H), 4.64–4.79 (m, 2H), 7.20–7.40 (m, 5H); ^{31}P NMR ($CDCl_3$, H_3PO_4 as external reference) δ 150.28 and 151.45 (1:1).

General procedure for the preparation of (−)-7aAc, (−)-7bAc, (−)-7cAc, (−)-7aMe, (−)-7bMe, and (−)-7cMe. To a solution of (−)-**6** (15 mg, 0.013 mmol) and 1*H*-tetrazole (2.2 mg, 0.031 mmol) in anhydrous CH_2Cl_2 (2 mL) was added a solution of the aforementioned phosphoramidite (0.016 mmol) in CH_2Cl_2 (1 mL) under an atmosphere of argon. The resulting mixture was stirred at 25 °C for 2 h, then cooled to −40 °C and a solution of *m*-chloroperoxybenzoic acid (*m*-CPBA) (9.5 mg, 57%, 0.016 mmol) in CH_2Cl_2 (1 mL) was added. The resulting mixture was stirred at −40 °C for 1 h, diluted with CH_2Cl_2, washed with saturated aq $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated. The residue was purified by column chromatography (silica gel; hexane: ether, 1:2) to provide the coupled product. (−)-1-*O*-(1-*O*-Octyl-2-*O*-acetyl-*sn*-glycero-3-benzyloxyphosphoryl)-2,6-di-*O*-benzyl-*myo*-inositol-3,4,5-tris-*O*-(dibenzylphosphate) [(−)-**7aAc**]: colorless syrup (16.2 mg, 80%); $[\alpha]_D^{20}$ −4.1° (*c* 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.26 (br s, 10H), 1.42–1.60 (m, 2H), 2.05 (s, 3H), 3.82–3.96 (m, 1H), 4.04–4.11 (m, 1H), 4.20–4.34 (m, 1H), 4.40–4.52 (m, 1H), 4.56–5.08 (m, 23H), 6.94–7.40 (m, 45H); ^{31}P NMR ($CDCl_3$, H_3PO_4 as external reference) δ −0.95, −1.13 (d), −1.38, −1.74 (d). (−)-1-*O*-(1-*O*-Hexadecyl-2-*O*-acetyl-*sn*-glycero-3-benzyloxyphosphoryl)-2,6-di-*O*-benzyl-*myo*-inositol-3,4,5-tris-*O*-(dibenzylphosphate) [(−)-**7bAc**]: colorless syrup (18.0 mg, 80%); $[\alpha]_D^{20}$ −3.3° (*c* 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.26 (br s, 26H), 1.44–1.56 (m, 2H), 2.06 (s, 3H), 3.80–3.94 (m, 1H), 4.04–4.12 (m, 1H), 4.20–4.34 (m, 1H), 4.40–4.52 (m, 1H), 4.56–5.08 (m, 23H), 6.92–7.40 (m, 45H); ^{31}P NMR ($CDCl_3$, H_3PO_4 as external reference) δ −0.89, −1.13 (d), −1.33, −1.68 (d). (−)-1-*O*-(1-*O*-Octadecyl-2-*O*-acetyl-*sn*-glycero-3-benzyloxyphosphoryl)-2,6-di-*O*-benzyl-*myo*-inositol-3,4,5-tris-*O*-(dibenzylphosphate) [(−)-**7cAc**]: colorless syrup (17.0 mg, 78%); $[\alpha]_D^{20}$ −3.0° (*c* 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.9 Hz, 3H), 1.26 (br s, 30H), 1.44–1.58 (m, 2H), 2.06 (s, 3H), 3.81–3.95 (m, 1H), 4.02–4.12 (m, 1H), 4.21–4.34 (m, 1H), 4.42–4.52 (m, 1H), 4.56–5.10 (m, 23H), 6.92–7.42 (m, 45H); ^{31}P NMR ($CDCl_3$, H_3PO_4 as external reference) δ −0.89, −1.13 (d), −1.38,−1.68 (d). (−)-1-*O*-(1-*O*-Octyl-2-*O*-methyl-*sn*-glycero-3-benzyloxyphosphoryl)-2,6-di-*O*-benzyl-*myo*-inositol-3,4,5-tris-*O*-(dibenzylphosphate) [(−)-**7aMe**]: colorless syrup (16.0 mg, 81%); $[\alpha]_D^{20}$ −3.8° (*c* 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 10H), 1.42–1.56 (m, 2H), 3.28 (s, 3H), 3.81–3.96 (m, 1H), 4.04–4.12 (m, 1H), 4.20–4.34 (m, 1H), 4.41–4.55 (m, 1H), 4.58–5.12 (m, 23H), 6.95–7.44 (m, 45H); ^{31}P NMR ($CDCl_3$, H_3PO_4 as external reference) δ −0.89, −1.14 (d), −1.28, −1.69 (d). (−)-1-*O*-(1-*O*-Hexadecyl-2-*O*-methyl-*sn*-glycero-3-benzyloxyphosphoryl)-2,6-di-*O*-benzyl-*myo*-inositol-3,4,5-tris-*O*-(dibenzylphosphate) [(−)-**7bMe**]: colorless syrup (17.0 mg, 82%); $[\alpha]_D^{20}$ −2.2° (*c* 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 26H), 1.41–1.54 (m, 2H), 3.28 (s, 3H), 3.83–3.96 (m, 1H), 4.04–4.12 (m, 1H), 4.20–4.34 (m, 1H), 4.40–4.56 (m, 1H), 4.58–5.12 (m, 23H), 6.95–7.44 (m, 45H); ^{31}P NMR ($CDCl_3$, H_3PO_4 as external reference) δ −0.92, −1.14 (d), −1.26, −1.69 (d); HRMS: Calcd *m*/*z* for $C_{89}H_{110}O_{20}P_4$: 1622.654. Found: 1645.665 (M + Na). (−)-1-*O*-(1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-3-benzyloxyphosphoryl)-2,6-di-*O*-benzyl-*myo*-inositol-3,4,5-tris-*O*-(dibenzylphosphate) [(−)-**7cMe**]: colorless syrup (18.0 mg, 81%); $[\alpha]_D^{20}$ −2.1° (*c* 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, *J* = 6.9 Hz, 3H), 1.25 (br s, 30H), 1.41–1.52 (m, 2H), 3.28 (s, 3H), 3.83–3.96 (m, 1H), 4.04–4.12 (m, 1H), 4.20–4.34 (m, 1H), 4.40–4.56 (m, 1H), 4.58–5.12 (m, 23H), 6.96–7.44 (m, 45H); ^{31}P NMR ($CDCl_3$, H_3PO_4 as external reference) δ −0.94, −1.18 (d), −1.28, −1.69 (d).

General procedure for the preparation of C_8Ac-PIP$_3$, C_{16}Ac-PIP$_3$, C_{18}Ac-PIP$_3$, C_8Me-PIP$_3$, C_{16}Me-PIP$_3$, and C_{18}Me-PIP$_3$. A mixture of the aforementioned compound (0.008 mmol) and Pd black (10 mg) in 85% ethyl alcohol (4 mL) was shaken under H_2 (50 psi) for 16 h. The catalyst was removed by filtration, and the filtrate was concentrate in vacuo. The residue was diluted with distilled water, and lyophilized to yield the final product in acid form. (+)-1-*O*-(1-*O*-Octyl-*O*-2-acetyl-*sn*-glycero-3-phosphoryl)-*myo*-inositol 3,4,5-trisphosphate [**C_8Ac-PIP$_3$**]: amorphous powder (5.4 mg, 96%); $[\alpha]_D^{20}$ +6.3° (*c* 0.2, CH_3OH); ^{1}H NMR (CD_3OD) δ 0.89 (t, *J* = 6.9 Hz, 3H), 1.21–1.38 (m, 10H), 1.48–1.59 (m, 2H), 2.06 (s, 3H), 3.40–3.56 (m, 5H), 3.95–4.04 (m, 1H), 4.08–4.37 (m, 5H), 4.42 (br s, 1H), 4.62–4.54 (m, 1H); ^{31}P NMR (CD_3OD, H_3PO_4 as external reference) δ −0.59, −0.92, −1.30, −2.20. (+)-1-*O*-(1-*O*-Hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphoryl)-*myo*-inositol 3,4,5-trisphosphate [**C_{16}Ac-PIP$_3$**]: amorphous powder (6.4 mg, 96%); $[\alpha]_D^{20}$ +3.9° (*c* 0.2, CH_3OH); ^{1}H NMR (CD_3OD) δ 0.89 (t, *J* = 6.9 Hz, 3H), 1.28 (br s, 26H), 1.48–1.59 (m, 2H), 2.06 (s, 3H), 3.41–3.74 (m, 5H), 3.95–4.04 (m, 1H), 4.10–4.37 (m, 5H), 4.42 (br s, 1H), 4.62–4.54 (m, 1H); ^{31}P NMR (CD_3OD, H_3PO_4 as external reference) δ −0.68, −1.01, −1.38, −2.31. (+)-1-*O*-(1-*O*-Octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphoryl)-*myo*-inositol 3,4,5-trisphosphate [**C_{18}Ac-PIP$_3$**]: amorphous powder (6.6 mg, 96%); $[\alpha]_D^{20}$ +3.6° (*c* 0.2, CH_3OH); ^{1}H NMR (CD_3OD) δ 0.89 (t, *J* = 6.9 Hz, 3H), 1.28 (br s, 30H), 1.48–1.60 (m, 2H), 2.06 (s, 3H), 3.41–3.74 (m, 5H), 3.95–4.05 (m, 1H), 4.10–4.37 (m, 5H), 4.42 (br s, 1H), 4.62–4.54 (m, 1H); ^{31}P NMR (CD_3OD, H_3PO_4 as external reference) δ −0.59, −0.92, −1.30, −2.21. (+)-1-*O*-(1-*O*-Octyl-2-*O*-methyl-*sn*-glycero-3-phosphoryl)-*myo*-inositol 3,4,5-trisphosphate [**C_8Me-PIP$_3$**]: amorphous powder (5.8 mg, 96%); $[\alpha]_D^{20}$ +5.6° (*c* 0.2, CH_3OH); ^{1}H NMR (CD_3OD) δ 0.89 (t, *J* = 6.9 Hz, 3H), 1.28 (br s, 10H), 1.49–1.61 (m, 2H), 3.41–3.74 (m, 8H), 3.95–4.05 (m, 1H), 4.10–4.37 (m, 5H), 4.42 (br s, 1H), 4.62–4.54 (m, 1H); ^{31}P NMR (CD_3OD, H_3PO_4 as external reference) δ −0.50 (2P), −0.97, −1.94. (+)-1-*O*-(1-*O*-Hexadecyl-2-*O*-methyl-*sn*-glycero-3-phosphoryl)-*myo*-inositol 3,4,5-trisphosphate [**C_{16}Me-PIP$_3$**]: amorphous powder (6.5 mg, 96%); $[\alpha]_D^{20}$ +4.3° (*c* 0.2, CH_3OH); ^{1}H NMR (CD_3OD) δ 0.89 (t, *J* = 6.9 Hz, 3H), 1.28 (br s, 26H), 1.49–1.60 (m, 2H), 3.38–3.58 (m, 8H), 3.95–4.03 (m, 1H), 4.06–4.34 (m, 5H), 4.42 (br s, 1H), 4.62–4.74 (m, 1H); ^{31}P NMR (CD_3OD, H_3PO_4 as external reference) δ −0.27 (2P), −0.78, −1.76. HRMS: Calcd *m*/*z* for $C_{26}H_{56}O_{20}P_4$: 812.224. Found: 811.234 (M−H). (+)-1-*O*-(1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphoryl)-*myo*-inositol 3,4,5-trisphosphate [**C_{18}Me-PIP$_3$**]: amorphous powder (6.7 mg, 96%); $[\alpha]_D^{20}$ +3.4° (*c* 0.2, CH_3OH); ^{1}H NMR (CD_3OD) δ 0.89 (t, *J* = 6.9 Hz, 3H), 1.28 (br s, 30H), 1.49–1.60 (m, 2H), 3.38–3.60 (m,

8H), 3.95–4.04 (m, 1H), 4.06–4.36 (m, 5H), 4.42 (br s, 1H), 4.61–4.74 (m, 1H); ^{31}P NMR (CD_3OD, H_3PO_4 as external reference) δ −0.18 (2P), −0.72, −1.65.

Fluorescence spectrophotometric measurement of intracellular Ca^{2+}

$[Ca^{2+}]$i was monitored by changes in the fluorescence intensity of fura-2-loaded cells. Jurkat T cells (1×10^7 cells/mL), suspended in assay buffer consisting of 4.3 mM Na_2HPO_4, 24.3 mM NaH_2PO_4, 4.3 mM K_2HPO_4, 113 mM NaCl, 5 mM glucose, pH 7.4, were incubated with 10 μM fura-2 AM in dark for 1 h at 37 °C. The cells were then pelleted by centrifugation at $1000\times g$ for 10 min, washed with assay buffer twice, and resuspended at approximately 8×10^5 cells/mL in the same buffer containing 1 mm Ca^{2+}. The effect of various inositol lipids and PIP_3 derivatives on $[Ca^{2+}]$i was examined by fura-2 fluorescence in a Hitachi F-2000 spectrofluorimeter at 37 °C with excitation and emission wavelengths at 340 and 510 nm, respectively. The maximum fura-2 fluorescence intensity (F_{max}) in Jurkat cells was determined by adding A23187 (1 μM), and the minimum fluorescence (F_{min}) was determined following depletion of external Ca^{2+} by 5 mM EGTA. The $[Ca^{2+}]$i was calculated according to the equation $[Ca^{2+}]i = K_d(F-F_{min})/(F_{max}-F)$, where K_d denotes the apparent dissociation constant (= 224 nM) of the fluorescence dye–Ca^{2+} complex.[26]

Acknowledgements

This work was supported by National Institutes of Health grants R01 GM53448.

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