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THE STRUCTURAL REQUIREMENT OF PHOSPHATIDYLINOSITOLS AS SUBSTRATE OF PHOSPHATIDYLINOSITOL 3-KINASE

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Abstract: Synthetic distearate phosphatidylinositol (PI) was not phosphorylated by PI 3-kinase. The fatty acids at glycerol *sn*-2 must be as short as octanoic acid or less to act as a substrate of PI 3-kinase. © 1999 Elsevier Science Ltd. All rights reserved.

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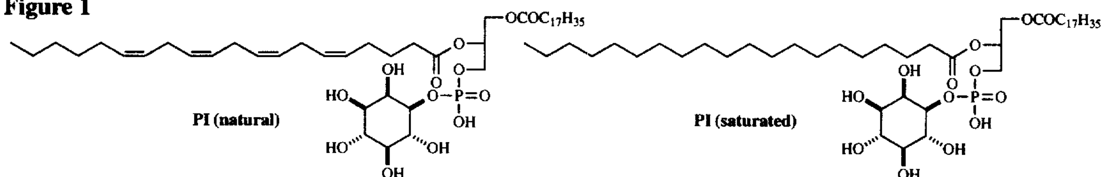
Phosphatidylinositol 3-kinase (PI 3-kinase) is a key enzyme in the signaling pathways of 3-phosphorylated polyphosphoinositides.[1] *In vitro,* PI 3-kinase phosphorylates phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI 4-P), and phosphatidylinositol 4,5-bisphosphate (PI 4, 5-P_2). The major substrate *in vivo* is assumed to be PI 4, 5-P_2, generating phosphatidylinositol 3,4,5-trisphosphate (PI 3, 4, 5-P_3), a putative second messenger which plays pivotal roles in activating GTP-GDP exchanging factor for Rac or ARF1, serine-threonine kinases such as PDK1, nPKCs, and Tec family tyrosine kinases.[2] PI 3, 4, 5-P_3 is dephosphorylated by specific PIP_3 5-phosphatases to give phosphatidylinositol 3,4-bisphosphate (PI 3, 4-P_2).[1] Though several reports describe the importance of the fatty acids in the diacylglycerols (DAG) substructure, systematic analysis focusing on their enzymatic reactions has not been carried out by other groups.[3]

The natural PI purified from bovine liver, which mainly contains arachidonate at the *sn*-2 position, is an excellent substrate of PI 3-kinase (Figure 1). On the other hand, we unexpectedly have found that synthetic distearate PI, synthetically hydrogenated natural PI and PI 4, 5-P_2 were not phosphorylated by PI 3-kinase.[4,5] The *sn*-2 side chain of DAG might play a critical role in the enzymatic reaction. In attempts to develop phosphatidylinositol analogs as biochemical probes and/or synthetic second messenger molecules, the saturated DAG substructure should provide a feasible basis for molecular design.[5,6] In the previous article, we reported the dephosphorylation

Figure 1



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of synthetic PI 3, 4, 5-P_3 with varied *sn*-2 side chains by PIP_3 5-phosphatases.[7] Here, we describe the optimization of the *sn*-2 fatty acids of PI by evaluating the phosphorylation reaction with PI 3-kinase.

First, a standard fatty acids mixture ($PI_{mixture}$) of PI_{C4}, PI_{C6}, PI_{C8}, PI_{C10}, PI_{C12}, PI_{C14} and PI_{C16} at the *sn*-2 center was synthesized from an equimolar mixture of seven carboxylic acids (C4, C6, C8, C10, C12, C14, C16). Respective PI with varied fatty acids was also synthesized independently (Scheme 1). The above standard PI mixture was subjected to the enzymatic reaction with PI 3-kinase.[8,9] Negative ion fast atom bombardment (FAB) mass spectrometric analysis[10,11] of the crude enzymatic reaction mixture using a matrix (triethanolamine:glycerol = 3:1) showed the apparent ion peaks of monophosphorylated PI_{C4}, PI_{C6} and PI_{C8} analogs (Figure 2). The phosphorylated ions derived from the longer chain analogs such as PI_{C16} were detected only at trace levels.

Scheme 1

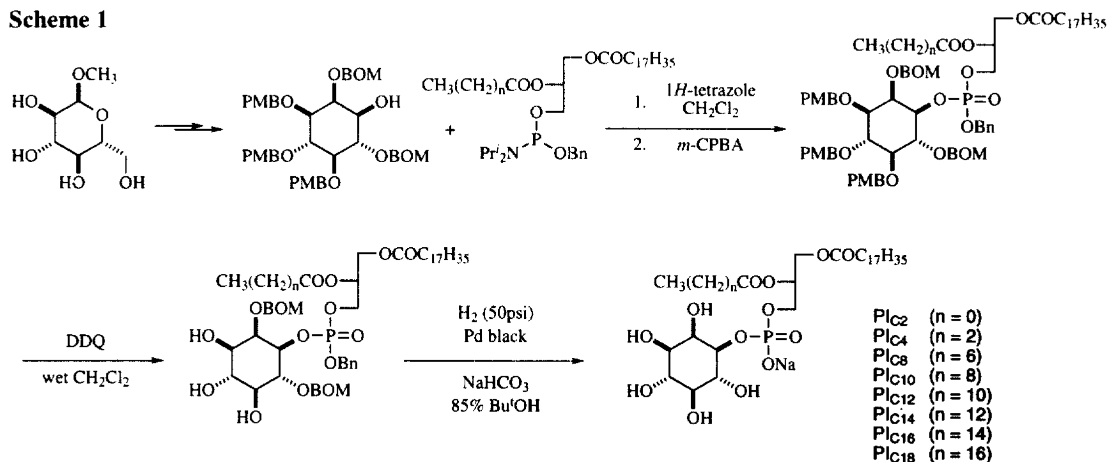
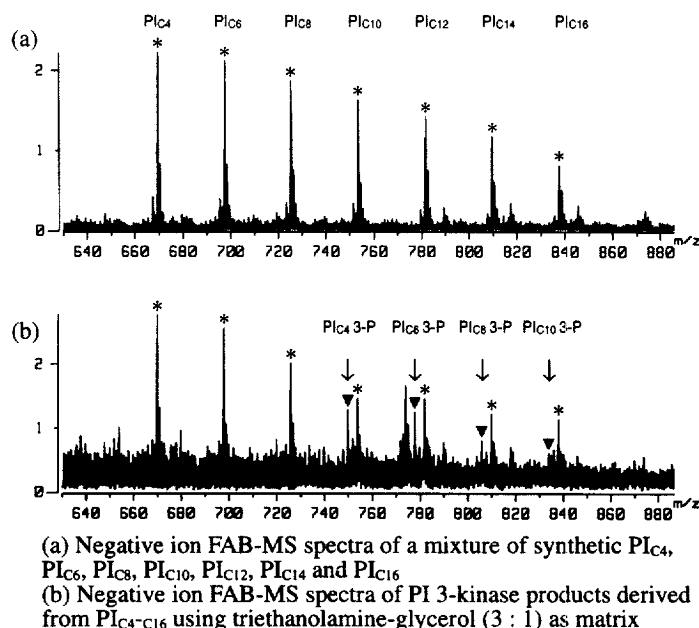
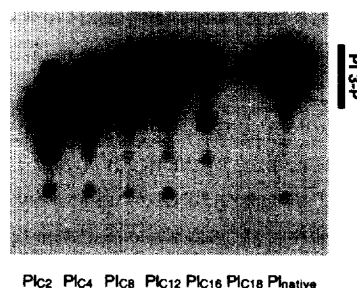


Figure 2



(a) Negative ion FAB-MS spectra of a mixture of synthetic PI_{C4}, PI_{C6}, PI_{C8}, PI_{C10}, PI_{C12}, PI_{C14} and PI_{C16}
(b) Negative ion FAB-MS spectra of PI 3-kinase products derived from PI_{C4}-$_{C16}$ using triethanolamine-glycerol (3 : 1) as matrix

Figure 3

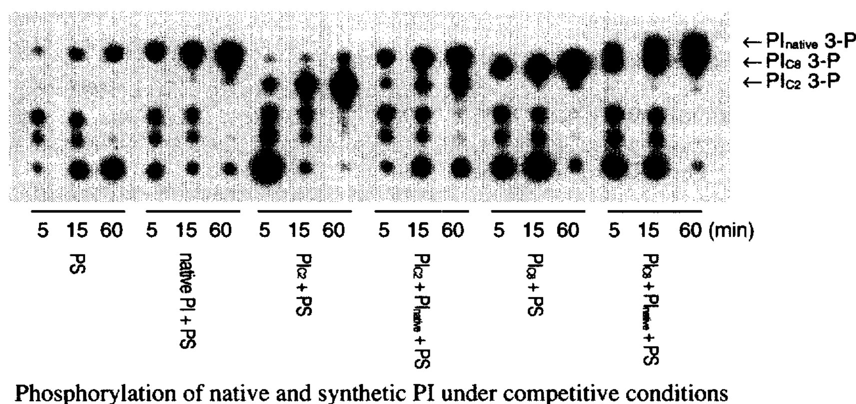


Independent phosphorylation of unsaturated native PI and saturated unnatural PIs with PI 3-kinase and [γ-^{32}P] ATP

Second, we found that short *sn*-2 fatty acid analogs such as PI_{C4} and PI_{C8} are excellent substrates of PI 3-kinase by means of independent phosphorylation experiments with synthetic PI_{C2}, PI_{C4}, PI_{C8}, PI_{C12}, PI_{C16} and PI_{C18} (Figure 3).[12] It is intriguing that the phosphorylation of both PI_{C4} and *sn*-2-epimer of PI_{C4} by PI 3-kinase proceeded without any distinction (data not shown).

Third, the reactivity of PI_{C2} and PI_{C8} was directly compared with natural PI by competitive phosphorylation as follows. A 1 : 1 mixture of both PIs was subjected to phosphorylation by PI 3-kinase in the same reaction vessel. Micelles containing PI_{C8} and natural PI were prepared using phosphatidylserine (PS) as a carrier. After PI 3-kinase reaction with [γ–^{32}P] ATP, the resulting products were analyzed by TLC. As the mobility of PI_{C8} 3-P was smaller than that of native PI 3-P, the radioactivity of respective PI 3-P was quantitated using imaging analyzer (Figure 4). The relative amounts of 3-[^{32}P]-phosphorylated PI_{C8} and natural PI (PI_{C8} 3-P / natural PI 3-P) were determined to be 1.17 / 1 (kinase reaction: 5 min), 2.52 / 2.64 (15 min), and 4.02 / 4.54 (60 min).

Figure 4



Phosphorylation of native and synthetic PI under competitive conditions

The structural requirement for saturated PI to act as a substrate of PI 3-kinase is thus as follows: the fatty acids at glycerol *sn*-2 must be as short as octanoic acid or less, and the absolute configuration of the glycerol moiety is not crucial. Considering the folding and free rotation of arachidonate side chains, the spherical size of the whole DAG substructure is more important than the number of methylene units of the *sn*-2 fatty acid.[13] The present results open a new window on the design of phosphatidylinositols with saturated *sn*-2 fatty acids, which are apparently more stable than natural ones, and may have a wide range of applications in creating artificial second messenger molecules, enzyme inhibitors, affinity probes to find specific binding proteins and photoaffinity labeling probes.[5]

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8. The PI (20-30 μg/mL) and phosphatidylserine (200-300 μg) were dissolved in DMSO. Micelles were formed by dispersing the above solution in a PI 3-kinase assay buffer. The reaction was started by adding ATP (10 μM), $MgCl_2$ (5 mM final) and purified PI 3-kinase, and the mixture was incubated for 1 hr. The lipid was extracted with $CHCl_3$ and dried *in vacuo*.
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11. FAB mass spectra were recorded on a JEOL JMS-HX110 double-focusing mass spectrometer of EBE arrangement with a JMS-DA7000 data system. Ion acceleration voltage was 10 kV.
12. Synthetic PI_{cn} with the chain lengths indicated in Figure 3 were tested. The reaction was done as described in ref. 8 except that [γ-^{32}P] ATP (5 μCi/reaction) was added to the reaction mixture. The resulting phospholipids were analyzed by TLC using Silica Gel 60 glass plates pretreated with MeOH - 1% aqueous potassium oxalate(1 : 1) and activated at 70°C. Development was performed with a solvent system of H_2O : AcOH : MeOH : acetone : $CHCl_3$ = 7 : 12 : 13 : 15 : 40.
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