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A THIOPHOSPHATE ANALOG OF DIMYRISTOYLPHOSPHATIDYL-INOSITOL-4-PHOSPHATE IS A SUBSTRATE FOR MAMMALIAN PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C

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Abstract

1,2-Dimyristoyloxypropane-3-thiophosphate(*rac*-1-*myo*-inositol-4-phosphate), a thiophosphate analog of dimyristoyl phosphatidylinositol-4-phosphate was synthesized as a substrate for mammalian phosphoinositide-specific phospholipase C. Its activity with Δ(1-132)-PI-PLC-δ_1 (a deletion mutant with the N-terminal pleckstrin homology domain removed) was studied in sonicated dispersions, with and without added Triton X-100. It had an initial activity of about 30 μmol min^{-1} mg^{-1}, which rapidly decreased due to substrate depletion in the vesicle or micelle. The slower rate of hydrolysis appeared limited by enzyme hopping or exchange of substrate between vesicles or micelles, which was more rapid in the presence of detergent.

Introduction

Phosphoinositide-specific phospholipase C (PI-PLC†; EC 3.1.4.11) from mammalian cells represents a large family of closely-related enzymes that play a key role in signal transduction through the hydrolysis of phosphatidylinositol bisphosphate to two second messengers, inositol trisphosphate and diacylglycerol.[1, 2, 3, 4] We previously reported the use of a thiophosphate analog of phosphatidylinositol, D-thio-DMPI, to assay PI-PLC from *Bacillus cereus*[5] and a deletion mutant (Δ1-132) of the mammalian enzyme.[6] The latter is a variant of PI-PLC-δ_1 with the N-terminal pleckstrin homology domain removed.[7] This enzyme gave a specific activity with D-thio-DMPI of about 4 μmol min^{-1} mg^{-1} [6] or one-fourth that with natural PI.[8] PI-PLC-δ_1 is most active with PIP_2, but will also catalyze the hydrolysis of PIP and PI at lower rates.[9]

Here we describe the synthesis of a thiophosphate analog of PIP, *rac*-thio-DMPIP, and its use as a substrate for Δ(1-132)-PI-PLC-δ_1. The reaction produces inositol-1,4-bisphosphate and a thiodiglyceride. The latter is coupled to a reaction with dithiobispyridine (DTP) to produce a chromophore that is detected at 324 nm (Scheme 1).

Experimental

Rac-thio-DMPIP was synthesized starting with 1,2,4,5-biscyclohexylidene-*myo*-inositol[10] (Scheme 1). The 3(1)-position was protected with a t-butyldiphenylsilyl ether[11] and a dimethylphosphate group was esterified at the 6(4)-position with $(CH_3O)_3PI^+$ generated in situ from $(CH_3O)_3P$ + I_2 as described by Stowell and

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† Abbreviations: PI-PLC, phosphoinositide-specific phospholipase C; D-thio-DMPI, 1,2-dimyristoyloxy-propane-3-thiophosphate(1D-1-*myo*-inositol); *rac*-thio-DMPIP, 1,2-dimyristoyloxypropane-3-thiophosphate-(*rac*-1-*myo*-inositol-4-phosphate); DTP, 4,4'-dithiobispyridine; PI, phosphatidylinositol; PIP, phosphatidyl-inositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate.

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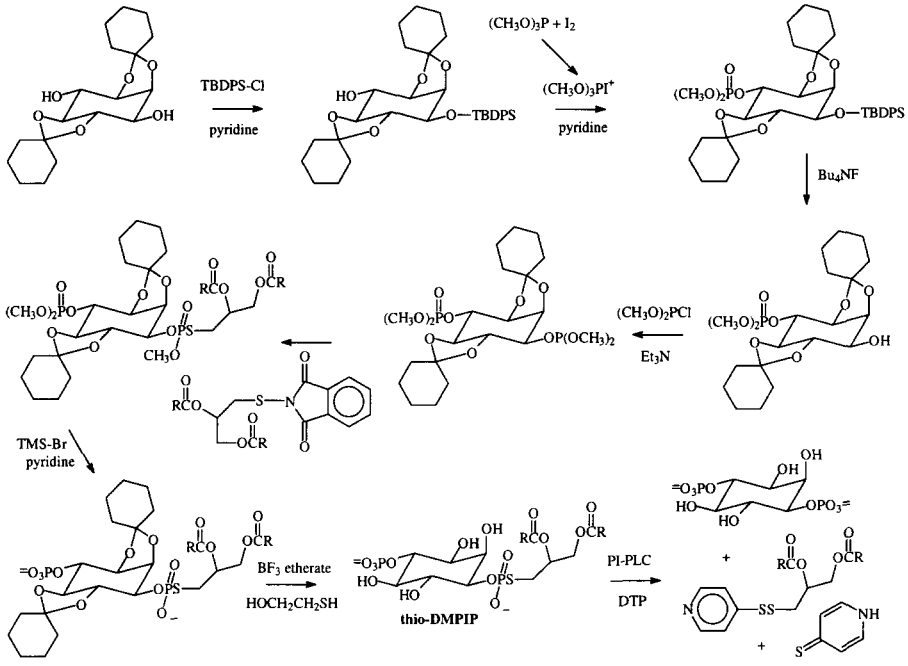

Scheme 1

Widlanski.[12] The silyl ether was removed using tetrabutylammonium fluoride and a 1,2-dimyristoyloxypropane-3-thiophosphoester was added to the free 3(1)-position by an Arbusov reaction of the dimethyl phosphite derivative with 1,2-dimyristoyloxypropane-3-thiophthalimide as described by Hendrickson et al.[13] The phosphate methyl esters were cleaved by treatment with trimethylsilyl bromide in methylene chloride and pyridine at room temperature for 24 h. The cyclohexylidino protecting groups were removed by treatment with BF_3-etherate in the presence of β-mercaptoethanol (2 h, rt) as described by Bushnev et al.[14] The final product was isolated as the triethylammonium salt and purified by chromatography on silica gel. It gave a single spot by TLC on silica gel (R_f = 0.38, $CHCl_3/CH_3OH/4N\ NH_3$, 9:7:2) and had a mass spectrum[15] and ^{1}H-NMR spectrum consistent with its structure. *Rac*-thio-DMPIP was not hydrolyzed by PI-PLC from *B. cereus*, which is specific only for PI. Incubation of the racemic substrate with excess mammalian enzyme resulted in 49% hydrolysis, indicating that the substrate is 98% pure.

PI-PLC[16] was assayed as previously described[6] with sonicated lipid dispersions of thiophosphate substrate analogs in the presence of DTP at 30 °C.[17] Plots of A_{324} vs. time were fitted to a double first-order rate equation $A_{324} = Limit1(1 - e^{-k_1 t}) + Limit2(1 - e^{-k_2 t})$ by nonlinear least-squares regression using the computer program Sigma Plot (Jandel Scientific, San Rafael, CA). The initial rate is equal to (Limit1 · k_1 + Limit2 · k_2). Limit1 and Limit2 represent the limiting values of A_{324} for each first-order rate term (k_1 and k_2 respectively).

Results and Discussion

Addition of PI-PLC to 0.4 mM *rac*-thio-DMPIP in the presence of DTP resulted in an initial rapid increase in absorbance followed by a slower increase (Fig. 1).[18] The data were fitted to a double first-order rate equation where k_1 = 0.116 sec^{-1}, k_2 = 0.0035 sec^{-1}, Limit1 = 0.0272, Limit2 = 0.0891. The initial rate was 30.9 μmol min^{-1} mg^{-1}. Addition of a second portion of enzyme resulted in a similar rapid and slow increase in absorbance. Enzyme activity calculated from the initial rates of five experiments (0.08 - 0.64 μg of enzyme) averaged 24.9 ± 4.9 μmol min^{-1} mg^{-1}. Limit1 represents the point where substrate depletion may become rate limiting and the rate is then dependent on exchange of substrate between vesicles or hopping of enzyme from one vesicle to

another. This limit increases with the amount of enzyme added (Fig. 2). Assuming about 4,500 lipid molecules per vesicle,[19] there is an excess of vesicles over enzyme molecules. The initial ratio of substrate molecules hydrolyzed to enzyme molecules (from Fig. 2) is 372; that would represent about 10-20 percent of the substrate on the outer surface of the vesicle hydrolyzed before substrate depletion becomes rate limiting.[19] This is consistent with a hypothetical model where one enzyme binds tightly to a lipid vesicle and hydrolyzes substrate in a processive or scooting mode.[20] The slow rate of hydrolysis after substrate depletion is then limited either by a slow hopping of enzyme from vesicle to vesicle or exchange of substrate between vesicles.

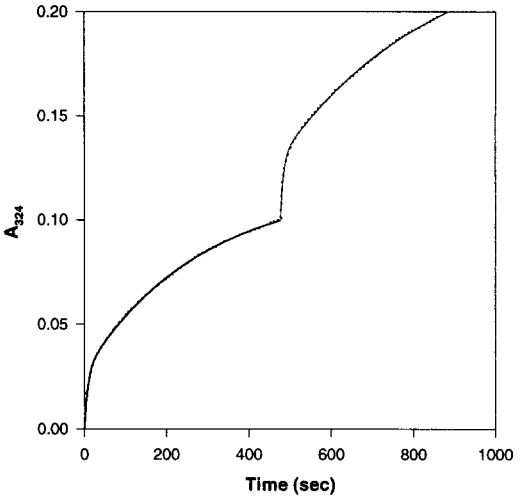

Figure 1. Time course for addition of PI-PLC to 0.4 mM *rac*-thio-DMPIP. Enzyme (80 ng, 1.14 pmol) was added at 0 and 477 sec. Dotted line, experimental curve; solid line, fit to a double first-order rate equation.

In Triton X-100 mixed micelles (0.4 mM *rac*-thio-DMPIP, 0.8 mM Triton X-100) the time course (Fig. 3) fitted a double first-order rate equation where $k_1 = 0.0262$ sec^{-1}, $k_2 = 0.00345$ sec^{-1}, Limit1 = 0.0445, and Limit2 = 0.319; the initial rate was 33.6 μmol min^{-1} mg^{-1}. Addition of a second portion of enzyme resulted in another rapid and slow increase in absorbance (data not shown) similar to that seen in Fig. 1. Enzyme activity calculated from the initial rates of four experiments (0.032 - 0.08 μg of enzyme) averaged 33.8 ± 2.1 μmol min^{-1} mg^{-1}. Limit1 represents 770 substrate molecules hydrolyzed per enzyme molecule. This larger limit indicates a greater rate of enzyme hopping or substrate exchange between micelles in the presence of detergent. The similar initial enzyme activity in the presence and absence of detergent indicates that enzyme activity is not greatly influenced by detergent.

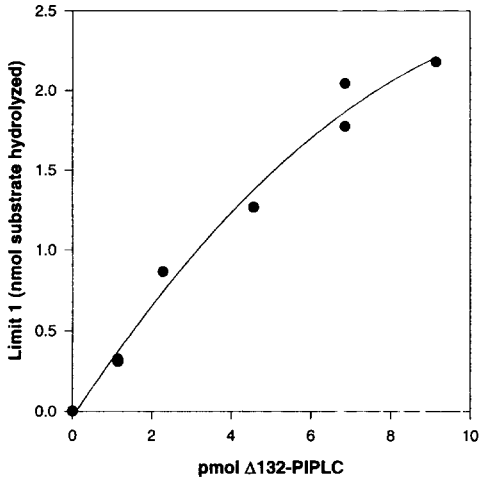

Figure 2. Limit1 of initial fast hydrolysis of 0.4 mM *rac*-thio-DMPIP as a function of the amount of enzyme added.

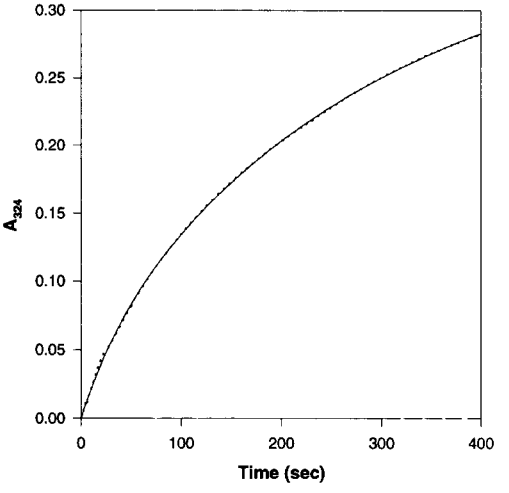

Figure 3. Time course for addition of PI-PLC to 0.4 mM *rac*-thio-DMPIP in the presence of 0.8 mM Triton X-100. Enzyme (48 ng, 0.69 pmol) was added at 0 sec. Dotted line, experimental curve; solid line, fit to a double first-order rate equation.

Mammalian PI-PLC appears to be at least moderately processive (scooting mode) with *rac*-thio-DMPIP. This is in contrast with its activity towards D-thio-DMPI, [6] which shows no indication of substrate depletion through the first few minutes of reaction.[21] The initial rate with *rac*-thio-DMPIP is not significantly influenced by the presence of detergent, although with detergent the slower rate due to enzyme hopping or exchange of lipid between vesicles is enhanced.

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15. Mass spectrum, negative ion electrospray: m/e 849.2, 424.4 (calc. for MH_2^-, $C_{37}H_{71}P_2SO_{15}$, m/e 849.4; calc. for MH^{2-}, $C_{37}H_{70}P_2SO_{15}$, m/e 424.2).
16. Δ(1-132)-PI-PLC-δ_1 (cloned from rat, 70 kDa) was a generous gift from Dr. R. L. Williams, MRC, Cambridge, UK.
17. Aliquots of lipid in chloroform-methanol were dried under a stream of nitrogen and then under high vacuum. Buffer (50 mM MES, pH 7, 0.1 M NaCl) was added and the solution was vortexed and sonicated to give a clear dispersion. Then Ca^{2+} (0.2 mM) was added and the dispersion was again sonicated. The standard assay contained 225 μL of 0.4 mM *rac*-thio-DMPIP, 0.2 mM $CaCl_2$, 0.1 M NaCl, and 50 mM MES, pH 7. DTP (5 μL of 50 mM in ethanol) was added and the background absorbance was recorded at 324 nm (30 °C), after which 5 μL of enzyme was added.
18. At higher calcium ion concentration (0.4 mM), in the presence of Triton X-100, the initial rate increased by about 30%, but the amount of curvature was about the same. This indicates that the curvature is not due to calcium ion depletion upon increased binding to product.
19. Assuming a diameter of 25 nm^2 for SUVs, 0.6 nm^2/molecule and 73% of molecules on the outer layer, there are 4,500 molecules per vesicle, and with racemic lipid, 1,600 (0.5 x 0.73 x 4,500) substrate molecules on the outer layer. A larger SUV of 40 nm^2 would have 15,000 molecules per vesicle and about 4,200 substrate molecules on the outer layer.
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21. With D-thio-DMPI the time course was linear up to about 10% hydrolysis of the total lipid substrate.