Effect of Thiophosphatidate on *in vitro* Protein Phosphorylation

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Phosphatidic acid (PA) stands at a branch point in glycerophospholipids biosynthesis and hence is a key intermediate in the formation of phosphoglyceride substances. PA does not accumulate because of phosphatidate phosphohydrolase (PAP) and cytidine diphosphate diacylglycerol (CDP-DG) synthase. These two pathways consume PA rapidly so that there exists always very small amount of PA in cells compared with other phospholipids. Numerous observations suggest that PA exerts a variety of biological effects. For example, PA correlates closely with secretion and superoxide generation in neutrophil,[1] membrane trafficking,[2] cell proliferation,[3] and actin polymerization.[4] Other important regulatory proteins whose activity can be activated directly by PA *in vitro* include phosphatidyl inositol 4-phosphate 5-kinase,[5] protein tyrosine phospatase 1C,[6] phospholipase Cγ,[7] protein kinase Cζ,[8] and Raf-1.[9]

In spite of importance of PA, the study of its physiological effect was hampered because exogenously added PA is destroyed rapidly by various metabolizing enzymes. The availability of long lasting analog of PA would provide means for investigating PA function *in vivo* as well as *in vitro*. In this regard phosphorothioate analog of PA is a particularly attractive candidate. A nonbridging oxygen atom in the phosphate group replaced by a sulfur atom makes thioPA resistant to the hydrolysis of phosphodiester bonds. Many phosphorothioate analogs of biomolecules have been constructed as reversible and irreversible inhibitors, transition state analogs, suicide substrates, or spectroscopic probes.[10] They include nucleoside phosphorothioates like ATP-γ-S, GTP-γ-S and phosphorothioate analogs of phospholipids such as thiophosphophatidylcholine, thiophosphatidylethanolamine, and thioPA. But the uses of thiophospholipids were limited to demonstrating stereoselectivity in the hydrolytic actions of isolated phospholipases A_2,[11] phospholipase C,[12] and phospholipase D.[13] For metabolic study, thioPA was used for elucidating its interaction with PAP and CDP-DG synthase.[14]

In this study, we synthesized thioPA as an analog of PA and checked its stability against PAP. To determine whether thioPA has any advantage in eliciting the effect of PA, we examined *in vitro* protein phosphorylation using cytosols of rat brain and heart. For more information on thioPA specificity, the phosphorylation profile was partially characterized with several effectors for the protein phosphorylations.

Experimental

ATP [γ^{32}P] was purchased from Dupont NEN (U.S.A.). 1, 2-dioleoyl-*sn*-glycerol and 1,2-dioleoyl phosphatidic acid were purchased from Sigma (U.S.A.). $PSCl_3$ was obtained from Aldrich (U.S.A.) and TLC plate (Silica gel 60 F_{254}) was from Merck (Germany). X-ray film was obtained from Kodak (CAT 871-5187). All other chemicals were of reagent grade commercially available.

ThioPA was synthesized as reported earlier by Bonnel *et al.*[14] and purified by normal phase HPLC according to the procedure of Keller *et al.*[15] with minor modification. A portion of 50 mg 1,2-dioleoyl-*sn*-glycerol (0.0746 mmole) dissolved in 0.5 mL dehydrated methylenechloride was added dropwise over a period of 30 min to 0.10 mmole $PSCl_3$ and 0.20 mmole pyridine in dehydrated methylenchloride (0.1 mL). After 12 hrs at room temperature, 0.1 mL pyridine/water (1 : 1 by volume) was added to the crude thiophosphoryl dichloride dissolved in 2.5 mL tetrahydrofuran. The opaque mixture was filtered to remove pyridinium chloride and the oily crop was dissolved in hexane/isopropanol/H_3PO_4 (90 : 10 : 0.03 by volume). The thioPA was purified by normal phase μ-porasil HPLC column and each peak was examined by TLC using ethylacetate/isooctane/acetic acid/water (130 : 20 : 30 : 100 by volume) as a developing solvent. The molecular weight of thioPA peak was further confirmed using FAB mass spectrometry.

Rats (female Wistar, 4 weeks old) were killed by a guillotine and organs (brain and heart) isolated were immediately placed in ice-cold solution containing 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, and 50 mM Tris-HCl (pH 7.4). The tissues were minced in this buffer, homogenized, and centrifuged at 37,000 × g for 20 min. The supernatants were used as enzyme sources. To disrupt lysosomes, in some cases, the supernatant was frozen at − 20 °C and thawed.

To compare the metabolic rate of thioPA with that of PA, turn-over rate of PA/thioPA was determined in the presence of radioactive ATP. The reaction mixture consisted of 20 mM Tris-HCl buffer (pH 7.4), 2 mM $MgCl_2$, 1 mM EGTA, 0.05 mM [γ^{32}P]ATP (600 cpm/pmol), 15 mM sodium fluoride, 450 μg rat brain cytosol proteins, and 200 μg PA or thioPA in a final volume of 0.23 mL. After incubating at 30 °C for 60 min, the reaction mixture was extracted with 2 mL $CHCl_3$/methanol (2 : 1 by volume). After washing with 0.1 M KCl, the organic mixture was dried, dissolved in 0.1 mL $CHCl_3$/methanol (2 : 1 by volume), and analyzed by TLC. TLC plate was developed using ethylacetate/isooctane/acetic acid/water (130 : 20 : 30 : 100 by volume) and visualized by I_2 vapor. The band of PA/thioPA was scraped and its radioactivity was determined.

Protein phosphorylation was performed as described by Boccking *et al.*.[16] Samples of supernatant fractions (180 μg

proteins for each tube) were incubated with 20 mM Tris-HCl (pH 7.4), 0.1 mM ATP (500 cpm/pmol), 3 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, and appropriate amount of effectors in a final volume of 100 μL. The reaction mixture was incubated for 10 min at 37 °C and the reaction was quenched by addition of 100 μL of 2× concentrated SDS/PAGE sampling buffer. SDS/PAGE was carried out and subjected to autoradiography with Kodak BioMax MR-1 film. Exposures were usually 24-48 h. In some experiments, the radioactive protein bands were excised from the gels and subject to liquid scintillation counting.

Result and Discussion

Resistance of thioPA to hydrolysis by PAP could be examined by phosphate group turnover-system exploiting cytosol as a source for PAP and DG kinase. In the presence of [γ-^{32}P]ATP, DG produced by PAP is rephosphorylated by DG kinase to make [^{32}P]PA. Once hydrolyzed by PAP, PA or thioPA gives the same product, dioleoyl-DG, hence the radioactive phosphorus incorporation rate should be identical. Therefore the radioactivity recovered in PA/thioPA is a good way of estimating the stability of the PA analog without preparing corresponding isotope labeled compound. Under the assay condition established, the radioactivity of the phosphate group of thioPA was 125 times lower than that of PA (Figure 1). This result confirmed the overwhelming (more than 100 times compared with PA) stability of thioPA against PAP present in the brain cytosol. This observation that thioPA undergoes much slower turnover is consistent with the previous report that thioPA acts as a competitive inhibitor for PAP activity.[14] Other phosphorothioate analogs of nucleosides and thiophosphorylated proteins were also shown to be resistant to hydrolysis by corresponding phosphatases.[10]

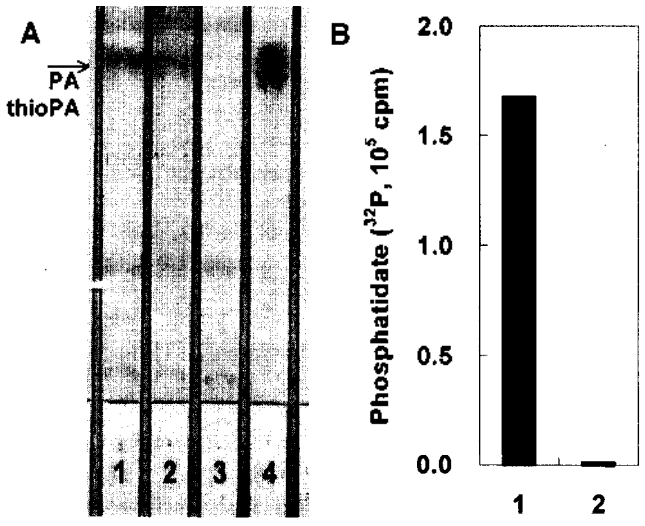

Figure 1. Comparison of *in vitro* turn-over rates of PA and thioPA in brain cytosol. Radiolabeling of PA/thioPA was carried out in the presence of [γ-^{32}P] ATP. A, same amount of thioPA or PA was incubated and total lipid extract was separated by TLC: lane 1, PA+cytosol; lane 2, thioPA+cytosol; lane 3, cytosol; lane 4, authentic PA. B, the radioactivity count of PA (lane 1) and thioPA (lane 2) bands.

As we have noted, thioPA undergoes much slower phosphate-hydrolysis and this property could make thioPA a long-lasting analog for testing the effect of PA. When thioPA was tested on its effect on protein phosphorylation in brain cytosol, the phosphorothioate analog doubled the total protein phosphorylation compared with that of PA (Figure 2). The two-fold enhancement effect was apparent even with the lysosome-disrupted enzyme source. The disruption of lysosomes by freeze-and-thawing released extra PAP and the increased PAP activity reduced the overall phosphorylation level. However thioPA-induced hyperphosphorylation was also observed.

To determine whether the protein phosphorylation induced by thioPA was selective or not, several factors known to affect phosphorylation were examined in the cytosol fractions of rat brain and heart. ThioPA and phosphatidyl serine (PS) as well as protein phosphatase inhibitors increased overall protein phosphorylation as expected (Figure 3). In heart cytosol, a 30 kDa protein was phosphorylated by PA, thioPA or PS (Figure 3A). Protein phosphatase inhibitors increased markedly phosphorylated proteins especially when microcystin, a serine/threonine

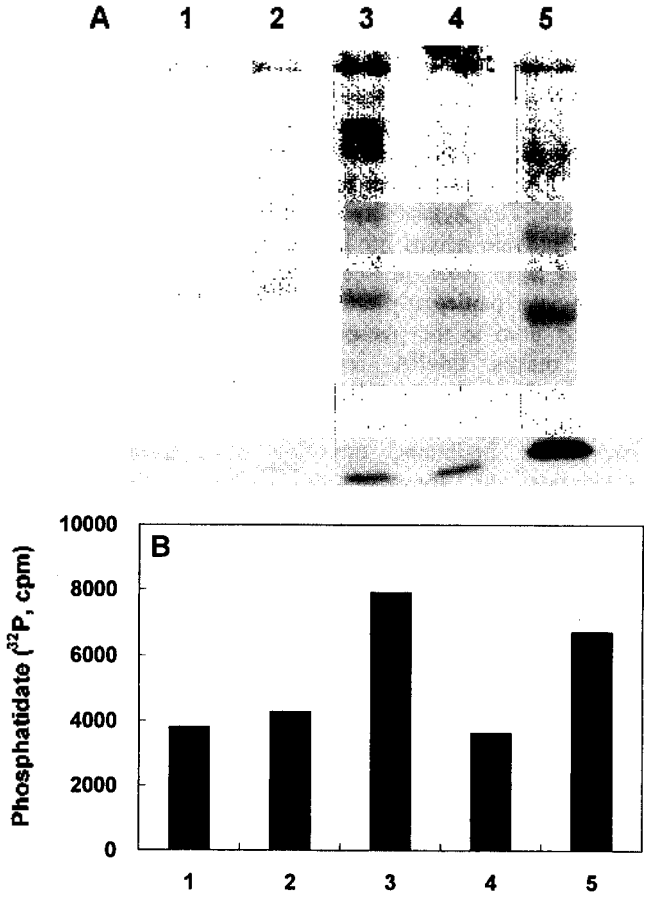

Figure 2. Enhancement of total protein phosphorylation by thioPA. Rat brain cytosol fraction was incubated with [γ-^{32}P]ATP (500 cpm/pmol) and the SDS/PAGE was carried out using 12.5% gel, 5 cm long (Hoefer® SE 250 Series). The exposure time was 24 h. A, autoradiography. B, total radioactivity: lane 1, control; lane 2, PA (100 μg/mL); lane 3, thioPA (100 μg/mL); lane 4, PA (100 μg/mL) with lysosome-disrupted enzyme source; lane 5, thioPA (100 μg/mL) with lysosome-disrupted enzyme source.

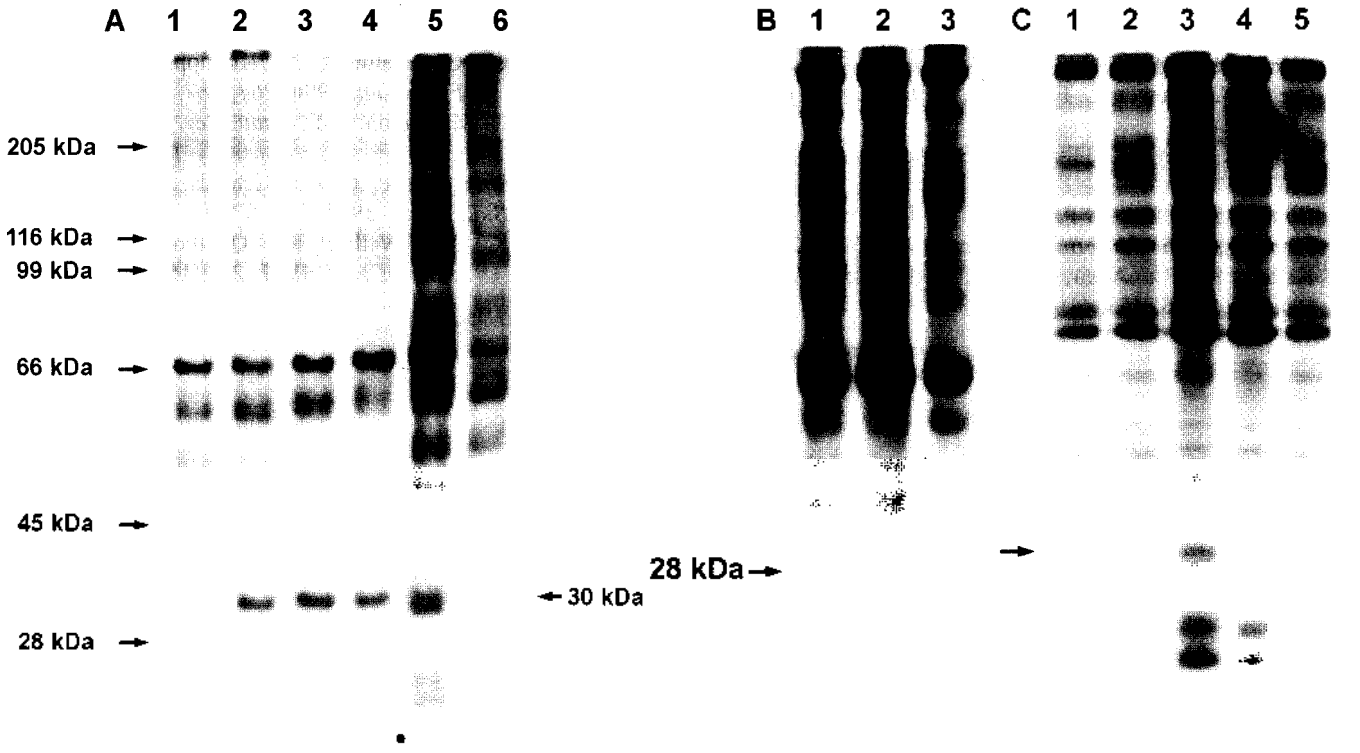

Figure 3. Autoradiography of *in vitro* protein phosphorylation induced by thioPA in the presence of some effectors. SDS-PAGE was performed using 12.5% gel, 14 cm long (Hoefer® SE 600 Series). The exposure time was 48 h. A, protein phosphorylation in rat heart cytosol: lane 1, control; lane 2, PA (100 μg/mL); lane 3, thioPA (100 μg/mL); lane 4, PS (100 μg/mL); lane 5, microcystin (1.5 μM); lane 6, vanadate (1 mM). B, selective phosphorylation of a 28 kDa protein in brain cytosol by thioPA: lane 1, thioPA (100 μg/mL); lane 2, microcystin (1.5 μM); lane 3, vanadate (1 mM). C, effect of protein phosphatase inhibitors on the thioPA induced phosphorylation in 28 kDa protein: lane 1, control; lane 2, thioPA (100 μg/mL); lane 3, thioPA (100 μg/mL)+microcystin (1.5 μM); lane 4, microcystin (1.5 μM); lane 5, thioPA (100 μg/mL)+vanadate (1 mM).

protein phosphatase inhibitor,[17] was added. However in the presence of vanadate, known as a tyrosine phosphatase inhibitor,[18] the phosphorylation of 30 kDa protein reduced significantly. The phosphorylation of 30 kDa protein in the heart cytosol is in good agreement with the previous report by Bocckino *et al.*.[16] In brain cytosol, we were able to observe phosphorylation of a 28 kDa brain protein that was induced by thioPA (Figure 3B). But none of protein phosphatase inhibitors themselves could increase the phosphorylation of 28 kDa protein. It seems therefore that the induction of phosphorylation by thioPA is different from simple inhibition of dephosphorylation. This observation is parallel to reports of existence of PA dependent protein kinase(s) in human platelet[19] and neutrophils.[20] In combination of thioPA and protein phosphatase inhibitors, the degree of phosphorylation in 28 kDa brain protein was found to be altered (Figure 3C). Microcystin brought additional increase in radioactivity (lane 3) but vanadate ion didn't have any effect (lane 5). This observation indicates that the radioactive phosphorus might be incorporated into serine/threonine residue of the protein because its thioPA-induced phosphorylation was protected by the inhibitor of serine/threonine protein phosphatase. Although we have not yet investigated the 28 kDa protein in detail, this protein could be an experimental manifestation of the action of long-lasting thioPA as an analog for PA effect in *in vitro* system.

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ERRATUM

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The previous table 3 on the page 1004 is corrected as shown below.

Table 3. Comparison of mole ratio Zn/Al for samples of H-Y and Na-A after the exposure to Zn vapor followed by oxidation in the air at 450°C

Exposure time (hour)		12	9	6	3	0
Zn/Al	H-Y	1.15	0.79	0.22	–	0.00
	Na-A	0.14	0.10	–	0.06	0.00