Biosynthetic Conversion of Phosphatidylglycerol to *sn*-1:*sn*-1′ Bis(monoacylglycerol) Phosphate in a Macrophage-like Cell Line†

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ABSTRACT: Bis(monoacylglycerol) phosphate has a unique stereoconfiguration of *sn*-glycero-1-phospho-1′-*sn*-glycerol and is synthesized from exogenous phosphatidylglycerol by macrophages. Previous work by our laboratory showed that the macrophage-like cell line RAW 264.7 synthesizes *sn*-glycero-1-phospho-1′-*sn*-glycerol bis(monoacylglycerol) phosphate. Here we describe studies using RAW 264.7 cells that examine the biosynthetic pathway by which bis(monoacylglycerol) phosphate is formed. Experiments were conducted using precursors that were specifically radiolabeled on the glycerol backbone in order to examine the stereoconfiguration of the intermediates and products formed in intact RAW 264.7 cells. The results of our studies indicate that a complex series of reactions are involved in the synthesis of bis(monoacylglycerol) phosphate. In this proposed pathway phosphatidylglycerol is hydrolyzed to form 1-acyllysophosphatidylglycerol which is then acylated on the headgroup glycerol to form the *sn*-glycero-1-phospho-1′-*sn*-glycerol enantiomer of bis(monoacylglycerol) phosphate. The *sn*-glycero-1-phospho-1′-*sn*-glycerol enantiomer of bis(monoacylglycerol) phosphate is then thought to undergo a stereoconversion that proceeds via the required removal of the acyl group at the *sn*-1 position. The resulting *sn*-glycero-1-phospho-1′-*sn*-glycerol enantiomer of lysophosphatidylglycerol with the acyl moiety on the original headgroup glycerol is then acylated to form *sn*-glycero-1-phospho-1′-*sn*-glycerol bis(monoacylglycerol) phosphate.

BMP,[1] a structural isomer of PG, normally comprises a small percentage of the total phospholipid mass in most tissues (Rouser et al., 1969; Mason et al., 1972). In certain pathologic states, however, BMP accumulation has been described (Rouser et al., 1968; Wherret & Huterer, 1972; Yamamoto et al., 1970). In contrast, normal alveolar macrophages have 18% of their phospholipid as BMP (Mason et al., 1972; Cochran et al., 1987) even though they do not synthesize BMP *de novo* (Waite et al., 1987). We and others have demonstrated that exogenous PG is converted to BMP, a process in which both glycerol moieties and the phosphate are retained (Somerharju & Renkonen, 1980). We postulated that BMP is synthesized by alveolar macrophages from PG present in the lung surfactant (Waite et al., 1987).

Although the existence of BMP in pig lung was described in 1967 (Body & Gray, 1967), it was not until 1974 that Brotherus et al. (1974) defined the unique *sn*-1:*sn*-1′ stereoconfiguration of BMP. Poorthuis and Hostetler (1976) demonstrated that BMP was synthesized from LPG as well as PG. This suggested that a phospholipase was involved in BMP synthesis. Further studies revealed that a transacylase catalyzed the transacylation of LPG to form BMP (Matsuzawa & Hostetler, 1978). In that study phosphatidylinositol acted as an acyl donor, a reaction that did not require added CoA or ATP (Matsuzawa & Hostetler, 1978). To study the specificity of the acyl donor further, Wherret and Huterer (1989) showed that exogenous phosphatidylcholine also served as an acyl donor when added to a crude subcellular fraction of alveolar macrophages. The accumulation of APG in subcellular fractions of a macrophage-like cell line, RAW 264.7, led Thornburg et al. (1991) to postulate that APG was an intermediate in BMP synthesis. The stereoconfiguration of the BMP synthesized in these *in vitro* studies, however, was not determined.

Joutti and Renkonen (1979) developed a system to analyze the stereoconfiguration of BMP formed by BHK-21 cells and found that 1-acyl-*sn*-3-LPG could be used as a precursor for *sn*-1:*sn*-1′-BMP. It was noted that *sn*-3:*sn*-1′-BMP was formed before *sn*-1:*sn*-1′-BMP accumulated. It was assumed that the syntheses of the two BMP enantiomers were independent but that *sn*-3:*sn*-1′-BMP was degraded and not a precursor of *sn*-1:*sn*-1′-BMP. Waite et al. (1987) have shown that the 1-acyl group of PG must be removed in the synthesis of *sn*-1:*sn*-1′-BMP in intact macrophages. Since both *sn*-3-PG and *sn*-3-LPG are precursors of *sn*-1-BMP, it is possible that the first step in the conversion of *sn*-3-PG to *sn*-1-BMP is through the action of a PLA_2 to form *sn*-3-LPG. It is difficult, however, to envisage that 1-acyl-*sn*-3-

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[1] Abbreviations: AC, adenylate cyclase; APG, acylphosphatidylglycerol; bGp, bis(glycerol) phosphate; BMP, bis(monoacylglycerol) phosphate; cAMP, cyclic adenosine 3′,5′-monophosphate; EDTA, ethylenediaminetetraacetate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LPG, lysophosphatidylglycerol; MEM, minimum essential medium; MeOH, methanol; MMA, monomethylamine reagent; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid; PLA_2, phospholipase A_2; PLD, phospholipase D; ROE, reorientation enzyme; *sn*-1:*sn*-1′, *sn*-glycero-1-phospho-1′-*sn*-glycerol; *sn*-3:*sn*-1′, *sn*-glycero-3-phospho-1′-*sn*-glycerol; TA, transacylase; TLC, thin-layer chromatography.

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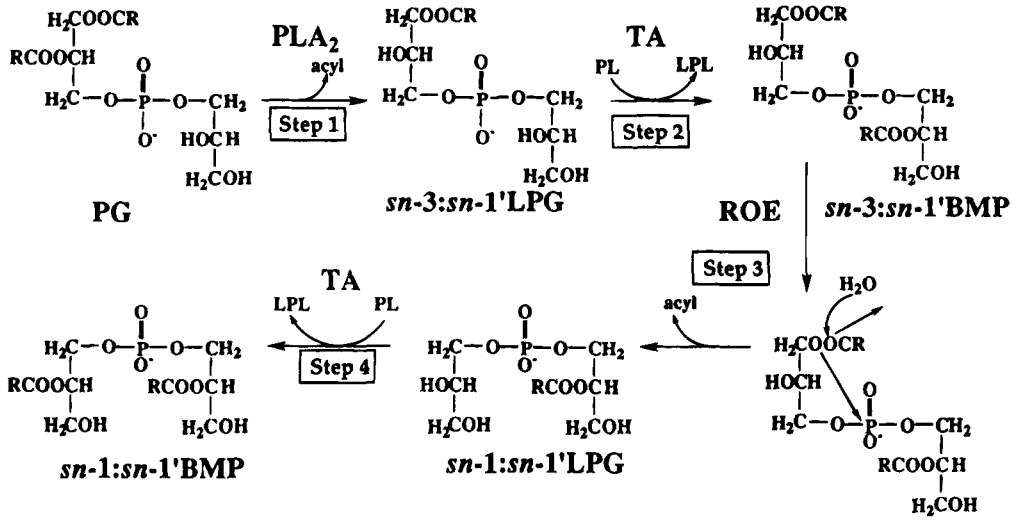
Scheme 1: Biosynthesis of BMP from PG[a]



[a] Phospholipase A_2 (PLA_2) hydrolyzes phosphatidylglycerol (PG) to form 1-acyl-LPG (step 1). LPG is then acylated by a transacylase (TA), using a phospholipid (PL) as the acyl donor, to form BMP that still retains the *sn*-3:*sn*-1′ stereoconfiguration of the original precursor PG (step 2). The glycerol backbone of the *sn*-3:*sn*-1′-BMP is reoriented by an enzymatic activity (ROE), whose mechanism is still unclear, to yield *sn*-1:*sn*-1′-LPG (step 3). The final *sn*-1:*sn*-1′-BMP is formed upon acylation of *sn*-1:*sn*-1′-LPG (step 4), possibly by the same transacylase that catalyzes step 2.

LPG is converted directly to *sn*-1:*sn*-1′-BMP without prior acylation of the headgroup glycerol. One fate of 1-acyl-*sn*-3-LPG in the synthesis of *sn*-1:*sn*-1′-BMP would be deacylation to bGp. This, however, is not a precursor for BMP synthesis (Somerharju & Renkonen, 1980). Since the 1-acyl group is lost in *sn*-1:*sn*-1′-BMP synthesis and since bGp is an unlikely intermediate, we postulate a mechanism that introduces an acyl moiety onto the glycerol headgroup prior to the removal of the 1-acyl group.

This report addresses the pathway in which both 1-acyl-*sn*-3-LPG and *sn*-3-PG are converted to *sn*-1:*sn*-1′-BMP in intact RAW 264.7 cells. A facile and rapid method for determining phospholipid stereoconfiguration is described that distinguishes between the two enantiomers of BMP as well as the two enantiomers of LPG formed. We also determined if *sn*-3:*sn*-1′-BMP is catabolized or is converted to *sn*-1:*sn*-1′-BMP and, therefore, is an intermediate in the conversion of *sn*-3-PG to *sn*-1:*sn*-1′-BMP (Scheme 1). The first step in this proposed pathway involves a PLA_2 that forms 1-acyl-*sn*-3-LPG. This is followed by acylation of the headgroup glycerol catalyzed by a transacylation step (TA, step 2). The location of this acyl group has not been established but has been depicted at the secondary position to reflect the high content of polyunsaturated fatty acids found in BMP (Huterer & Wherret, 1979; Wherret & Huterer, 1973; Cochran et al., 1985). The *sn*-3:*sn*-1′-BMP thus formed is the proposed substrate for the reorientation enzyme (ROE, step 3) that proceeds via the required removal of the acyl group at the *sn*-1 position. The resulting product, *sn*-1:*sn*-1′-LPG with the acyl group on the original headgroup glycerol, is postulated then to be acylated in a second transacylation step (step 4).

EXPERIMENTAL PROCEDURES

Materials. RAW 264.7 (murine monocytic, macrophage-like, Abelson leukemia virus transformed BALB/c) cells were obtained from the American Type Culture Collection. Tissue culture medium was bought from GIBCO, the fetal bovine serum was purchased from Flow Laboratories, and the gentamicin sulfate was obtained from Hazelton. PLA_2 (EC 3.1.1.4) of *Ophiophagus hannah* and porcine pancreas as well as ampicillin were purchased from Sigma. 18-*crown*-6 crown ether was obtained from Aldrich. NaB^3H_4 and [2-^{3}H]-glycerol were purchased from American Radiolabeled Chemicals. Silica gel G and H TLC plates were bought from Analtech. *Escherichia coli* BB26−36 strain was generously provided by Dr. D. Leuking, Michigan Technological University.

Synthesis of [1-^{3}H]Glycerol Phospholipids. [1-^{3}H]Glycerol was synthesized by reduction of L-glyceraldehyde by tritiated sodium borohydride. Briefly, 150 μmol of L-glyceraldehyde was dried *in vacuo* for 12 h and then dissolved in 1 mL of 2-propanol. Sodium [^{3}H]borohydride (50 μmol, 100 mCi/mmol) and 1 μg of 18-*crown*-6 crown ether (1,4,7,10,13,16-hexaoxacyclooctadecane) were added and allowed to react for 2 h; then excess sodium borohydride (450 μmol) was used to force the reaction to completion. The reduction was stopped by the addition of 60 μL of water. The 2-propanol was evaporated under a nitrogen stream, and the [1-^{3}H]glycerol was resuspended in ethanol/water (1:19, by volume) with an estimated specific radioactivity of 10 mCi/mmol.

The *E. coli* glycerol auxotroph BB26-36 was transformed with PUC-18 to allow for selection by ampicillin resistance (Sambrook et al., 1989). Bacteria cultures demonstrating growth inhibition in glycerol-deficient MEM were cultured in the presence of 50 μM [1-^{3}H]glycerol, supplemented with ampicillin (50 μg/mL). After overnight incubation at 37 °C, the bacteria were centrifuged at 10000*g* for 10 min. The pellet was extracted according to the method of Bligh and Dyer (1959), modified by the addition of 50 mM acetic acid used instead of water. Phospholipids were separated on silica gel H plates with chloroform/methanol/glacial acetic acid (65:35:8 v/v) as the mobile phase (system A; R_f values: PE, 0.27; PG, 0.56). Phospholipids were located using the Bioscan radioactivity imaging scanner and authentic standards, and these regions of silica were scraped and extracted as described above. This yielded 1,2-diacyl-[1-^{3}H]-*sn*-

glycero-3-phosphoethanolamine ([1-^{3}H]PE) and 1,2-diacyl-[1-^{3}H]-*sn*-glycero-3-phospho-1′-[1′-^{3}H]-*sn*-glycerol ([1-^{3}H]-*sn*-3:*sn*-1′-PG) with tritium in both glycerol moieties of the PG. A transphosphatidylation reaction catalyzed by Savoy cabbage PLD (Comfurius & Zwaal, 1977) was employed to convert [1-^{3}H]PE and glycerol to 1,2-diacyl-[1-^{3}H]-*sn*-glycero-3-phospho-*rac*-glycerol ([1-^{3}H]-*sn*-3:*rac*-PG). 1-Acyl-2-lyso-[1-^{3}H]-*sn*-glycero-3-phospho-*rac*-glycerol ([1-^{3}H]-*sn*-3:*rac*-LPG) was formed by PLA_2 (porcine pancreatic) hydrolysis of [1-^{3}H]-*sn*-3:*rac*-PG. Each of these compounds was isolated by TLC. The specific radioactivity of [1-^{3}H]-glycerol phospholipids was 9 mCi/mmol.

Synthesis of 1,2-Dioleoyl-[3-^{3}H]-sn-glycero-3-phospho-rac-glycerol. 1,2-Dioleoyl-*sn*-glycerol was oxidized by pyridinium dichromate to form 1,2-diacylglyceraldehyde, which was then eluted from a silicic acid column (silica gel 60, 25-mL bed volume) using 40 mL of hexane/diethyl ether (4:1, by volume). Unoxidized diglyceride and 1,3-diacylglycerone were eluted with 50 mL of hexane/diethyl ether (1:1, by volume). The reduction of the aldehyde moiety by sodium borotritide yielded the 1,2-diacyl-*sn*-glycerol that was specifically tritiated at the *sn*-3 position. The introduction of a phosphocholine group into 1,2-diacyl-*sn*-glycerol has been extensively analyzed (Bittman, 1993). Using 2-chloro-2-oxo-1,3,2-dioxaphospholane, a cyclic intermediate was formed which was then converted to form 1,2-diacyl-[3-^{3}H]-*sn*-glycero-PC by treatment with trimethylamine. A transphosphatidylation reaction catalyzed by Savoy cabbage PLD replaced the choline moiety with glycerol to form the desired 1,2-diacyl-[3-^{3}H]-*sn*-glycero-3-phospho-*rac*-glycerol ([3-^{3}H]-*sn*-3:*rac*-PG). The specific activity was determined to be 154 mCi/mmol (341 000 dpm/nmol). The details of the synthesis are in a manuscript that has been submitted for publication (unpublished experiments).

PG Metabolism by Intact RAW 264.7 Cells. RAW 264.7 cells [(3−4) × 10^6] were grown to confluency in 35 mm × 10 mm culture dishes. The substrate was prepared by evaporating solvent under a nitrogen stream from the substrate, PG, LPG, or BMP, which was then sonically dispersed in the cell culture medium before addition to cells (1.5 mL/culture dish). Cells were harvested by removing medium and washing with ice-cold saline twice before scraping them from the dish into methanol. The phospholipids were extracted by the modified method of Bligh and Dyer (1959) and resolved by TLC. The chloroform extractable products were isolated on silica gel H plates using chloroform/methanol/ammonium hydroxide (130:70:10 v/v) (system B; R_f values: PG, 0.40; BMP, 0.53). The products were located by scanning radioactivity, then scraped, and extracted from the silica and either quantitated for radioactivity by liquid scintillation or analyzed for stereoconfiguration. The methanol/water phase of the extraction was dried *in vacuo*, resuspended in methanol/chloroform (2:1, by volume), and resolved on TLC using system B as the mobile phase. This analysis revealed that more than 85% of the tritiated metabolites in the methanol/water phase was LPG. It was determined that the LPG isolated from the silica gel had the same stereo configuration as that purified from the aqueous phase of the modified extraction mixture of Bligh and Dyer. Therefore, the amount of LPG formed was calculated as a combination of water-soluble products and LPG isolated by TLC.

Analysis of Stereoconfiguration. The products resolved by TLC, as described above, were scraped and extracted from the silica gel to determine the amount of each enantiomer of either BMP or LPG present (Thornburg et al., 1991). The solvent was then removed from the phospholipid sample by evaporation under a nitrogen stream, and MMA was used to remove the acyl groups (Clarke & Dawson, 1981). After 90 min at 53 °C, MMA was evaporated, and products were extracted with 1.4 mL of methanol, 1.4 mL of water, and 2.1 mL of $CHCl_3$. The organic phase was removed, and to minimize $CHCl_3$ dissolved in the upper layer, 1.5 mL of sodium acetate (150 mM, pH 4.6) was added to the methanol/water phase. After centrifugation the upper aqueous phase was removed and added to 0.2 M sodium (meta)periodate and then left in the dark for 30 min. Glycerol (5 mg) was added as a carrier, and the reaction was left for another 30 min. The sodium (meta)periodate cleaves the carbon−carbon bond of the vicinal hydroxyl groups distal to the phosphate moiety, thereby forming formaldehyde and bis-(glycoaldehyde) phosphate. Formaldehyde was precipitated by the addition of 0.5 mL of 0.2 M sodium arsenite followed by 2 mL of a 5,5-dimethyl-1,3-cyclohexanedione (dimedone) solution (20 mg/mL ethanol/water, 1:1, by volume). After 12 h in the dark, the crystalline suspension was filtered by a Buchner suction filtration apparatus to separate the formaldehyde/dimedone precipitate from the bis(glycoaldehyde) phosphate remaining in solution. Radioactivity in the precipitate and the filtrate was quantitated by liquid scintillation counting. The *sn*-1:*sn*-1′-BMP formed from [1-^{3}H]-PG yielded tritiated bis(glycoaldehyde) phosphate, while *sn*-1:*sn*-1′-BMP from [3-^{3}H]PG released tritiated formaldehyde upon periodate cleavage.

Synthesis of sn-3:sn-1′-BMP. Incubation of PG with a lipid-enriched, low-density subcellular fraction of RAW 264.7 cells (fractions 1 and 3) (Waite et al., 1990) was used to synthesize *sn*-3:*sn*-1′-BMP. RAW 264.7 cells were grown to confluency in Dulbecco's MEM with high glucose, 10 mM Hepes, 10% heat-inactivated fetal bovine serum, and 50 mg/mL gentamicin sulfate and scraped into medium and centrifuged at 100*g* for 5 min. The cells were washed two times in ice-cold isotonic saline and resuspended in 5 mL of 1 mM $NaHCO_3$. After homogenization 50 times by a Dounce homogenizing apparatus, the crude homogenate was adjusted to 45% sucrose and fractionated as previously described using discontinuous sucrose flotation ultracentrifugation (Joutti & Renkonen, 1979). Fractions 1 and 3 were isolated from the 9/30% and 30/35% sucrose interfaces, respectively. Radiolabeled PG was sonically dispersed in a buffer containing 100 mM sodium acetate (pH 4.5) and 10 mM 2-mercaptoethanol. The pH was adjusted to 4.5 by the addition of 1 M sodium acetate/10 mM EDTA (pH 4.5) and incubated at 37 °C for 60−90 min with 50−100 μg of protein of the subcellular fraction. The incubations were stopped by the addition of $CHCl_3$/MeOH (1:2, by volume), and LPG, PG, BMP, and APG were isolated using silica gel H plates with system B. Metabolites were located and scraped and then extracted from the silica gel. The stereoconfiguration of BMP isolated was found to be >90% *sn*-3:*sn*-1′-BMP.

Analytical Methods. The lipid phosphorus was quantitated by the method of Chalvardjian and Rudnicki (1970).

Table 1: Stereoconfigurational Analysis of Stereospecifically Radiolabeled Phospholipids[a]

substrate	% filtrate	% precipitate
[1-^{3}H]PE	7	93
[1-^{3}H]-*sn*-3:*rac*-PG	10	90
[1-^{3}H]-*sn*-3:*sn*-1-PG	50	50
[2-^{3}H]-*sn*-3:*sn*-1-PG	95	5
[3-^{3}H]PC	88	12
[3-^{3}H]-*sn*-3:*rac*-PG	90	10

[a] Phospholipids were analyzed to determine the relative position of the radiolabel and the phosphodiester bond. [1-^{3}H]-*sn*-3-phosphatidylethanolamine ([1-^{3}H]PE) and natural [1-^{3}H]-*sn*-3:*sn*-1′-phosphatidylglycerol ([1-^{3}H]-*sn*-3:*sn*-1′-PG) were derived from *E. coli* incubated with [1-^{3}H]glycerol. [1-^{3}H]-*sn*-3:*rac*-PG and [3-^{3}H]-*sn*-3:*rac*-PG were synthesized by a transphosphatidylation reaction using [1-^{3}H]PE or [3-^{3}H]PC, glycerol, and Savoy cabbage PLD. [2-^{3}H]-*sn*-3:*sn*-1′-PG was bacterially derived from incubation of *E. coli* with [2-^{3}H]glycerol. [3-^{3}H]PC was synthesized as described in Experimental Procedures. Between 25 000 and 100 000 dpm of each precursor was analyzed. The results presented are the average of five determinations with a deviation of less than 5%. [3-^{3}H]-labeled precursors yield tritiated bis(glycoaldehyde) phosphate in the soluble filtrate, while [1-^{3}H]-labeled precursors produce a tritiated formaldehyde−dimedone precipitate. Although the [2-^{3}H]-*sn*-3:*sn*-1′-PG is radiolabeled on both glycerol moieties, it yields only a tritiated filtrate, because the radiolabeled secondary position is adjacent to the phosphodiester bond. The recovery on each assay was greater than 95%.

RESULTS

The major challenge in studying BMP synthesis has been distinguishing between the two enantiomers of BMP formed. Initial efforts to describe phospholipid stereoconfiguration used the stereospecific enzyme *sn*-glycerol-3-phosphate dehydrogenase that selectively oxidizes *sn*-glycerol 3-phosphate, but not *sn*-glycerol 1-phosphate, to dihydroxyacetone phosphate (Brotherus et al., 1974; Joutti & Renkonen, 1979; Somerharju et al., 1977; Joutti, 1979). Thornburg et al. (1991) reported a method that uses 1,2-dioleoyl-[1-^{14}C]-*sn*-glycero-3-phospho-*rac*-glycerol as the precursor for BMP synthesis. This approach allowed the relative position of the radiolabel and the phosphate to be determined since both glycerols and the phosphate are retained during the conversion of PG to BMP (Somerharju & Renkonen, 1980). We have devised two new and facile methods to synthesize stereospecifically tritiated phospholipids. The first approach involves the chemical synthesis of 1,2-dioleoyl-[3-^{3}H]-*sn*-glycero-3-phosphocholine as outlined in Experimental Procedures. The second method employs the glycerol auxotroph BB26-36 to biosynthesize 1,2-diacyl-[1-^{3}H]-*sn*-glycero-3-phosphoethanolamine ([1-^{3}H]PE) and 1,2-diacyl-[1-^{3}H]-*sn*-glycero-3-phospho-1′-[1′-^{3}H]-*sn*-glycerol ([1-^{3}H]-*sn*-3:*sn*-1′-PG) from exogenous [1-^{3}H]glycerol. The stereospecificity of the product PG is due to the known mechanism of glycerokinase (Boyer, 1970). Table 1 describes the positional specificity attained in each synthesis using the stereoconfigurational assay described in Experimental Procedures. Both [1-^{3}H]PE and [1-^{3}H]-*sn*-3:*rac*-PG yield tritiated formaldehyde that was recovered in the precipitate. The [1-^{3}H]-*sn*-3:*sn*-1′-PG from *E. coli* is radiolabeled on both glycerol moieties, and therefore half of the radiolabel is located in the precipitate and half is located in the filtrate. As a control for the stereo configuration assay, [2-^{3}H]-*sn*-3:*sn*-1′-PG was used because, regardless of the stereoconfiguration of the glycerol moiety, the radiolabel should be found in the filtrate. Analysis of the [3-^{3}H]PC and the subsequent [3-^{3}H]-*rac*-PG demonstrated that approximately 90% of the radiolabel was found in the filtrate while with [1-^{3}H]-*sn*-3:*rac*-PG, 90% of the radiolabel is recovered in the precipitate. These data show that the accuracy of this assay is roughly 90% for a pure enantiomer. Since the precursors used here have a racemic glycerol headgroup, we do not refer to the stereoconfiguration of that group in the following metabolic studies.

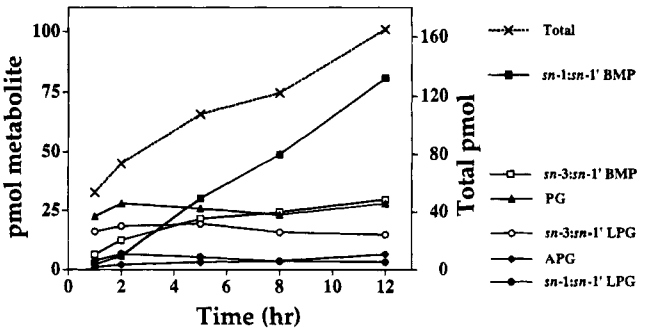


FIGURE 1: Time-dependent metabolism of 1,2-diacyl-[3-^{3}H]-*sn*-glycero-3-phospho-*rac*-glycerol ([3-^{3}H]-*sn*-3:*rac*-PG). RAW 264.7 cells were cultured with 0.75 nmol of [3-^{3}H]-*sn*-3:*rac*-PG for 12 h. At various times, the cells were harvested and washed, and phospholipids were extracted as described in Experimental Procedures. The stereoconfiguration of isolated BMP and LPG was determined. The amount of cell-associated radiolabeled product was calculated from the specific radioactivity of the [3-^{3}H]-*sn*-3:*rac*-PG. Substrate: PG, ▲. Products: sn-1-BMP, ■; *sn*-3-BMP, □; *sn*-1-LPG, ●; *sn*-3-LPG, ○; APG, ◆. Total PG uptake, ×.

In order to establish the pathway for BMP synthesis, we first determined the precursor−product relationships in the conversion of *sn*-3-PG to *sn*-1-BMP as a function of time (Figure 1). Over the course of the experiment there was continual uptake of PG by the cells. The level of both enantiomers of LPG remained fairly constant whereas the amount of *sn*-3 BMP continued to increase throughout the experiment. The final product, *sn*-1-BMP, was the major product after 5 h and continued to accumulate in a linear fashion over 12 h. Measurable amounts of APG were also recovered. These results show that the proposed intermediates of *sn*-1-BMP synthesis are formed from precursor *sn*-3-PG.

Preliminary data from our laboratory suggested that the exogenous concentration of PG affected the conversion of PG to BMP. To further explore this dependency, we cultured RAW 264.7 cells for 8 h in the presence of [3-^{3}H]-*sn*-3:*rac*-PG in varying concentrations of 0.5−31.5 μM. We found that as the extracellular concentration increased, so did the cell-associated radiolabeled lipid (Figure 2). As described in Figure 3, PG accumulated in increasing amounts at all concentrations of PG used. Up to a concentration of 15 μM PG, *sn*-1-BMP and *sn*-3-LPG were the major metabolites recovered. Above that concentration of *sn*-3-PG, no further increase in *sn*-1-BMP was observed, and *sn*-3-LPG was the major metabolite formed. The *sn*-1 enantiomer of LPG increased with higher concentrations of PG, as did the amount of *sn*-3-BMP. On the other hand, above 2.5 μM PG the percentage of each enantiomer of LPG was fairly constant (Figure 4).

The percentages of product in this experiment are shown in Figure 4. Over the increasing PG concentrations tested, the major shift in PG metabolites was a limitation of the final product *sn*-1-BMP. Accompanying the increase in

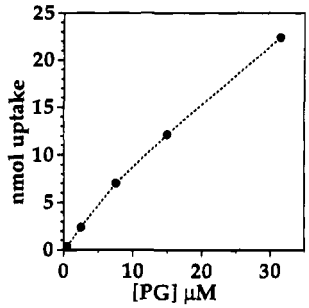


FIGURE 2: Concentration dependence of RAW 264.7 metabolism of PG. RAW 264.7 cells were cultured with varying concentrations of PG mixed with 2.5×10^5 dpm of [3-^{3}H]-*sn*-3:*rac*-PG for 8 h. Total uptake is determined by measuring the amount of radiolabel found as $CHCl_3$ and H_2O-soluble products from the modified extraction of Bligh and Dyer, followed by calculating the mass from the specific radioactivity at each concentration.

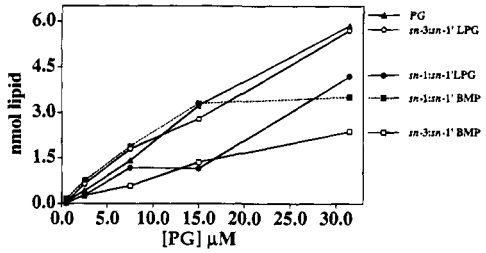


FIGURE 3: Concentration dependence of 1,2-diacyl-[3-^{3}H]-*sn*-glycero-3-phospho-*rac*-glycerol ([3-^{3}H]-*sn*-3:*rac*-PG) metabolism. RAW 264.7 cells were cultured with varying concentrations of PG mixed with 2.5×10^5 dpm of [3-^{3}H]*sn*-3:*rac*-PG for 8 h. At various times the cells were harvested and radiolabeled metabolites analyzed. The enantiomers of BMP and LPG were quantitated. The *x*-axis represents the concentration of extracellular PG while the *y*-axis represents the nanomoles of each lipid species. The results presented are representative of three experiments. Substrate: PG, ▲. Products: *sn*-1:*rac*-BMP, ■; *sn*-3:*rac*-BMP, □; *sn*-1:*rac*-LPG, ●; *sn*-3:*rac*-LPG, ○.

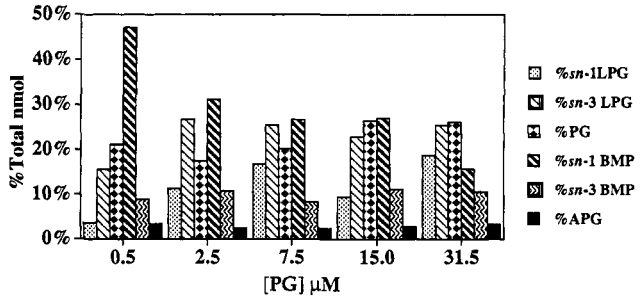


FIGURE 4: Concentration dependence of 1,2-diacyl-[3-^{3}H]-*sn*-glycero-3-phospho-*rac*-glycerol ([3-^{3}H]-*sn*-3:*rac*-PG) metabolism. RAW 264.7 cells were cultured with varying concentration of PG mixed with 2.5×10^5 dpm of [3-^{3}H]-*sn*-3:*rac*-PG for 8 h. At various times the cells were harvested and radiolabeled metabolites analyzed. The enantiomers of BMP and LPG were quantitated. The results are presented as a percentage of the total nanomolar uptake of the precursor PG.

extracellular PG was the accumulation of the LPG intermediate primarily. The increased accumulation of LPG suggests that the rate-limiting step may be the transacylation step. While the absolute amount of *sn*-1-BMP continued to increase (Figure 3), the percentage decreased (Figure 4).

We next studied the time-dependent metabolism of the first predicted intermediate, [1-^{3}H]-*sn*-3:*rac*-LPG, in RAW 264.7 cells at a fixed concentration. Figure 5 describes the results using 8 μM 1-acyl-*sn*-3:*rac*-LPG formed by the PLA_2 hydrolysis of [1-^{3}H]-*sn*-3:*rac*-PG. At the early time points,

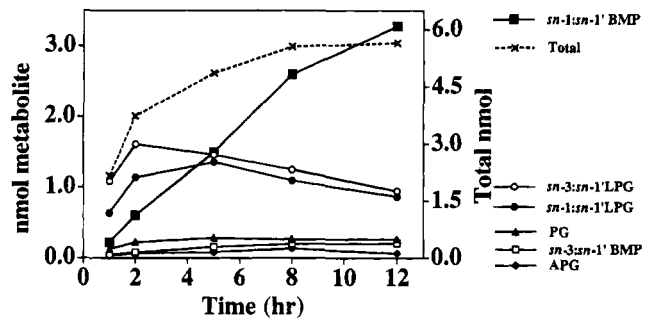


FIGURE 5: Time-dependent RAW 264.7 metabolism of 1-acyl-[1-^{3}H]-*sn*-glycero-3-phospho-*rac*-glycerol ([1-^{3}H]-*sn*-3:*rac*-LPG). RAW 264.7 cells were cultured with 12 nmol of [1-^{3}H]-*sn*-*rac*-LPG for 12 h. At various times the cells were harvested, and radiolabeled products were isolated as described before. Substrate: *sn*-3-LPG, ○. Products: PG, ▲; *sn*-1-BMP, ■; *sn*-3-BMP, □; *sn*-1-LPG, ●; APG, ◆. Total LPG uptake, ×.

we found that *sn*-3:*rac*-LPG was the predominant lipid recovered. By the fifth hour *sn*-1:*rac*-BMP and *sn*-1:*rac*-LPG equaled the amount of *sn*-3:*rac*-LPG. At this point *sn*-1-BMP became the predominant product recovered, similar to the finding with *sn*-3-PG as the precursor (Figure 1). From the fifth hour on, both enantiomers of LPG continued to decrease equally. Small amounts of *sn*-3-BMP, -PG, and -APG also accumulated. These results are similar to those seen under conditions of high PG precursor concentration where both enantiomers of LPG accumulate (Figure 3). The slow formation of PG suggests that acylation of a second hydroxyl group of the glycerol backbone does not readily occur. Further, the low accumulation of APG from *sn*-3-PG (Figure 1) or 1-acyl-*sn*-3-LPG argues that APG is not a major metabolite in *sn*-1-BMP synthesis.

Previous work by our laboratory showed that the acyl group at the *sn*-1 position must be released to enable the phosphodiester linkage to shift from the *sn*-3 position to the *sn*-1 position of the glycerol backbone (Waite et al., 1987; Thornburg et al., 1991). Since PG is not re-formed from 1-acyl-*sn*-3-LPG and little APG is recovered, some other acylated intermediate of 1-acyl-*sn*-3-LPG should be synthesized. A transacylase capable of acylating 1-acyl-LPG to form both enantiomers of BMP has been demonstrated (Poorthuis & Hostetler, 1975; Brotherus et al., 1974; Joutti & Renkonen, 1979; Somerharju et al., 1977; Huterer et al., 1993). It is likely, therefore, that *sn*-3-BMP is the intermediate formed from *sn*-3-LPG. To test the role of *sn*-3-BMP as an intermediate, RAW 264.7 cells were cultured with 3.33 μM *sn*-3-BMP and 3.33 μM *sn*-1-BMP for 18 h (Table 2). There was a 45% conversion of *sn*-3-BMP to *sn*-1-BMP while there was no alteration in the stereoconfiguration of the *sn*-1-BMP. Roughly one-third of the products from *sn*-3-BMP was LPG. Further, *sn*-1-BMP was not significantly degraded to bGp. Figure 6 describes the time course of the conversion of *sn*-3-BMP to *sn*-1-BMP. At all time points *sn*-1-BMP was the major product found. The predicted intermediate *sn*-1-LPG was formed rapidly and reached a maximum by the fifth hour. We postulate that the *sn*-1-LPG was formed by the concomitant reorientation and deacylation of *sn*-3-BMP. It is unlikely that the *sn*-1-LPG was formed from the deacylation of *sn*-1-BMP since it has been demonstrated that *sn*-1-BMP is stable in the RAW 264.7 cell (Table 2). Probably the formation of *sn*-3-LPG from *sn*-3-BMP is the result of direct deacylation by a phospho-

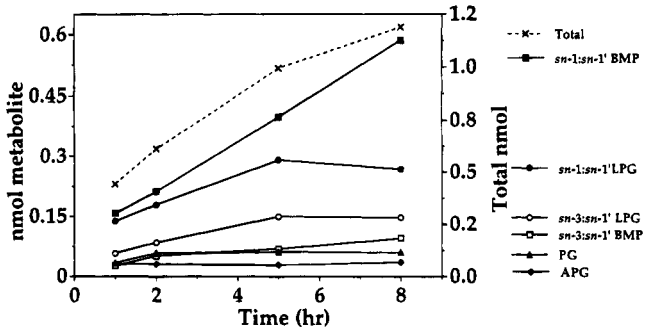


FIGURE 6: Time-dependent RAW 264.7 metabolism of 1-acyl-[1-^{3}H]-*sn*-glycero-3-phospho-1′-acyl-*sn*-[1′-^{3}H]-glycerol ([1-^{3}H]-*sn*-3:*rac*-BMP). RAW 264.7 cells were cultured with 5 nmol of [1-^{3}H]-*sn*-3:*sn*-1′-BMP for 8 h. At various times the cells were harvested, and radiolabeled products were isolated as described before. Substrate: sn-3:*rac*-BMP, hashed boxes; *sn*-3-BMP, □. Products: PG, ▲; *sn*-1-BMP, ■; *sn*-1:*rac*-LPG, ●; *sn*-3:*rac*-LPG, ○; APG, ◆. Total BMP uptake, ×.

Table 2: BMP Metabolism by RAW 264.7 Cells after 18 h[a]

precursor	% cell associated[b]	% cell-associated DPM		
		sn-1-BMP	*sn*-3-BMP	LPG
sn-3:*sn*-1′-BMP	55	45	23	32
sn-1:*sn*-1′-BMP	58	86	5	9

[a] RAW 264.7 cells were cultured with [1-^{3}H]-*sn*-3-BMP or [1-^{3}H]-*sn*-1-BMP. After 18 h, the medium was removed, and the cells were washed with ice-cold saline, before being scraped from the culture dish. Phospholipids were extracted and resolved by TLC as outlined in Experimental Procedures. Radioactivity found with the cells after the saline wash was designated as cell-associated radioactivity. BMP was extracted from the silica gel and analyzed for its stereoconfiguration. The amount of LPG was determined as a combination of LPG resolved by TLC and water-soluble counts. [b] Percentage of total radioactivity found in the cells.

lipase. APG and PG accounted for only a small portion of the recovered lipid.

DISCUSSION

We have investigated the synthetic pathway of *sn*-1-BMP in the RAW 264.7 cell using stereospecifically radiolabeled PG synthesized by two facile methods. We find that the first metabolites to accumulate during the conversion of *sn*-3-PG to *sn*-1-BMP are sequentially *sn*-3-LPG and *sn*-3-BMP (Figure 1). PG is not re-formed from *sn*-3-LPG (Figure 5) or *sn*-3-BMP (Figure 6). It is likely that the *sn*-3-LPG formed from PG is the result of the previously described PLA_2 (Franson & Waite, 1973) which does not alter the stereo configuration and leaves the acyl group in the *sn*-1 position. Huterer et al. (1993) proposed that 1-acyl-LPG was the intermediate in BMP synthesis, although they did not address the question of product stereoconfiguration. We have identified two enantiomers of LPG and demonstrated that RAW 264.7 cells can convert *sn*-3-BMP to *sn*-1-BMP (Table 2 and Figure 6). During this conversion of *sn*-3-BMP to *sn*-1-BMP a majority of the LPG formed was the *sn*-1-LPG enantiomer, as predicted in Scheme 1. This does not rule out the possibility that some of the *sn*-1-LPG was formed by the deacylation of *sn*-1-BMP (Huterer & Wherret, 1982). However, roughly 90% of the *sn*-1-BMP remains intact after 18 h in the cell. Another possibility may be that PG is reoriented to form *sn*-1-LPG but this most likely represents a minor pathway for two reasons. First, *sn*-3-LPG is the major LPG enantiomer formed from exogenous PG, and PG is not re-formed from 1-acyl-*sn*-3-LPG. *sn*-3-LPG also produced only two metabolites, *sn*-1-LPG and *sn*-1-BMP. Therefore, we postulate that *sn*-1-LPG is formed by the reorientation of the glycerol backbone of *sn*-3-BMP. We have not studied the acylation of *sn*-1-LPG due to a lack of its accumulation. However, a transacylase has been reported that acylates LPG to form BMP, although the enantiomeric form was not determined (Poorthuis & Hostetler, 1975; Brotherus et al., 1974; Joutti & Renkonen, 1979; Somerharju et al., 1977; Huterer et al., 1993).

Thornburg et al. (1991) demonstrated that the conversion of *sn*-3-PG to *sn*-1-BMP is an intramolecular reorganization. These studies showed that the phosphate migrates from the *sn*-3 position to the *sn*-1 position in a unidirectional fashion. The reorientation of the glycerol backbone to form the *sn*-1:*sn*-3 stereoconfiguration is unique in phospholipid metabolism. There is no information available on the mechanism of this stereoconversion. However, analogous reactions may occur with non-lipid substrates. One reaction is ribonuclease A (Walsh, 1979), which is a phosphodiesterase that cleaves the 3′ to 5′ phosphoester bond between ribonucleotides. This cleavage begins with the base hydrolysis of the phosphodiester bond to form a 2′,3′-cyclic phosphate intermediate. The phosphorus undergoes a proposed pentacovalent transition state prior to water hydrolysis of the cyclic phosphate in a stereospecific fashion. The 2′-hydroxide ion is protonated, and the phosphate is re-formed on the 3′ carbon. During this hydrolysis the phosphate is left stereospecifically on the 3′ position and does not randomly distribute between the 2′ and the 3′ carbons.

The mechanism of cAMP formation by AC is still unclear, but the synthesis of cAMP yields a six-membered ring composed of the 3′, 4′, and 5′ carbons and the phosphodiester bond linking the 3′ and the 5′ carbons. Upon cleavage of the phosphoester bond at the 3′ position by a specific cAMP phosphodiesterase, 5′-AMP is formed (Stryer, 1988). As with ribonuclease A, the phosphate is esterified to a specific carbon, the 5′ carbon of ribose, and not randomly distributed between two positions.

Using the models of ribonuclease A and AC/cAMP phosphodiesterase, we postulated that the reorientation occurs through a phosphorane ring intermediate (Scheme 1). This intermediate, which is most likely transitory, may be a six-membered ring composed of a phosphodiester bond linking the *sn*-1 and sn-3 positions of the glycerol.

In summary, we believe that our data strongly support the pathway proposed in Scheme 1 as the major route of BMP synthesis. Our data also leave open the possibility that variations on this theme exist, but these variations would represent minor metabolic routes. We focused on the conversion of PG to BMP in the macrophage-like cell since we postulate that macrophage metabolism of lung surfactant PG accounts for the high level of BMP in the alveolar macrophage (Waite et al., 1987). Our previous observation that APG accumulated when a subcellular fraction of RAW 264.7 cells was incubated with PG is confirmed here (Waite et al., 1990). However, it is a minor metabolite and can be formed from BMP directly and probably is not a significant intermediate in the synthesis of *sn*-1-BMP (Huterer et al., 1993). The synthesis of stereospecifically radiolabeled PG has allowed our laboratory to distinguish between the enantiomers generated during BMP synthesis. Studies are

underway to isolate the enzymes involved in BMP synthesis and define the mechanism of the enzyme(s) responsible for the reorientation step, a step that is unique in phospholipid metabolism.

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