# Decrease in cardiac phosphatidylglycerol in streptozotocin-induced diabetic rats does not affect cardiolipin biosynthesis: evidence for distinct pools of phosphatidylglycerol in the heart

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Biosynthesis of phosphatidylglycerol (PG) and cardiolipin (CL) were investigated in perfused hearts of diabetic rats 4 days or 28 days after streptozotocin injection. Sham-injected and insulin-treated diabetic rats were used as controls. In addition, another group of rats fasted for 54 h was examined. Isolated rat hearts from these groups were perfused for 30 min with [$^{32}$P]$P_i$, and the radioactivity incorporated into PG and CL and their pool sizes were determined in heart ventricles. There was no difference in the amount of radioactivity incorporated into CL, PG or other phospholipids between all groups. In addition, the pool sizes of CL and other phospholipids were unaltered. However, a striking decrease in the pool size of PG was observed in both diabetic and fasted rats compared to sham- and insulin-treated controls at 4 days after streptozotocin injection. The decrease in PG mass in diabetic rats was rapid (within 24–48 h) and was localized to cardiac membranes. Diabetes did not affect the activity of the enzymes of PG and CL biosynthesis in the mitochondrial fraction, or phospholipase A activity in subcellular fractions prepared from rat heart homogenates. In addition, pulse–chase experiments confirmed that diabetes did not affect the rate of new PG or CL biosynthesis. Since radioactivity associated with PG was unaltered in continuous-pulse perfusion experiments, a calculated 1.8-fold increase in the specific radioactivity of cardiac PG was observed in the hearts of acute diabetic rats compared with controls. Since the radioactivity incorporated into PG and CL, and the rate of CL biosynthesis, were unaltered in diabetic-rat hearts compared with controls, new CL was probably synthesized from newly synthesized PG. We postulate the existence of distinct pools of PG in the heart, and that the pool of newly synthesized PG used for CL biosynthesis does not appear to mix immediately with the pre-existing pool of PG in the isolated intact rat heart.

## INTRODUCTION

The polyglycerophospholipids bis(monoacylglycero)phosphate, cardiolipin (CL) and phosphatidylglycerol (PG) are the principal polyglycerophospholipids observed in mammalian tissues [1]. Recently, a new polyglycerophospholipid, bis-phosphatidic acid, has been identified, and a putative role for this phospholipid in signal transduction proposed [2]. CL and PG are the major polyglycerophospholipids in the rat heart, together comprising approx. 16.5% of the entire phospholipid mass of this organ [3,4]. PG has been implicated in the modulation of the activity of several membrane enzymes [5]. CL is required for the activity of many mitochondrial enzymes involved in energy metabolism. For example, CL in the mitochondrial inner membrane is the receptor for creatine kinase [6] and is required for the activity of cytochrome *c* oxidase [7]. In addition, CL was demonstrated to play a critical role in the respiratory electron-transport chain of Chinese-hamster ovary cells [8]. PG biosynthesis in mammalian tissues occurs via the Kennedy pathway [9]. Phosphatidic acid (PA) is converted into CDP-diacylglycerol (CDP-DG) by the enzyme CTP:PA cytidylyltransferase. CDP-DG is then converted into PG phosphate (PGP) and then PG by the sequential action of PGP synthase and PGP phosphatase. CL is synthesized when another molecule of CDP-DG is condensed with PG [10]. The major site of PG and CL biosynthesis appears to be the mitochondria [10,11].

Clinical, experimental and pathological studies all support the existence of a specific or primary cardiomyopathy associated with diabetes mellitus [12]. Although the exact cause of diabetic heart-muscle disease is not known, several mechanisms may contribute to it, including disturbed myocardial energy metabolism [13]. In diabetes, altered cardiac mitochondrial oxidative phosphorylation is documented [14]. In the cardiac sarcolemmal membranes of rats that had been made diabetic with streptozotocin the mass of CL was shown to be decreased [15], whereas in the cardiac sarcoplasmic reticulum of these animals the CL mass was increased [16]. Thus, we investigated the possibility that CL biosynthesis was altered in the isolated hearts of diabetic animals. In this paper we demonstrate that streptozotozin (STZ)-induced diabetes causes a rapid decrease in the pool size of cardiac PG which persists up to at least 28 day of diabetes, but this decrease in PG mass does not affect the biosynthesis of new PG or CL. Our results suggest that newly synthesized PG is utilized for new CL biosynthesis and that distinct pools of PG may exist in the intact rat heart.

## MATERIALS AND METHODS

### Animals and materials

Male Sprague–Dawley rats (190–200 g) were used throughout the study. [$^{32}$P]$P_i$ was obtained from DuPont, Canada. Whatman t.l.c. plates (silica-gel G, 0.25 mm thickness) and Ecolite scintillation cocktail were obtained from Canlab, Winnipeg, Canada. Ames Diastix were obtained from Miles Canada Inc., Etobicoke, Canada. NPH insulin (bovine and pig) was obtained from Connaught Novo Ltd., Willowdale, Canada. Chemstrip bG was obtained from Boehringer Mannheim, Canada. All other biochemicals were of analytical grade and obtained from either

Abbreviations used: CL, cardiolipin; PG, phosphatidylglycerol; PA, phosphatidic acid; CDP-DG, CDP-diacylglycerol; STZ, streptozotocin; PGP, PG phosphate; PLA, phospholipase A; KHB, Krebs–Henseleit buffer.

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Fisher Scientific, Edmonton, Canada, or Sigma Chemical Co., St. Louis, MO, U.S.A., or Canlab Division of Baxter Co., Winnipeg, Canada.

### Preparation of animals and confirmation of the diabetic state

After an overnight fast, rats were injected in the tail vein with STZ (75 mg/kg body wt.) dissolved in freshly prepared 0.1 M citrate buffer (pH 4.5), buffered with 1 M NaOH. Control rats were injected with 0.1 M citrate only. Each rat was placed in a metabolic cage for the determination of daily urine output and food intake and kept in a temperature- and light-controlled room. Rats were fed with pellet chow and water *ad libitum*. At 24 h after injection, glucosuria was confirmed in the diabetic rats by using Ames Diastix. Urine of the diabetic rats contained $> 2$ g/dl or $> 111$ mmol/l glucose (estimated from the Diastix). One group of animals were injected daily (14:00 h) with 3 units of NPH insulin. Within 24 h of insulin treatment the diabetic animals had no glucosuria. Blood glucose levels of rats were estimated on day 4 just before cardiac perfusion, by using Chemstrip bG (glucose oxidase). Only animals that had a blood glucose concentration $> 21 \pm 6$ mmol/l ($> 380 \pm 10$ mg/dl) were used in the diabetic group. Another group of animals, fasted for 54 h, was examined.

### Perfusion of isolated rat hearts in the Langendorff mode with [$^{32}$P]$P_i$ and isolation and analysis of radioactive phospholipids

Each animal was killed by decapitation and the heart quickly removed, cleaned of extraneous tissue, cannulated via the aorta and perfused with Krebs–Henseleit buffer (KHB) [17] in the Langendorff mode [18] as described previously [4]. Initial approaches involved using [1,3-$^3$H]glycerol, but were abandoned because cellular glycerol is generated and secreted into the perfusate in perfused diabetic-rat hearts [19], complicating interpretation of the radioisotopic incorporation into phospholipids. Since short-term perfusion of diabetic-rat hearts does not alter intracellular $P_i$ concentration [20], we perfused hearts with [$^{32}$P]$P_i$. Thus the observed labelling of phospholipids with [$^{32}$P]$P_i$ in the diabetic-rat heart should not be the result of an altered specific radioactivity of $P_i$. In continuous-pulse experiments, hearts were perfused with 12.5 ml of KHB containing 1.4 mM [$^{32}$P]$P_i$ (40 $\mu$Ci/ml) in the perfusate for 30 min. In pulse–chase experiments hearts were perfused with 12.5 ml of KHB containing 1.4 mM [$^{32}$P]$P_i$ (50 $\mu$Ci/ml) for 15 min, followed by perfusion with 12.5 ml of KHB for up to 60 min. After perfusion, the atria were cut away from the heart and the ventricles were rapidly frozen in liquid $N_2$. The ventricles were freeze-dried overnight and dry weight was determined. Radioactivity incorporated into phospholipids was determined 48 h after perfusion of the hearts.

The organic phase was isolated from the freeze-dried ventricles as described previously [4], dried under a stream of $N_2$ gas and resuspended in 100 $\mu$l of chloroform:methanol (2:1, v/v). 10 $\mu$l was taken for the determination of radioactivity associated with the organic phase. A 25 $\mu$l portion of the organic phase was placed on a thin-layer plate and phospholipids were separated by the two-dimensional t.l.c. procedure described in [3]. Silica-gel areas corresponding to PG and CL were removed from the plate, and radioactivity was determined in a Beckman model LS 3801 scintillation counter with internal standards.

### Assay of enzymes

Mitochondrial fractions were prepared from isolated rat heart as described previously [4]. PA:CTP cytidylytransferase, PGP synthase, PGP phosphatase and CL synthase were assayed as described in the respective references [21–24]. The phospholipase A (PLA) activity in the 10000 *g* and 100000 *g* pellets and the 100000 *g* supernatant (cytosol) was measured in a total volume of 0.5 ml. The reaction mixture contained 100 $\mu$l of 0.25 M Tris/HCl, pH 8.5, 100 $\mu$l of 25 mM $CaCl_2$, 100 $\mu$l of 0.2 mM phosphatidyl[U-$^{14}$C]glycerol (sp. radioactivity 0.3 $\mu$Ci/pmol), water and 0.1 mg of protein, except that the pH of the cytosolic PLA incubation was 8.0. Radioactive substrate PG was synthesized as described in [4], mixed with stock PG and dried under $N_2$. Water was added, and the mixture was vortex-mixed and then sonicated for 10 min in a bath sonicator before use. The reaction was initiated by addition of [$^{14}$C]PG. The mixture was incubated at 37 °C for 30 min and terminated by addition of 2 ml of chloroform, followed by addition of 1 ml of 0.1 M HCl in methanol and 1 ml of 0.73 % NaCl. The mixture was centrifuged at full speed in a bench-top centrifuge, and the aqueous phase was removed by suction and the organic phase dried under $N_2$. The organic phase was resuspended in 25 $\mu$l of chloroform/methanol (2:1, v/v) and a 20 $\mu$l sample was separated on thin-layer plates in the solvent system chloroform/methanol/7 M $NH_4OH$ (30:15:2, by vol.) [2]. A sample of lyso-PG was placed on each plate as standard, and, after development of the plates, the lyso-PG spot detected with iodine was removed and the radioactivity in lyso-PG determined. For determination of phospholipid mass and radioactivity in subcellular fractions, hearts from control and diabetic animals were removed, and a 10 % (w/v) homogenate in 10 mM Tris/HCl (pH 7.4)/0.25 M sucrose was prepared and subcellular fractions were isolated as described in [4]. Marker-enzyme analysis revealed that the mitochondrial fraction was contaminated with 10 % microsomal material and the microsomal fraction contained 5 % mitochondrial particles [25]. PG and CL masses were determined in samples (1 mg of protein) of the membrane fractions. Extraction of phospholipids was performed as described above for the freeze-dried tissue.

### Other analyses

Phospholipid mass determinations were performed as described in [4]. Protein was determined by the dye-binding method [26]. Results in this study are depicted as mean $\pm$ S.D. Student's *t* test or Dunnet's *t* test for multiple comparisons with a single control were used for the determination of significance. The level of significance was defined as either $P < 0.05$ or $P < 0.001$.

## RESULTS

Diabetes was confirmed by measurement of glucosuria and blood glucose levels in STZ-treated rats. As shown in Table 1, over a 28-day period, diabetic rats exhibited significant 10-fold and 8-fold increases in urine output compared with control and insulin-treated diabetic rats respectively. Over a 28-day period,

**Table 1 Daily food intake and urine output of control and treated animals**

Average daily food intakes and urine outputs of control, diabetic and insulin-treated diabetic rats over a 28-day period are shown. Data represent means $\pm$ S.D. (number of animals): * $P < 0.001$ for control versus diabetic; † $P < 0.001$ for diabetic versus insulin-treated diabetic.

| | Food intake (g) | Urine output (ml) |
|---|---|---|
| Control | 29 $\pm$ 3 (20) | 12 $\pm$ 3 (10) |
| Diabetic | 50 $\pm$ 4 (20)*† | 175 $\pm$ 56 (10)*† |
| Insulin-treated diabetic | 31 $\pm$ 7 (10) | 11 $\pm$ 3 (5) |

**Table 2 Body and ventricular weight of control and treated animals**

Body weights and ventricular weights of control, diabetic, insulin-treated diabetic and 54 h-fasted rats are shown. Data represent means ± S.D. (number of animals): * $P < 0.001$ for control versus diabetic; † $P < 0.001$ for diabetic versus insulin-treated diabetic.

| | 4 days | 28 days |
|---|---|---|
| Body wt. (g) | | |
| Control | 244 ± 11 (20) | 393 ± 28 (10) |
| Diabetic | 207 ± 15 (20)*† | 280 ± 43 (10)*† |
| Insulin-treated diabetic | 243 ± 10 (10) | 387 ± 21 (5) |
| Fasted | 195 ± 12 (4)*† | – |
| Freeze-dried ventricular wt. (g) | | |
| Control | 0.164 ± 0.008 (20) | 0.223 ± 0.019 (10) |
| Diabetic | 0.146 ± 0.005 (20)* | 0.166 ± 0.016 (10)*† |
| Insulin-treated diabetic | 0.156 ± 0.009 (10) | 0.225 ± 0.018 (5) |
| Fasted | 0.147 ± 0.015 (4) | – |

**Table 3 Radioactivity incorporated into PG and CL in hearts from control and treated animals**

Hearts from control, diabetic, insulin-treated diabetic and fasted rats were perfused for 30 min with [$^{32}$P]$P_i$ as described in the Materials and methods section. After perfusion, the radioactivity incorporated into PG and CL was determined. Data represent means ± S.D. (number of hearts): N.D., not determined.

| | $10^{-4}$ × Incorporation (c.p.m./g of freeze-dried ventricles) | | | |
|---|---|---|---|---|
| | Control | Diabetic | Insulin-treated diabetic | Fasted |
| 4 days | | | | |
| PG | 4.0 ± 0.6 (10) | 4.5 ± 0.3 (10) | 4.0 ± 0.2 (5) | 3.8 ± 0.5 (4) |
| CL | 0.9 ± 0.1 (10) | 0.8 ± 0.2 (10) | 0.8 ± 0.1 (5) | 0.8 ± 0.2 (4) |
| 28 days | | | | |
| PG | 4.6 ± 1.0 (10) | 4.5 ± 0.8 (10) | 4.1 ± 1.4 (5) | N.D. |
| CL | 0.8 ± 0.1 (10) | 0.7 ± 0.1 (10) | 0.7 ± 0.1 (5) | N.D. |

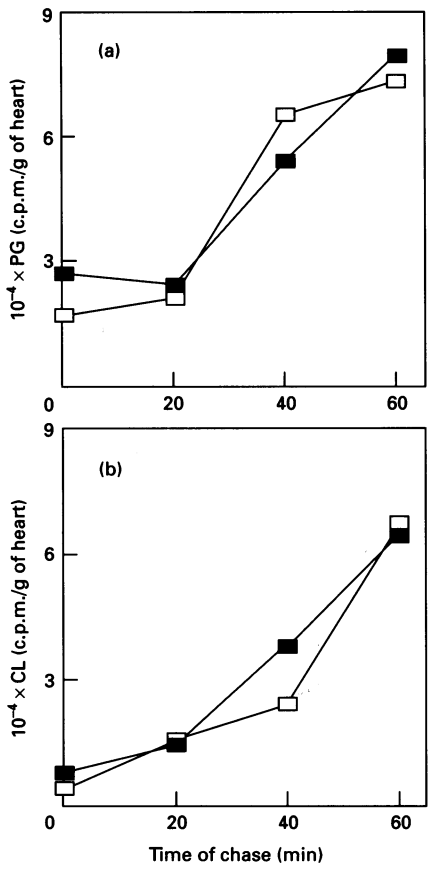


**Figure 1 Pulse–chase study of PG and CL biosynthesis in diabetic-rat hearts**

Isolated hearts from control (■) and diabetic (□) rats were perfused for 15 min with [$^{32}$P]$P_i$ and then perfused for up to 60 min with non-radioactive buffer. After perfusion, the radioactivity incorporated into PG (**a**) and CL (**b**) was determined. Each point represents the mean of two hearts.

diabetic rats showed significant 72% and 61% increases in food intake compared with control and insulin-treated diabetic rats respectively. As shown in Table 2, body weights of diabetic rats were significantly less compared with controls. In addition, ventricular weights of diabetic-rat hearts were decreased significantly compared with controls. The ratio (ventricular weight/body weight) × 1000 was 0.64–0.70 and was similar for all groups. Thus these data, in combination with glucosuria and elevated blood glucose levels (see the Materials and methods section), indicate that the rats used in this study exhibited the biochemical and metabolic characteristics associated with insulin-dependent (Type 1) diabetes [27]. In addition, these effects could be reversed upon treatment with insulin. Furthermore, rats fasted for 54 h exhibited a decrease in body and ventricular weight, with the ratio (ventricular/body weight) × 1000 equal to 0.75.

### Effect of STZ-induced diabetes on the synthesis of PG and CL

To investigate whether biosynthesis of PG or CL was altered in diabetes, hearts from 4- and 28-day-diabetic and control rats, insulin-treated rats or rats fasted for 54 h were perfused with [$^{32}$P]$P_i$ for 30 min and the radioactivity incorporated into PG and CL was determined. We chose 30 min because significant and reproducible amounts of radioactivity could be observed in CL at this time. The total amount of radioactivity incorporated into the hearts of 4- and 28-day-diabetic rats was $(2.0 \pm 0.2) \times 10^7$ c.p.m./g of freeze-dried heart and was unchanged compared with controls. In the hearts of both control and rats made diabetic, the radioactivity incorporated into the organic phase, expressed as a percentage of the total radioactivity in the heart, was approx. 10%. As shown in Table 3, there was no significant difference in the radioactivity incorporated into PG or CL in the hearts of diabetic animals compared with controls at either 4 or 28 days. In addition, no significant difference in the radioactivity incorporated into other phospholipids was observed (results not shown). To determine if diabetes altered the rate of biosynthesis of new CL, hearts from control or 4-day-diabetic animals were perfused for 15 min with KHB containing [$^{32}$P]$P_i$ and then subsequently chased for up to 60 min with KHB. As shown in Figure 1, the rate of synthesis of both new PG and CL was unaltered in the diabetic-rat hearts compared with controls. Thus the rate of biosynthesis of PG and CL *de novo* from [$^{32}$P]$P_i$ appeared to be unaltered in acute diabetes.

### STZ-induced diabetes causes a rapid decrease in cardiac PG

The mass of PG and CL was determined in the hearts of control, diabetic and insulin-treated diabetic rats and rats fasted for 54 h.

**Table 4 Ventricular pool sizes of PG and CL from control and treated animals**

Hearts were perfused as described in the Materials and methods section, and the ventricular pool sizes of PG and CL from 4-day and 28-day control, diabetic, insulin-treated diabetic and fasted rats were determined. Data represent means ± S.D. (number of hearts): * $P < 0.001$ for control versus diabetic; † $P < 0.001$ for diabetic versus insulin-treated diabetic. N.D., not determined.

| | Pool (μmol/g of freeze-dried ventricles) | | | |
|---|---|---|---|---|
| | Control | Diabetic | Insulin-treated diabetic | Fasted |
| 4 days | | | | |
| PG | 1.31 ± 0.12 (10) | 0.84 ± 0.07 (10)*† | 1.14 ± 0.10 (5) | 0.97 ± 0.06 (4)* |
| CL | 10.4 ± 0.5 (10) | 11.4 ± 0.7 (10) | 10.7 ± 0.3 (5) | 10.2 ± 0.6 (4) |
| 28 days | | | | |
| PG | 1.21 ± 0.06 (10) | 0.97 ± 0.07 (10)*† | 1.19 ± 0.06 (5) | N.D. |
| CL | 12.0 ± 0.1 (10) | 12.5 ± 0.6 (10) | 12.4 ± 0.5 (5) | N.D. |

**Table 5 Subcellular location of PG and CL in diabetic-rat hearts**

Hearts from control and 4-day diabetic animals were removed and homogenized in 0.25 M sucrose/5 mM Tris/HCl, pH 7.4, and the PG and CL contents determined in the 10000 *g* pellet and the 100000 *g* pellet. Data represent means ± S.D. (number of hearts): * $P < 0.05$ versus control.

| | Content (nmol/mg of protein) | |
|---|---|---|
| | Control | Diabetic |
| PG | | |
| 10000 *g* pellet | 5.1 ± 0.2 (3) | 3.5 ± 0.2 (3)* |
| 100000 *g* pellet | 1.9 ± 0.2 (3) | 1.4 ± 0.2 (3)* |
| CL | | |
| 10000 *g* pellet | 41 ± 2 (3) | 38 ± 2 (3) |
| 100000 *g* pellet | 4.3 ± 0.1 (3) | 3.8 ± 0.5 (3) |

**Table 6 Time course of ventricular pool size of PG after treatment of rats with STZ**

Rats were injected with STZ, and the ventricular pool size of PG was determined as described in the Materials and methods section. Data present means ± S.D. (number of hearts): * $P < 0.05$ versus control.

| Time after onset of diabetes (days) | PG (μmol/g of freeze-dried ventricles) |
|---|---|
| 0 | 1.35 ± 0.09 (4) |
| 1 | 0.94 ± 0.10 (3)* |
| 2 | 0.80 ± 0.05 (3)* |
| 3 | 0.77 ± 0.04 (3)* |
| 4 | 0.75 ± 0.11 (3)* |

Diabetes did not alter the mass of CL compared with controls at 4 and 28 days (Table 4). The total phospholipid phosphorus mass, expressed as μmol/g dry wt. of heart, was 63 ± 7 and 69 ± 4 at 4 and 28 days respectively, and was unaltered in diabetic hearts compared with controls. In addition, the mass of other major membrane phospholipids was unaltered (results not shown). In contrast, a significant 36% and 20% decrease in the mass of PG was observed in 4-day- and 28-day-diabetic hearts respectively, compared with controls (Table 4). The pool sizes of PG and CL were determined in the 10000 *g* and 100000 *g* pellets of homogenates prepared from hearts of 4-day control and diabetic animals. We observed a significant 31% decrease ($P < 0.05$) in PG mass in the 10000 *g* pellet and a 26% decrease ($P < 0.05$) in the 100000 *g* pellet in diabetic hearts compared with controls (Table 5). The mass of CL in subcellular fractions was unaltered in either group. No significant PG or CL mass was detected in the 100000 *g* supernatant. Thus the decrease in PG mass was specific to membranes and was consistent with the decrease in whole heart PG. In another set of experiments, hearts from control and diabetic animals were perfused for 30 min with [$^{32}$P]$P_i$, and the radioactivity incorporated into PG was determined in a 2–2.5 mg portion of mitochondrial or microsomal fraction prepared from the heart homogenate. Radioactivity incorporated into PG was 77 ± 12 d.p.m./mg in mitochondrial fractions and 94 ± 14 d.p.m./mg in microsomal fractions and was unchanged in diabetic hearts compared with controls. Thus the amount of radioactive PG observed in both control and diabetic subcellular fractions was similar to control values.

As a further control, we perfused hearts from rats which had been fasted for 54 h as above and determined the radioactivity associated with PG and the PG mass. As shown in Tables 3 and 4, the results were similar to those for the diabetic animals, i.e. the mass of PG was decreased but the radioactivity incorporated into PG and CL was unaltered compared with controls. These results indicate that the decreased PG observed in diabetes is not due to the toxic effect of STZ, but is possibly due to an accelerated catabolism similar to that observed in fasting. We performed a time-course study on the decrease in PG mass in diabetic animals. As shown in Table 6, the cardiac PG mass was decreased as early as 1 day after injection of STZ, and was highly significant and maximum by 2 days compared with controls.

The decrease in PG mass may have been due to a change in the activity of one of the PG- and CL-biosynthetic enzymes. Hearts from control and 4-day-diabetic rats were isolated, and the activity of the biosynthetic enzymes were assayed in the 10000 *g* pellet prepared from the homogenates of these hearts. The activities of PA:CTP cytidylyltransferase, PGP synthase and CL synthase were 44 ± 8, 130 ± 24, 4.3 ± 1.4 pmol/min per mg of protein respectively, and were unaltered in diabetic hearts compared with controls, In addition, the activity of PGP phosphatase was 1.04 ± 0.34 nmol/min per mg of protein and was unaltered in diabetic hearts compared with controls. Thus the decrease in PG mass was not due to an alteration in the rate of PG or CL biosynthesis. The decrease in PG mass might have

been due to an increased PLA activity directed towards PG. Such an increase in PLA activity directed towards phosphatidylcholine has been observed in muscle homogenates of diabetic ketotic rats [28]. PLA activity was assayed in subcellular fractions prepared from controls and rats made diabetic for 4 days, and was $4.2 \pm 0.7$, $3.1 \pm 0.2$ and $1.3 \pm 0.4$ nmol/min per mg of protein in the 10000 *g* pellet, 100000 *g* pellet and 100000 *g* supernatant respectively, and values were unaltered in diabetic hearts compared with controls.

## DISCUSSION

The objective of this study was to determine if cardiac PG and CL biosynthesis were affected by diabetes. We utilized perfusion of hearts in the Langendorff mode to investigate PG and CL biosynthesis in the diabetic rat heart. The major findings of this paper are (1) the biosynthesis of new cardiac PG and CL does not appear to be affected by acute or chronic STZ-induced diabetes in short-term heart perfusion experiments, (2) the mass of PG is rapidly decreased and maintained in diabetic-rat hearts, (3) newly synthesized CL is synthesized from newly synthesized PG in the intact heart and (4) the heart appears to contain distinct pools of PG.

Our previous studies indicated that PG and CL were actively synthesized in the isolated perfused rat heart from both radioactive glycerol and phosphate [4]. It was not known if the biosynthesis of these important phospholipids was altered in the diabetic heart. Hearts from 4- and 28-day control, diabetic, or insulin-treated diabetic rats that were perfused for 30 min with [$^{32}$P]$P_i$ exhibited no differences in the radioactivity associated with either PG or CL. In addition, the activities of the biosynthetic enzymes involved in PG and CL biosynthesis were unaffected by diabetes. These data clearly indicate that the biosynthesis of new PG and CL is unaltered, at least in short-term heart perfusion experiments, in diabetic hearts compared with controls.

We were most intrigued to find that the pool size of PG was rapidly decreased in the hearts of diabetic rats. The radioactivity incorporated into PG and CL remained similar in 4- and 28-day-diabetic-rat hearts compared with controls, but the pool size of PG was significantly decreased in the diabetic-rat hearts. Thus, the resulting specific radioactivity of PG in the 4-day diabetic-rat hearts was increased 1.8-fold, from $3.05 \times 10^4$ to $5.36 \times 10^4$ c.p.m./$\mu$mol. In addition, the specific radioactivity of PG in the subcellular fractions of diabetic hearts was increased, since the radioactivity incorporated into PG was unaltered but the pool size of PG in these fractions was decreased compared with controls. If the specific radioactivity of the precursor PG had increased and if the rate of CL biosynthesis was unaltered, then an increase in radioactivity associated with CL should have occurred. However, an increased incorporation of radioactivity into CL was not observed, and CL synthase activity was unaltered in 4-day diabetic-rat hearts compared with controls. An attractive hypothesis to explain the data is the possibility that newly synthesized PG was preferentially used for CL biosynthesis in the heart. Although we cannot eliminate the possibility that at least some pre-existing PG was used for CL biosynthesis, the fact that the radioactivity incorporated into PG and CL remained unaltered in diabetic hearts makes this possibility rather remote. Another implication of these findings is that the pools of newly synthesized PG and pre-existing PG used for CL biosynthesis do not readily mix in the heart. Evidence for this hypothesis were the pulse–chase studies with $^{32}$P. In these studies the rate of increase in radioactivity into CL in 4-day diabetic-rat hearts paralleled that of control hearts. If both newly synthesized and pre-existing pools of PG had mixed before the biosynthesis of new CL, then the rate of radioactivity incorporation into CL would have increased, due to the increased specific radioactivity of PG.

The functional significance of separate PG pools in the heart remains to be elucidated. The pool size of PG could not be decreased further after 4 days of diabetes, suggesting that this non-labile (or less labile) pool of PG is critical for cellular functions other than CL biosynthesis. PG has been implicated in the regulation of several enzymes [5]. In light of the recent observation that PG is released into perfusates of rat liver and may play a role in attenuation of platelet aggregation [29], it is possible that another distinct pool of PG (extra-mitochondrial) may contribute to such a function outside the cardiac myocyte.

PG is localized in mitochondrial and non-mitochondrial membrane domains of the cell [1] and can readily transverse bilayers [30], possibly making it accessible to cytosolic as well as membrane PLAs. The PG hydrolysed must then be exposed at sites which are readily accessible to the PLA. The presence of enhanced phospholipase $A_2$ activity towards arachidonic acid-containing phosphatidylcholine in ketotic diabetic-rat muscle homogenates has recently been demonstrated [28]. In our study, significant PLA activity towards PG was detected in all subcellular fractions, but alteration in the activity was not observed. Rat heart cytosol was shown to contain an active PLA with principally $A_1$ activity, which required $Ca^{2+}$ for optimum activity and exhibited some similarity to the solubilized sarcoplasmic-reticulum enzyme [31]. A $Ca^{2+}$-stimulated phospholipase $A_2$ was identified in the cytosolic fraction of several mammalian tissues [32]. In that study cytosolic $Ca^{2+}$ concentrations as low as 0.32 $\mu$M caused a translocation of this phospholipase to membranes. Chemically induced diabetes is associated with decreases in $Ca^{2+}$ uptake into $Ca^{2+}$-sequestering organelles, resulting in increased cytosolic $Ca^{2+}$ levels [14]. The cytosolic enzyme in the present study required $Ca^{2+}$ for optimal activity. Thus it is possible that in the presence of elevated concentrations of $Ca^{2+}$ in the cytosol this enzyme may hydrolyse exposed PG in heart membranes.

In summary, we clearly show that the first major diacyl phospholipid molecule to show gross decreases in acute STZ-induced diabetes in the heart appears to be PG, yet this does not affect the biosynthesis of new CL in short-term heart perfusion experiments. In addition, the decrease in PG mass was also observed after a brief fasting, suggesting a possible relationship to nutritional events. Thus the observed decrease in PG mass may not necessarily be diabetes-dependent. This indicates that the ventricular mass of PG, although markedly decreased in diabetes, may not be used as a marker for the presence of Type-1 diabetes, but possibly of metabolic conditions associated with altered catabolism.

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