Cardiolipin biosynthesis in the isolated heart

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The pathway for the biosynthesis of new cardiolipin was investigated in the isolated perfused intact rat heart. Isolated rat hearts were perfused in the Langendorff mode for up to 60 min with Krebs–Henseleit buffer containing 0.1 μ M [U- 14 C]glycerol. Analysis of radioactivity incorporated into phospholipids in the organic phase revealed an increase in radioactivity incorporated into phosphatidylglycerol, cardiolipin and other phospholipids with time of perfusion. This was associated with a loss of radioactivity from phosphatidic acid. In contrast, perfusion of hearts for up to 60 min with 0.1 mM [1,(3)- 3 H]glycerol in the perfusate revealed an increased radioactivity associated with phosphatidic acid as well as cardiolipin, phosphatidylglycerol and other phospholipids. Perfusion of hearts for up to 60 min with [32 P]P_i in the perfusate revealed a time-dependent increase in radioactivity associated with all phospholipids. Perfusion of hearts for up to 60 min with 0.1 μ M or 0.1 mM glycerol in the perfusate did not affect the concentration of phosphatidic acid, cardiolipin or phosphatidylglycerol. To determine the rate-limit-

ing step of cardiolipin biosynthesis, hearts were pulsed for 5 min with 0.1 μ M [1,(3)- 3 H]glycerol and chased for up to 60 min with 0.1 μ M glycerol in the perfusate. Radioactivity was maximum at the start of the chase in phosphatidic acid (and 1,2-diacylglycerol), and was subsequently chased into phosphatidyl-inositol, phosphatidylglycerol and cardiolipin (and other phospholipids). Significant radioactivity in phosphatidylglycerol phosphate was not detected. Radioactivity in CDP-*sn*-1,2-diacylglycerol remained constant throughout the chase. The activities of the enzymes of the Kennedy pathway for cardiolipin biosynthesis in the heart were determined. On the basis of continuous-pulse and pulse-chase labelling studies it is postulated that the cardiac polyglycerophospholipids phosphatidylglycerol and cardiolipin are actively synthesized from newly synthesized phosphatidic acid via the Kennedy pathway. In addition, the results suggest that the rate-limiting step of cardiolipin biosynthesis in the intact heart is probably the conversion of phosphatidic acid into CDP-*sn*-1,2-diacylglycerol.

INTRODUCTION

Cardiolipin (CL), the first polyglycerophospholipid discovered, was isolated from bovine heart by Mary Pangborn in 1942 [1]. In mammalian tissues, CL is characteristically associated with the mitochondrial inner membrane [2] and is required for the activity of, or intimately associated with, a number of key mitochondrial enzymes, including cytochrome *c* oxidase [3]. In rat heart CL is a major phospholipid component comprising approx. 15% of the entire cardiac phospholipid mass [4].

The pathway for biosynthesis of CL was first elucidated by Kennedy and co-workers for *Escherichia coli* [5,6]. Regulation of the enzymes and the mechanisms which govern CL biosynthesis in bacteria [7,8] and in yeast [9,10] are well documented. In mammalian tissues biosynthesis of CL occurs via CDP-*sn*-1,2-diacylglycerol (CDP-DG), catalysed by CTP:phosphatidic acid (PA) cytidyltransferase (EC 2.7.7.41) [11]. This reaction occurs in both mitochondrial and microsomal fractions [12]. It is not known if newly synthesized or pre-existing PA is used for CL biosynthesis in mammalian heart. The committed step of CL biosynthesis involves the conversion of CDP-DG into phosphatidylglycerol (PG) by sequential action of phosphatidylglycerol phosphate (PGP) synthase (EC 2.7.8.5) and PGP phosphatase (EC 3.1.3.27). PGP synthase has been partially purified from mammalian liver mitochondria [13]. None of the above enzymes has been purified to homogeneity from any mammalian source. In the final step of the pathway, PG is then converted into CL via condensation with CDP-DG catalysed by CL synthase. CL synthase was recently purified to homogeneity from rat liver mitochondria by Schlame and Hostetler [14]. The

enzyme is localized to the mitochondrial inner membrane [15]. The rate-limiting step of this pathway for CL biosynthesis in mammalian heart is unknown.

Although CL plays an important role in the maintenance of mitochondrial function and membrane integrity, studies on the biosynthesis of this important phospholipid in intact organs have been largely ignored. Most studies on polyglycerol phospholipids and their biosynthesis in mammalian tissues have been performed in tissue subcellular fractions [11,16]. There are no detailed studies on CL biosynthesis in the isolated intact rat heart. In fact, a previous study demonstrated that isolated rat heart mitochondrial fractions did not have the ability to synthesize CL from radioactive *sn*-glycerol 3-phosphate (GP) [17]. It could be argued that the biosynthesis of CL in the mammalian heart should be similar to that of the liver, lung and brain subcellular fractions. However, the liver and lung actively secrete phospholipids in the form of lipoproteins (liver) and surfactant (lung) and have much lower concentration (> 2.6-fold and > 19-fold, respectively) of CL [4]. In addition, the brain content of CL is > 45-fold lower than that of heart [18]. Thus their phospholipid and CL biosynthesis might be different from that in an organ which has a greater content of CL and does not secrete phospholipid. The objective of the present study was to investigate CL biosynthesis in the intact heart. In this study, the pathway for new CL biosynthesis in the isolated perfused intact rat heart is identified. In addition, we demonstrate that newly synthesized PA is used for CL biosynthesis and that the rate-limiting step of CL biosynthesis is likely to be the conversion of PA into CDP-DG in the isolated perfused heart.

MATERIALS AND METHODS

Male Sprague-Dawley rats (100–125 g) were used throughout the study. [1,3-(³H)]Glycerol was obtained from Amersham, Oakville, Canada. [5-(³H)]CTP, [U-(¹⁴C)]glycerol 3-phosphate, and [U-(¹⁴C)]glycerol were obtained from DuPont, Winnipeg, Canada. [³²P]P_i was obtained from ICN Biochemicals, Mississauga, Ontario, Canada. Dimethyldichlorosilane and t.l.c. (silica gel 60, 0.25 mm thickness) plates were obtained from BDH, Toronto, Canada. Ecolite scintillation cocktail was obtained from Canlab. All other biochemicals were of analytical grade, obtained from Fisher Scientific, Edmonton, Canada, Sigma Chemical Co., St. Louis, MO, U.S.A., or CanLab Division of Baxter Co., Winnipeg, Canada.

Preparation of rat heart mitochondrial fractions

Animals were killed by decapitation, and the heart was rapidly removed and cannulated via the aorta and 10–20 ml of ice-cold 0.145 M NaCl was perfused through the heart to remove blood. Subsequent procedures were performed at 4 °C. A 5% (w/v) homogenate was prepared in 0.145 M NaCl by a 5 s burst of a Polytron generator (Kinematika, Lucerne, Switzerland), and was then centrifuged at 1000 *g* (Sorvall RC-5 Superspeed refrigerated centrifuge with SS-34 rotor) for 5 min. The resulting supernatant was centrifuged at 10000 *g* for 15 min. The resulting pellet was resuspended in 1 ml of 0.145 M NaCl by 15 strokes of a hand-held tissue grinder and used as the source of mitochondrial fraction for assay of enzymes. The supernatant from this centrifugation was re-centrifuged at 105000 *g* (Beckman model L8-70 centrifuge with Ti 70.1 rotor) for 60 min. The resulting pellet was resuspended in 1 ml of 0.145 M NaCl and designated the microsomal fraction. In some experiments, for assay of CL synthase, two hearts were homogenized and the resulting 10000 *g* pellet was resuspended in 0.5 ml of 0.145 M NaCl to produce a higher mitochondrial protein yield.

In other experiments, a 10% homogenate of rat liver (typically 1–3 g) was prepared in 0.25 M sucrose/0.1 mM EDTA, pH 7.4, with a hand tissue grinder. The homogenate was centrifuged at 1000 *g* (SS-34 rotor) for 10 min. The resulting supernatant was centrifuged at 3300 *g* for 10 min and the speed was then increased to 8000 *g* for another 2 min. The resulting mitochondrial pellet was washed three times and finally resuspended in 1 ml of the homogenizing buffer. This resuspended mitochondrial fraction was the enzyme source for synthesis of both phosphatidyl-[¹⁴C]glycerol phosphate ([¹⁴C]PGP) and phosphatidyl-[¹⁴C]glycerol ([¹⁴C]PG).

Preparation of [¹⁴C]PG and [¹⁴C]PGP

[¹⁴C]PGP was prepared by a modification of the procedure of Macdonald and McMurray [13]. The incubation mixture contained 150 mM Tris/HCl, pH 7.4, 2 mM *p*-chloromercuribenzoic acid, 1.0 mM CDP-DG and 0.5 mM [¹⁴C]GP (40 μ Ci/ μ mol) and 10 mg of rat liver mitochondrial protein in a final volume of 0.7 ml. The mixture was incubated for 4.5 h at 37 °C and terminated by addition of 5.6 ml of chloroform/methanol/HCl (500:500:3, by vol.). This mixture was centrifuged at 1000 *g* for 10 min to pellet protein, and the supernatant was transferred to 16 mm $\times$ 100 mm screw-cap tubes. Then 2 ml of chloroform and 0.7 ml of 0.9% NaCl were added to cause phase separation. The mixture was re-centrifuged and the aqueous phase removed by suction. The lower phase was washed with 3 $\times$ 5 ml of theoretical upper phase (chloroform/

methanol/0.9% NaCl, 1:45:47, by vol.), except that the first wash contained 0.1 mM GP. Subsequent steps for purification of [¹⁴C]PGP were performed exactly as described previously [13].

[¹⁴C]PG was prepared essentially as described above for [¹⁴C]PGP, except that *p*-chloromercuribenzoic acid was excluded from the incubation mixture. After termination of the reaction, [¹⁴C]PG was isolated exactly as described for [¹⁴C]PGP [13], except that [¹⁴C]PG was eluted from the silicic acid column in the 25% fraction of methanol in chloroform. A sample of the final column fraction was separated on thin-layer plates by using the two-dimensional system described [4] and on plates which had been previously soaked in 0.4 M oxalic acid then air-dried overnight and developed in a solvent system containing chloroform/methanol/HCl (435:65:1, by vol.). In both t.l.c. systems only [¹⁴C]PG was detected and co-migrated with PG markers. Final purification of [¹⁴C]PG involved t.l.c. on the oxalate-treated plates with a separate lane for standard PG. The plate was covered with Cellophane, except for the standard lane, and stained with iodine vapour. [¹⁴C]PG was eluted from the silica gel by the method of Arvidson [19].

Assay of enzymes of CL biosynthesis

CTP:PA cytidyltransferase was assayed by a modification of the method of Carman and Kelley [20]. To a 16 mm $\times$ 100 mm test tube (all test tubes were treated with dimethyldichlorosilane, 2% in 1,1,1-trichloroethane) was added, in this order, 0.05–0.1 mg of mitochondrial protein and 0.145 M NaCl to a volume of 60 μ l, 10 μ l of 0.5 M Tris/maleate, pH 6.5 (Tris buffered with 1.0 M maleic acid solution), 10 μ l of 10 mM [5-(³H)]CTP (sp. radioactivity 12000 d.p.m./nmol), 10 μ l of 0.15 M Triton X-100, 5 mM PA (prepared by sonicating for 20 min a weighed sample of PA in the Triton solution in a silane-treated 16 mm $\times$ 100 mm tube). The reaction was started by addition of 10 μ l of 0.2 M MgCl₂·6H₂O. The mixture was incubated at 30 °C for 5 min and terminated by addition of 0.5 ml of 0.1 M HCl in methanol. Subsequent steps were performed exactly as described previously [20]. Enzyme activity was linear with time up to at least 10 min with 0.1 mg of protein.

PGP synthase was assayed by a modification of the method of Carman and Belunis [21]. The incubation mixture contained enzyme (50–100 μ g) and 0.145 M NaCl added to a volume of 50 μ l, 10 μ l of 0.5 M Tris/HCl, pH 7.0, 10 μ l of β -mercaptoethanol (prepared fresh) and 10 μ l of 5.0 mM [U-(¹⁴C)]GP (sp. radioactivity 12500 d.p.m./nmol). The reaction was initiated by addition of 10 μ l of 100 mM MgCl₂·6H₂O. The mixture was incubated at 30 °C for 10 min and terminated by addition of 0.5 ml of 0.1 M HCl in methanol, and subsequent steps were performed exactly as described previously [21]. Enzyme activity was linear with time up to at least 15 min with 0.1 mg of protein.

PGP phosphatase activity was assayed by a modification of the method of Macdonald and McMurray [13]. The incubation mixture contained 10 μ l of 0.5 M Tris/maleate, pH 6.5, 10 μ l of 0.1 M β -mercaptoethanol, 10 μ l of 40 μ M [¹⁴C]PGP (sp. radioactivity 15000 d.p.m./nmol), 0.05–0.1 mg of protein and 0.145 M NaCl in a total volume of 100 μ l. Incubation was at 37 °C for 5 min. The reaction was terminated by addition of 2 ml of chloroform/methanol/HCl (500:500:3, by vol.). Then 1 ml of chloroform and 1.5 ml of 0.73% NaCl were added to effect phase separation. Tubes were vortex-mixed and then centrifuged at 2000 *g* for 5 min. The aqueous phase was removed and the organic phase was dried down under N₂ gas and resuspended in 25 μ l of chloroform/methanol (2:1, v/v), and 20 μ l of this was placed on an oxalate-treated thin-layer plate and developed in a solvent system containing chloroform/methanol/HCl (435:65:1,

by vol.). Standard PG was added to each lane for identification. Silica gel corresponding to PG was removed and the radioactivity determined. Reaction was linear with time up to at least 7.5 min with 0.1 mg of protein.

CL synthase was assayed exactly as described by Schlame and Hostetler [22], except that the assay contained 0.05–0.1 mg of protein, the pH of the assay was 8.5 and the samples were sonicated for 10 s in a Branson model 1200 sonicator before incubation. Incubation was at 37 °C for 60 min with [^{14}C]PG (sp. radioactivity 45000 d.p.m./nmol). CL synthase activity was linear for up to at least 70 min with 0.1 mg of protein. This assay could be performed with mitochondrial fraction which was previously frozen at –20 °C, without significant loss of activity.

Perfusion of isolated rat hearts in the Langendorff mode with radioactive glycerol and P_i

The animal was killed by decapitation and the heart quickly removed, cleaned of extraneous tissue, and cannulated via the aorta. Then 5 ml of Krebs–Henseleit buffer (KHB) [23] was perfused through the heart with a syringe to remove blood, and the heart was subsequently placed on the perfusion apparatus and perfused in the Langendorff mode [24] with KHB (5 ml/min) saturated with O_2/CO_2 (19:1) at 37 °C for 5 min or until electrical stabilization was achieved. An electrode was placed on the aorta and another electrode immersed in the buffer solution bathing the apex of the heart, and the viability of the heart was monitored by electrocardiac recording. This procedure allows assessment of both ventricular and atrial activities simultaneously. The $p\text{O}_2$ of the buffer was 69.6 ± 2.0 kPa. Under these conditions the heart remained electrically stable, and therefore viable, for up to 4 h of perfusion [25]. In continuous-pulse experiments, after stabilization, the hearts were perfused with 12.5 ml of KHB containing 0.1 mM [1,(3)- ^3H]glycerol (2 $\mu\text{Ci}/\text{ml}$), or 0.1 μM [$\text{U}-^{14}\text{C}$]glycerol (1 $\mu\text{Ci}/\text{ml}$) or 1.4 mM [^{32}P] P_i (16 $\mu\text{Ci}/\text{ml}$) in the perfusate for up to 60 min. This concentration of P_i is found in KHB. In pulse-chase experiments, hearts were perfused with 12.5 ml of KHB containing 0.1 μM [1,(3)- ^3H]glycerol (5 $\mu\text{Ci}/\text{ml}$) for 5 min and subsequently perfused with 12.5 ml of KHB containing 0.1 μM glycerol for up to 60 min. After perfusion, 10 ml of air was forced through the heart to remove the residual perfusate in the vessels. The heart was cut open, blotted dry, and the wet weight determined. The homogenization, extraction and t.l.c. were performed within 48 h, and radioactivity incorporated into phospholipids was determined immediately thereafter.

Isolation and analysis of radioactive phospholipids

The heart (typically 0.40–0.45 g) was perfused as described above, and after perfusion the heart was homogenized (20 s burst of Polytron homogenizer) in 5 ml of chloroform/methanol (2:1, v/v) containing butylated hydroxytoluene (0.05 mg/ml). Tissue left on the generator probe was removed and homogenized in another 5 ml of the chloroform/methanol. The homogenates were combined and centrifuged at 1000 g for 20 min in a bench-top centrifuge to pellet debris. A 100 μl sample of the homogenate was taken (with a Hamilton syringe) for the determination of total radioactive glycerol associated with the heart. The homogenate was transferred to 16 mm $\times$ 125 mm screw-cap tubes, and 5 ml of 0.73 % NaCl was added to the homogenate to initiate phase separation. The tubes were shaken for 5 min and then centrifuged at 1000 g (model TJ-6 bench-top centrifuge) for 20 min. The aqueous phase was removed and 5 ml of theoretical

upper phase (chloroform/methanol/0.9 % NaCl, 3:48:47, by vol.) was added to wash the organic phase. The tubes were mixed and centrifuged as described above, and the aqueous phase was removed and combined with that of the first centrifugation. The organic phase was dried under a stream of N_2 gas and resuspended in 100 μl of chloroform/methanol (2:1, v/v). A 10 μl sample was taken for determination of radioactivity associated with the organic phase. A 20 μl 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 [4]. The thin-layer plates were prepared by spraying the plate lightly with 0.4 M boric acid, blotting dry the excess boric acid and then air-drying the plates overnight. PG and PA standards were placed on the plate before chromatography, except when lipid phosphorus analysis was performed. In some experiments, a 10 μl portion of the organic phase was placed on a thin-layer plate with a sample of CDP-DG standard and separated from other lipids as described for CDP-DG [26]. In other experiments a 10 μl sample was placed on a thin-layer plate with a portion of PGP standard and separated as described [27]. Oxalate-treated plates were used for PGP separation. In the pulse-chase experiments a 10 μl sample of the organic phase was placed on a thin-layer plate, and DG was separated in a solvent system containing diethyl ether/benzene/ethanol/acetic acid (225:250:10:1, by vol.). The lipids were revealed with iodine vapour and removed into 7 ml scintillation vials. Then 0.5 ml of water was added and the vials were sonicated for 5 min to disperse the silica gel. Finally, 5 ml of Ecolite scintillation cocktail was added. The radioactivity was determined in a Beckman model LS 3801 scintillation counter with internal standards.

Other procedures

Non-perfused hearts and hearts perfused for 60 min with KHB containing either 0.1 μM or 0.1 mM glycerol were analysed for P present in phospholipids. The lipid-extraction procedure was the same as described above. A 35 μl sample of the organic phase was placed on the borate-treated thin-layer plates, and phospholipids were separated in the two-dimensional system [4]. The phospholipid spots were removed and the P content of phospholipids were determined by the method of Rouser et al. [28]. Protein concentration was determined by the method of Bradford [29]. Results in this study are depicted as means $\pm$ S.D.

RESULTS

Enzyme activities *in vitro* involved in cardiolipin biosynthesis

The activities *in vitro* of the enzymes involved in CL biosynthesis were investigated in subcellular fractions prepared from rat heart. A 5 % homogenate was prepared in 0.145 M NaCl and subcellular fractions were obtained by differential centrifugation. The activities of these enzymes were assayed under optimal conditions. CTP:PA cytidyltransferase activity was localized to both crude mitochondrial (Table 1) and microsomal fractions. Microsomal PA:CTP cytidyltransferase activity was 61 ± 9 pmol/min per mg of protein. PGP synthase, PGP phosphatase and CL synthase were localized to the mitochondrial fraction (Table 1). PGP phosphatase activity has been identified in the soluble fraction of baby-hamster kidney cells [30]. No such extra-mitochondrial activity was found in the heart.

CL biosynthesis from glycerol

Since glycerol is a common precursor for phospholipid biosynthesis, the contribution of the Kennedy pathway to CL

Table 1 Enzyme activities *in vitro* involved in cardiolipin biosynthesis

The mitochondrial fraction was prepared as described in the Materials and methods section and the enzymes were assayed under optimal conditions. Values represent means $\pm$ S.D. of three hearts assayed in duplicate.

Enzyme	Activity (pmol/min per mg of protein)
PA: CTP cytidyltransferase	62 $\pm$ 7
PGP synthase	166 $\pm$ 15
CL synthase	3.1 $\pm$ 0.8
PGP phosphatase	1.96 $\pm$ 0.11*

* nmol/min per mg of protein.

biosynthesis was evaluated in the isolated rat heart. Hearts were perfused for various times with 0.1 μ M [U - 14 C]glycerol (1 μ Ci/ml). Total uptake of radioactive glycerol was 2.4×10^6 d.p.m./g of heart, and was maximal by 15 min of perfusion and remained constant throughout the pulse period up to 60 min of perfusion. In addition, radioactivity associated with the organic phase was 0.35×10^6 d.p.m./g of heart, and was maximum by 15 min of perfusion and remained constant throughout the perfusion. Radioactivity incorporated into CL increased with time of perfusion (Figure 1a). In addition, radioactivity incorporated into PG increased with time of perfusion (Figure 1b). By 15 min of perfusion, the earliest time point examined, radioactivity in PA was maximum and decreased with time of perfusion, consistent with radioactivity being incorporated into PG and CL (Figure 1b). Significant radioactivity could not be detected in PGP. This was not surprising, since PGP is not observed *in vivo* [5]; however, see [16]. Radioactivity in CDP-DG was $(2.16 \pm 0.68) \times 10^2$ d.p.m./g of heart at 15 min of perfusion and remained constant throughout the perfusion. The radioactivity incorporated into other phospholipids was determined. By 60 min of perfusion 14 C radioactivity in phosphatidylcholine (PC) [$(7.2 \pm 0.5) \times 10^4$ d.p.m./g of heart], phosphatidylethanolamine (PE) [$(4.4 \pm 0.31) \times 10^4$ d.p.m./g of heart], phosphatidylserine (PS) [$(3.0 \pm 0.4) \times 10^3$ d.p.m./g of heart] and phosphatidylinositol (PI) [$(1.7 \pm 0.3) \times 10^4$ d.p.m./g of heart] was observed. Clearly, these data indicate that the isolated perfused rat heart actively synthesizes new CL from newly synthesized PA.

Subsequent glycerol-labelling studies were performed with [1,(3)- 3 H]glycerol. Hearts were perfused for up to 60 min with 0.1 mM [1,(3)- 3 H]glycerol (2 μ Ci/ml) in the perfusate. This is representative of the circulating plasma concentration of glycerol in the rat [31]. The total radioactivity associated with the hearts was $(3.45 \pm 0.31) \times 10^6$ d.p.m./g of heart by 15 min of perfusion and remained constant thereafter. This was calculated to be 6.2% of the radioactivity in the starting perfusate. In addition, the radioactivity associated with the organic phase increased from $(3.0 \pm 0.4) \times 10^5$ to $(1.1 \pm 0.1) \times 10^6$ d.p.m./g of heart by 60 min of perfusion. In contrast with hearts perfused with 0.1 μ M glycerol, the radioactivity in PA in these hearts was increased with time of perfusion (Figure 2a). This was likely to be due to the continuing incorporation of radioactive glycerol into the organic phase. Radioactivity in PG (Figure 2a) and CL (Figure 2b) was increased with time of perfusion. In addition, radioactivity in other phospholipids was increased; PC, to $(2.4 \pm 0.3) \times 10^5$ d.p.m./g of heart; PE, to $(1.5 \pm 0.2) \times 10^5$

Figure 1 Radioactivity incorporated into PA, PG and CL in hearts perfused with 0.1 μ M [U - 14 C]glycerol

Isolated rat hearts were cannulated via the aorta and perfused with KHB containing 0.1 μ M [U - 14 C]glycerol for up to 60 min. (a) After perfusion, the hearts were homogenized in chloroform/methanol (2:1, v/v) and the homogenate was fractionated into organic and aqueous phases. Radioactivity in CL in the organic phase was determined. (b) The radioactivity in PG (black symbols) and PA (white symbols) in the organic phase was determined. Results are means $\pm$ S.D. of at least three hearts.

d.p.m./g of heart; PS, to $(8.0 \pm 1.2) \times 10^3$ d.p.m./g of heart; PI, to $(4.9 \pm 0.8) \times 10^4$ d.p.m./g of heart by 60 min of perfusion. No significant radioactivity in PGP was detected.

CL synthesis from P_i

Since P_i is required for the biosynthesis of all phospholipids, the synthesis of CL was monitored with time in hearts perfused with [32 P] P_i . Hearts were perfused for various times with 1.4 mM [32 P] P_i (16 μ Ci/ml). This is representative of the P_i concentration in the KHB. Uptake of radioactive P_i increased with time of perfusion to $(1.11 \pm 0.11) \times 10^7$ c.p.m./g of heart of 60 min. Radioactive P_i in the organic phase increased to $(1.21 \pm 0.13) \times 10^6$ c.p.m./g of heart by 60 min of perfusion. At all time points of perfusion the ratio of radioactive P_i in the organic phase to the total radioactivity in the heart was approx. 1:10–11. Radioactivity incorporated into phospholipids in the organic phase was determined. As indicated in Figure 3, CL, PG

Figure 2 Radioactivity in PA, PG and CL in hearts perfused with 0.1 mM [1,3- ^3H]glycerol

Isolated rat hearts were perfused for up to 60 min with 0.1 mM [1,3- ^3H]glycerol in the perfusate, and the radioactivity in PA, PG and CL in the organic phase was determined. (a) PA (white symbols); PG (black). (b) CL. Results are means $\pm$ S.D. of four hearts.

and PA were actively synthesized with time in hearts perfused with [^{32}P]P_i in the perfusate. Radioactivity in other phospholipids was increased with time up to 60 min of perfusion: PC to $(2.72 \pm 0.31) \times 10^5$, PE to $(1.83 \pm 0.52) \times 10^5$, PS to $(9.21 \pm 0.61) \times 10^3$, sphingomyelin to $(1.77 \pm 0.10) \times 10^3$, and PI to $(4.38 \pm 0.34) \times 10^5$ c.p.m./g of heart. Radioactivity in lysoPA increased to $(7.30 \pm 0.61) \times 10^3$ c.p.m./g of heart. At all time points of perfusion, the ratio of radioactivity in PA to that in lysoPA was approx. 27–30:1. This indicates that newly synthesized lysoPA was rapidly converted into PA. Radioactivity in PI greatly exceeded that incorporated into the three major cardiac phospholipids, CL, PE and PC. The large amount of radioactivity associated with PI was likely due to the incorporation of [^{32}P]P_i into PI phosphates and PI bisphosphates, in addition to the PI glycerol phosphate backbone. The amount of radioactivity recovered in all the major phospholipids was representative of approx. 95–97% of the radioactivity in the organic phase.

Pool sizes of PA, PG and CL

The pool sizes of CL, PG and PA were determined in non-perfused hearts and in hearts perfused for 60 min with

Figure 3 Radioactivity in PA, PG and CL in hearts perfused with [^{32}P]P_i

Isolated rat hearts were perfused for up to 60 min with 12.5 ml of KHB containing [^{32}P]P_i (16 $\mu\text{Ci/ml}$) and the radioactivity in (a) CL, (b) PG and (c) PA in the organic phase was determined. Results are means $\pm$ S.D. of three hearts.

either 0.1 μM or 0.1 mM glycerol. Hearts were homogenized in chloroform/methanol as described in the Materials and methods section, and after phase separation the organic phase was dried under N₂ gas and resuspended in 100 μl of chloroform/methanol (2:1, v/v) and a 5 μl sample was removed for the determination of total lipid P. A 35 μl sample of the organic phase was placed on a thin-layer plate, and PA, PG and CL were separated by two-dimensional t.l.c. and the P contents determined. CL (2529 ± 217 nmol/g of heart), PG (262 ± 42 nmol/g of heart) and PA (48 ± 7 nmol/g of heart) represented approx. 15%, 1.6% and 0.28% of the total phospholipid mass in these hearts respectively. The results for percentage of total lipid P agree with those reported previously by Poorthuis et al. [4] for PG and CL and by Simon and Rouser [18] for PA. The pool sizes of PA, PG

Figure 4 Pulse-chase study of CL biosynthesis with $0.1 \mu\text{M}$ glycerol in the perfusate

Isolated rat hearts were perfused for 5 min with $0.1 \mu\text{M}$ $[1, (3)\text{-}^3\text{H}]$ glycerol and then perfused for up to 60 min with $0.1 \mu\text{M}$ glycerol. Subsequently, the radioactivity in CL, PG and PA was determined. (a) PA (white symbols); PG (black symbols). (b) CL. Results are means $\pm$ S.D. of three hearts.

and CL were unaltered after perfusion with $0.1 \mu\text{M}$ or 0.1mM glycerol compared with non-perfused hearts (results not shown).

The rate-limiting step of CL biosynthesis in heart

A pulse-chase protocol was established to determine the rate-limiting step of CL biosynthesis in the heart. Hearts were perfused for 5 min with $0.1 \mu\text{M}$ $[1, (3)\text{-}^3\text{H}]$ glycerol ($5 \mu\text{Ci/ml}$), and subsequently the radioactivity was chased with $0.1 \mu\text{M}$ glycerol in the perfusate for up to 60 min. Total radioactivity associated with the hearts was decreased from $(8.82 \pm 1.01) \times 10^6$ d.p.m./g of heart at the start of the chase to $(4.43 \pm 0.77) \times 10^6$ d.p.m./g of heart by 60 min of chase. The radioactivity lost from the hearts was quantitatively recovered in the perfusate and identified as glycerol. Radioactivity associated with the organic phase was $(1.9 \pm 0.2) \times 10^6$ d.p.m./g of heart, was maximum at the beginning of the chase and remained constant throughout the chase. Radioactivity in PA was maximum at the beginning of the chase and decreased with time (Figure 4a). This was accompanied by an increase in radioactivity associated with PG and CL with time of chase (Figures 4a and 4b). Radioactivity in CDP-DG was

$(7.13 \pm 1.47) \times 10^2$ d.p.m./g at the start of the chase and remained constant thereafter. Radioactivity in DG was $(5.7 \pm 0.8) \times 10^5$ d.p.m./g of heart at the beginning of the chase and decreased to $(0.5 \pm 0.1) \times 10^5$ d.p.m./g of heart by 60 min of perfusion. Radioactivity in PE, PC, PS and PI was increased from $(2.2 \pm 0.3) \times 10^5$, $(1.7 \pm 0.3) \times 10^5$, $(7.2 \pm 0.8) \times 10^3$ and $(7.1 \pm 0.7) \times 10^4$ d.p.m./g of heart at the start of the chase to $(5.7 \pm 0.5) \times 10^5$, $(5.6 \pm 0.7) \times 10^5$, $(3.8 \pm 0.5) \times 10^4$ and $(1.4 \pm 0.2) \times 10^5$ d.p.m./g of heart at the end of the chase, respectively. Significant radioactivity in PGP was not detected.

DISCUSSION

The objective of this study was to identify the major pathway for the biosynthesis of new CL in the isolated rat heart. To mimic the conditions of the beating heart *in vivo*, isolated rat hearts were perfused in the Langendorff mode [24] with KHB [23] containing radioactive glycerol or P_i . This method allowed the simultaneous monitoring of the incorporation of radioactive glycerol or P_i into CL and the electrocardiac activity of the beating heart. Under these conditions, no change in the electrocardiac function of the beating heart up to and including 60 min of perfusion was observed. Thus the viability of the heart was maintained throughout the perfusion experiments.

In mammalian tissues exogenous glycerol has been widely used to study phospholipid biosynthesis [32–34]. When hearts were perfused for up to 60 min with $0.1 \mu\text{M}$ $[\text{U-}^{14}\text{C}]$ glycerol in the perfusate, a decreased radioactivity in PA was accompanied by an increase in radioactivity incorporated into CL and other phospholipids. These studies are consistent with an earlier observation by Åkesson et al. [34]. In that study rats were injected intraportally with radioactive glycerol, and the livers were removed at various times after injection and the radioactivity incorporated into lipids was determined. The radioactivity in PA was maximum by 20–30 s and declined as radioactivity was incorporated into other phospholipids and triacylglycerols. To confirm the active synthesis of CL, we utilized a different phospholipid precursor, $[\text{P}_i\text{-}^{32}\text{P}]$. Perfusion of hearts with $[\text{P}_i\text{-}^{32}\text{P}]$ revealed that CL could be actively synthesized from P_i as a substrate precursor in the isolated perfused intact rat heart. Since the radioactivity from both $[\text{P}_i\text{-}^{32}\text{P}]$ and $[\text{U-}^{14}\text{C}]$ glycerol was incorporated rapidly into PA and more slowly into CL, these data clearly demonstrate that the rat heart can actively synthesize CL, and that this synthesis most likely occurs via the Kennedy pathway.

In the continuous pulse-labelling studies with radioactive glycerol, radioactivity in the heart was maximal by 15 min of perfusion with radioactive $0.1 \mu\text{M}$ or 0.1mM glycerol in the perfusate. These data suggest that the isolated perfused rat heart must have a great capacity to take up glycerol. The circulating plasma concentration of glycerol in the rat is approx. 0.1mM [31], and cell membranes are freely permeable to glycerol. Therefore plasma glycerol must rapidly enter the heart during perfusion, and in the present study a rapid equilibrium of radiolabelled glycerol must have occurred within 15 min.

Once inside the cell, glycerol can be converted into GP by glycerol kinase. A previous study had indicated that the rat heart contained no glycerol kinase activity [35]. However, a subsequent study demonstrated the activity to be present in the myocardium [36]. Radioactivity associated with PA rapidly reached a maximum by 15 min of perfusion when $0.1 \mu\text{M}$ glycerol was present in the perfusion medium. These data indicate that the isolated perfused rat heart can rapidly synthesize PA from this exogenous glycerol source. Evidence in support of this was the studies with $[\text{P}_i\text{-}^{32}\text{P}]$. In these studies, the relative rate of incorporation of ^{32}P

radioactivity into PA was approx. 27–30-fold higher than into lysoPA. This indicates that the lysoPA produced in the heart from radioactive GP must be rapidly converted into PA. In hearts perfused with radioactive glycerol, radioactivity increased with time into PA when 0.1 mM glycerol was present in the perfusate, but was lost out of PA when 0.1 μ M glycerol was present in the perfusate; yet in the presence of either glycerol concentration, radioactivity was incorporated with time into all other phospholipids. This was likely due to the increased incorporation of radioactive glycerol into the organic phase in the presence of 0.1 mM glycerol in the perfusate, whereas glycerol incorporation into the organic phase was maximum by 15 min of perfusion with 0.1 μ M glycerol. This was consistent with a previous study in liver slices [37]. In that study, the rate of glycerol incorporation into the lipid fraction was shown to differ with various glycerol concentrations in the incubation medium. The differential labelling of PA, in the presence of the two different glycerol concentrations, compared with other phospholipids is explained by the fact that PA lies at a branch point in glycerolipid synthesis and that both CDP-DG and DG, required for the synthesis of all the major phospholipids, are produced from this key phospholipid [38]. Thus, since radioactivity in the organic phase was maximal by 15 min in the hearts perfused with 0.1 μ M glycerol, then the time-dependent movement of radioactivity would have been out of PA and into other phospholipids.

On the basis of continuous-pulse-labelling studies with 0.1 μ M glycerol in the perfusate, the rate-limiting step of PG and CL biosynthesis in the isolated heart appeared to be the conversion of PA into CDP-DG. Since the pool size of PA did not change when hearts were perfused for up to 60 min with 0.1 μ M glycerol in the perfusate, a pulse-chase protocol was implemented to confirm this hypothesis. In these studies, a decreased radioactivity associated with PA (and DG) was accompanied by an increase in radioactivity incorporated into PI, PG and CL (and other phospholipids). Radioactivity in CDP-DG remained constant throughout the chase. Thus it appeared that some of the radioactive CDP-DG produced was rapidly converted into PI, PG and CL. CTP:PA cytidyltransferase activity has been demonstrated in both mitochondrial and microsomal fractions of rat liver homogenates [12]. In the liver the activity of the microsomal enzyme exceeded the activity of the mitochondrial enzyme [39]. In the present study, the activities in these two fractions were similar. This might be due to the greater requirement of the heart for CL biosynthesis, since the content of CL is almost 2.6-fold that of the liver, whereas the content of PI, which is synthesized in the endoplasmic reticulum [12], is almost 3.6-fold higher in the liver than the heart [4]. We did not observe any accumulation of radioactivity in PGP. This was not surprising, since PGP phosphatase activity was almost 12-fold greater than the PGP synthase activity, and thus any PGP produced should be rapidly converted into PG. The small amount of CL synthase activity observed might suggest that this step should be rate-limiting for CL biosynthesis in the heart. Indeed, regulation of rat liver CL synthase activity by thyroid hormone has been documented [40], and this may be associated with alterations in liver CL mass [41]. The data in the present paper do not refute the possibility that the CL synthase is rate-limiting. However, at least under the conditions of the present study, the rate-limiting step of new CL biosynthesis in the isolated perfused heart is likely to be the conversion of PA into CDP-DG.

Another pathway of CL biosynthesis, via phospholipase D, was postulated [42]. In these experiments, exogenous cabbage phospholipase D and radioactive PG produced CL. The 10000 g fraction of rat ventricular myocardium was shown to contain a small amount of phospholipase D activity towards PC [43].

However, phospholipase D activity towards PG has not been reported in rat heart mitochondria. Thus this pathway likely contributes little, if at all, to new CL biosynthesis in the heart. A previous study reported that PGP and PG could be synthesized *in vitro* from radioactive GP in mitochondrial fractions prepared from rat heart [17]. No radioactive CL was detected. In another study, radioactive CL was synthesized *in vitro* from radioactive GP in guinea-pig heart mitochondrial fractions [44]. Finally, those same authors demonstrated that mitochondrial fractions from adult heart mitochondria synthesize *in vitro* some radioactive CL from [14 C]PG and [3 H]CDP-DG [45]. However, the above studies could not indicate if CL was synthesized from newly synthesized or pre-existing PA. The present study demonstrates unequivocally that new CL may be actively synthesized in the isolated perfused intact rat heart via the Kennedy pathway from newly synthesized PA.

In summary, we have utilized a model, the isolated perfused intact rat heart, for the study of CL biosynthesis. We demonstrated that new CL is actively synthesized from newly synthesized PA via the Kennedy pathway. In addition, we have shown that the rate-limiting step of CL biosynthesis in the isolated perfused intact rat heart is probably the conversion of PA into CDP-DG.

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