CARDIOLIPIN, ITS PREFERENTIAL DEACYLATION IN MAMMALIAN MYOCARDIA. MINI REVIEW AND CHROMATOGRAPHIC– COMPUTATIONAL ANALYSIS

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SUMMARY

Comparative correlative TLC analysis conducted on whole-tissue homogenate of control and in-vitro-incubated samples of mammalian (guinea-pig, rat, rabbit, mouse, pig, and hamster) myocardia and bullfrog cardiac muscle and thigh skeletal muscle revealed:
- (a) a noticeable, high level of CL in all mammalian myocardia which was greatly reduced after in-vitro incubation, with concurrent production of a high level of MLCL;
- (b) the extent of deacylation of CL and concurrent formation of MLCL is age-related, being lowest in fetal heart and much higher in the young adult heart and aged heart;
- (c) the level of CL was much lower in bullfrog cardiac muscle than in mammalian myocardia, and a small amount of MLCL is produced by in-vitro incubation; and
- (d) the level of CL was lowest in bullfrog thigh skeletal muscle and no MLCL was produced by in-vitro incubation.

Some sense of order is emerging in the lipolytic action of these tissues on their endogenous CL. This is probably related to the function of the tissues in vivo.

INTRODUCTION

Cardiolipin (CL), a phospholipid which occurs solely in the mitochondria, is associated with the enzymes of oxidative metabolism and contains mostly 18:1, 18:2, and 18:3 fatty acids [1–3]. Mammalian myocardia contain uniquely high levels of CL compared with other tissues [4,5].

We have previously reported diminution of CL in experimentally produced ischemic dog myocardium and infarcted cardiac muscle of cat,

rabbit, and man, with subsequent appearance of lyso derivatives of CL, and mitochondrial swelling [5,6]. More recently, Lesnefsky et al. have shown that myocardial ischemia selectively deacylated CL in rabbit heart subsarcolemmal mitochondria with no changes in the amounts of other phospholipids [7]. In 2000 Vreken et al. reported that Barth's syndrome (a genetic mutation in an X-linked gene) results in CL depletion, mitochondrial dysfunction, and cardiomyopathy [8]. It has also been reported that CL was substantially depleted in diabetic mouse myocardium [9] and in aged rat [10], although treatment of rats with thyroxine has been shown to elevate the biosynthesis and amount of CL in the heart [11].

We recently reported that the endogenous CL of mammalian myocardia and pectoral muscle of some birds is preferentially deacylated by an endogenous phospholipase (PLA_1 and/or PLA_2) during in-vitro incubation for 60 min at pH 7.4 and 38°C, producing monolysocardiolipin (MLCL) and fatty acids, with concurrent diminution of CL [12–14]. More recently we found that after similar treatment of whole-tissue homogenate of heart, pectoral muscle and kidney from seven-day-old chicks CL deacylation was most prominent in the cardiac muscle and significantly low in the pectoral muscle [15]. Noticeable hydrolysis of sphingomyelin (SM) and concurrent formation of ceramide were most conspicuous in the kidney. In contrast, similarly treated whole-tissue homogenate of rabbit spleen selectively deacylated phosphatidylethanolamine plasmalogen (PE), producing lyso PE [16].

These data clearly illustrate tissue specificity in relation to their lipolytic capabilities on their respective endogenous phospholipids and seem to emphasize that CL has a unique position in the metabolism of phospholipids that is not yet well defined and seems to be related to the energy requirement of the myocardium; this should be investigated further.

In an attempt to learn more about the actual role of CL and its function in in-vitro deacylation, referred to above, we conducted in-vitro incubation of whole-tissue homogenate as a source of enzymes (phospholipases A_1 and A_2) and substrates (phospholipids) from:
  (a) mammalian (guinea-pigs, rabbit, rat, mouse, pig, and hamster) cardiac muscle;
  (b) guinea-pig and rabbit cardiac muscle (fetal, young adult, and aged);
  (c) amphibian cardiac muscle and thigh skeletal muscle (bullfrog).

These tissues are known to contain numerous mitochondria and hence high levels of CL. Results from TLC–densitometric analysis of the

in-vitro response of these tissues, and their correlation with in-vivo metabolism, will be discussed.

EXPERIMENTAL

Our focus in the study was on TLC–densitometric analysis of the endogenous CL of control samples and their respective incubated samples to identify the products, e.g. lysocardiolipin, formed during in-vitro incubation.

Incubation and Extraction of Tissue

Homogenate of whole tissue from a variety of mammalian cardiac muscle, bullfrog cardiac muscle, and thigh skeletal muscle, as sources of enzymes (phospholipases) and substrates (phospholipids) was incubated in vitro for 60 min at pH 7.4 and 38°C to determine the lipolytic properties of the tissues. Cardiac muscle and skeletal muscles were thinly sliced, promptly placed in aluminum foil, and flash-frozen between two 1-cm-thick slabs of pre-cooled aluminum maintained at −20°C or below. The tissue was then freeze-dried in vacuo and pulverized with a mortar and pestle. Samples (100 mg) were transferred to 13-mm × 100-mm test tubes with PTFE-lined screw caps and solutions of Tris buffer (Trizma hydrochloride solution, pH 7.4, Sigma, USA; 0.05 M, 2 mL) and $CaCl_2$ (0.1 M, 20 μL) were added. The samples were then incubated at 38°C, with frequent vortex mixing, for 60 min, when incubation was terminated by rapid freezing of the test-tube contents (in situ). The samples were then freeze-dried and extracted with chloroform–methanol, 2:1 (v/v), 2 mL).

Control samples were prepared by direct extraction (chloroform–methanol, 2:1 (v/v), 2 mL).

Thin-Layer Chromatography

Chloroform–methanol extracts obtained from the freeze-dried tissue homogenate were analyzed by thin-layer chromatography on silica gel, with 1-propanol–chloroform–ethyl acetate–methanol–water 50:50:50:21:18 as mobile phase, to identify the phospholipid profile of the tissue. Ten-microliter volumes of the extracts were applied to the plates. Experimental details of the chromatography have been described elsewhere [17]. Because comparison of the phospholipid profiles of the extracts obtained from controls and from their respective incubated samples will reveal products re-

sulting from the incubation, the endogenous phospholipase(s) activated could be identified.

Differential staining of the phospholipids (CL and its lyso derivatives) on the TLC plates with thionine reagent, which furnishes a lavender spot for MLCL, is described in detail elsewhere [18].

The plates were scanned densitometrically at 600 nm (thionine λ_{max} ≈ 600 nm) with a Camag scanning densitometer. Data were processed with the CATS software package and Microsoft Excel software for comparative quantification of the phospholipid components of control and incubated samples.

RESULTS

This comparative and correlative study focused on TLC–densitometric analysis of the relative concentrations of CL in cardiac muscle (guinea-pig, rabbit, rat, mouse, pig, and hamster), bullfrog cardiac muscle and thigh skeletal muscle and the products resulting from in-vitro incubation of whole-tissue homogenate of these tissues at pH 7.4 and 38°C for 60 min. The principal results obtained are presented in Figs 1a–1f, 2a and 2b, and 3a and 3b as one-dimensional chromatograms.

Figure 1 reveals the high level of CL in all the young adult mammalian cardiac muscle studied compared with the much lower level in bullfrog heart (Fig. 3a) and the minute amount in bullfrog thigh muscle (Fig. 3a). The amount of MLCL formed during in-vitro incubation was noticeably high in all mammalian myocardia (Figs 1a–1f, I lanes). The amount was much less in bullfrog heart and none was produced by bullfrog thigh muscle (Figs 3a and 3b). The concurrent reduction in the amount of CL is compatible with the amount of MLCL formed in these organs (Figs 1a–1f, I lanes, and Figs 3a and 3b). Fetal guinea-pig heart and fetal rabbit heart also deacylated CL during this incubation procedure (Figs 2a and 2b). The concentration of CL in the fetal heart was less than that in the adult and aged hearts (Fig. 2a, C lanes). The level of MLCL was very low in fetal guinea-pig heart compared with the much higher levels in both young adult and aged hearts (Fig. 2a, I lanes) indicating a relationship between the deacylation products and the age of the animal.

In conclusion, comparative and correlative analysis of whole-tissue homogenate of control and in-vitro-incubated samples of mammalian myocardia and bullfrog cardiac muscle and thigh skeletal muscle revealed:
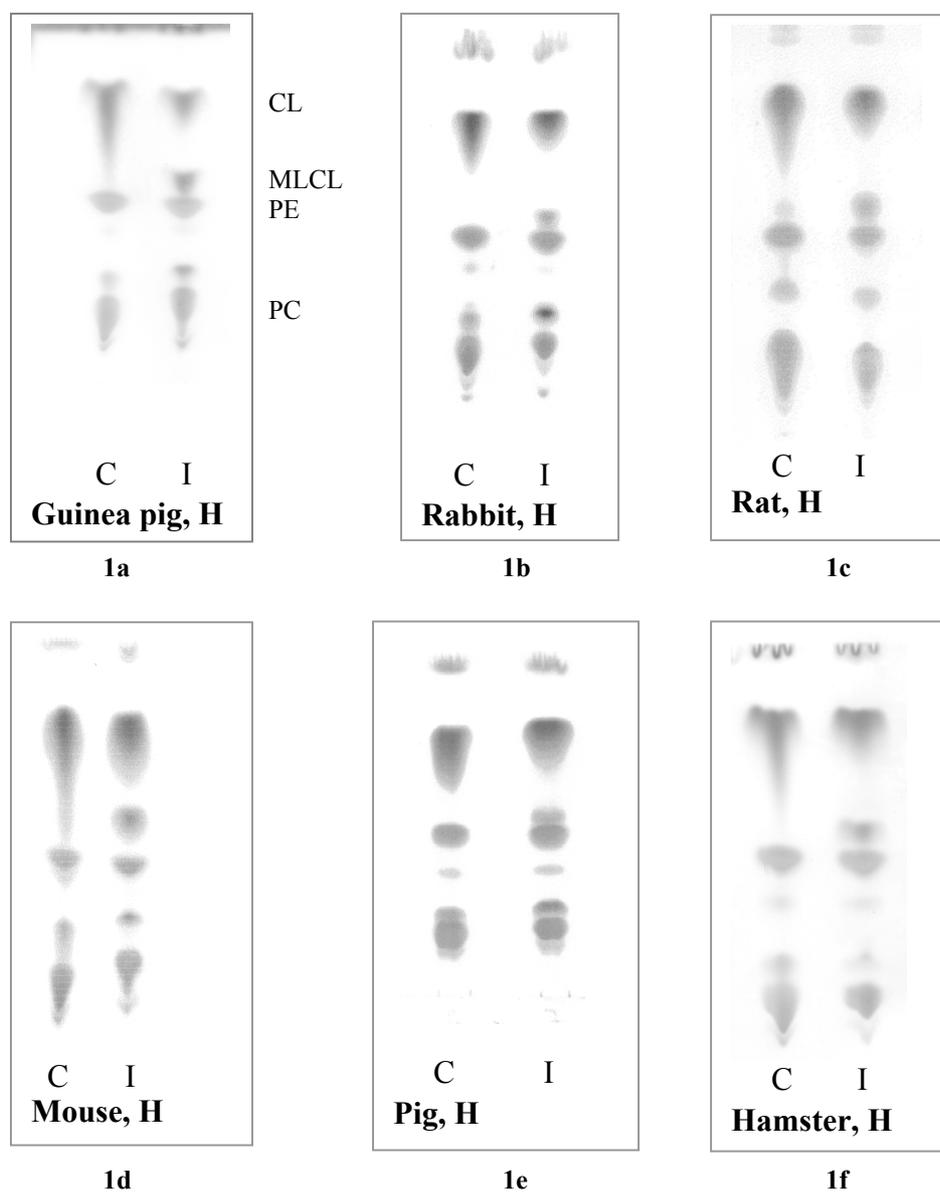
Fig. 1

TLC chromatograms obtained from chloroform–methanol extracts of control (C lanes) and incubated (I lanes) samples showing the high levels of CL in all the control samples. Formation of MLCL with concomitant reduction in CL levels is apparent for all the incubated samples. CL, cardiolipin; MLCL, monolysocardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine
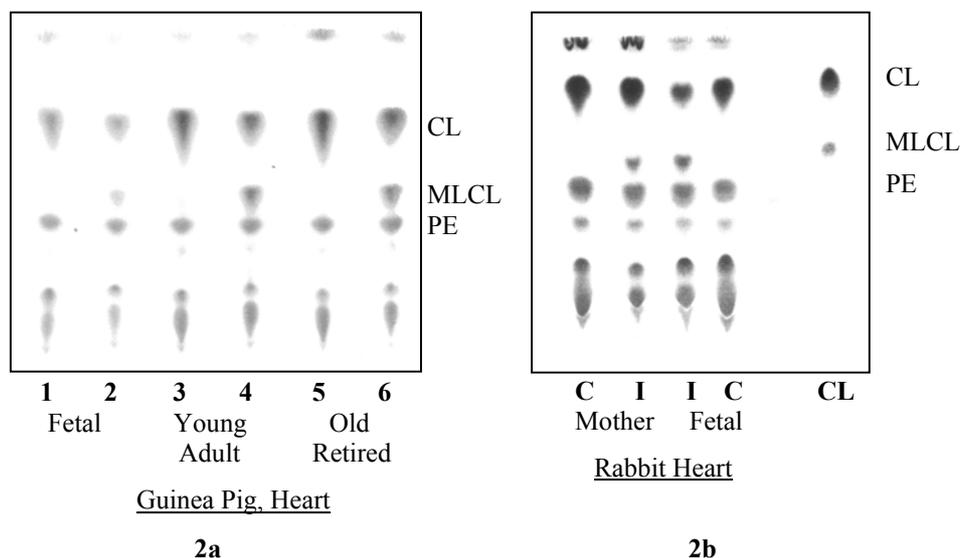
Fig. 2

(a) TLC chromatograms obtained from chloroform–methanol extracts of control (odd lanes) and incubated (even lanes) samples of guinea-pig heart (fetal, young adult, and aged). CL levels are very low in the fetal heart of guinea-pig compared with young adult and aged guinea-pig heart. After incubation the MLCL level in fetal heart is very low compared with levels in both young adult and aged heart. (b) TLC chromatograms obtained from chloroform–methanol extracts of control (C lanes) and incubated (I lanes) samples of rabbit heart (mother and fetus). Deacylation of CL and formation of MLCL during in-vitro incubation is similar for both mother and fetal heart samples. CL (cardiolipin) and MLCL (monolysocardiolipin) are standards

(a) a noticeably high level of CL in all mammalian cardiac muscle, which was greatly reduced by in-vitro incubation, in which a high level of MLCL is produced concurrently;

(b) the extent of deacylation of CL and the amount of MLCL formed is age-related, being lowest for fetal heart and much higher for heart from young adults and aged animals;

(c) much less CL was present in bullfrog cardiac muscle than in mammalian myocardium, and a small amount of MLCL was produced during in-vitro incubation; and

(d) the amount of CL was lowest in bullfrog thigh skeletal muscle and no MLCL was produced during in-vitro incubation.

Some sense of order is emerging in the lipolytic action of these tissues (from different animal species) on their respective endogenous CL. This is probably related to the function of the tissues in vivo.
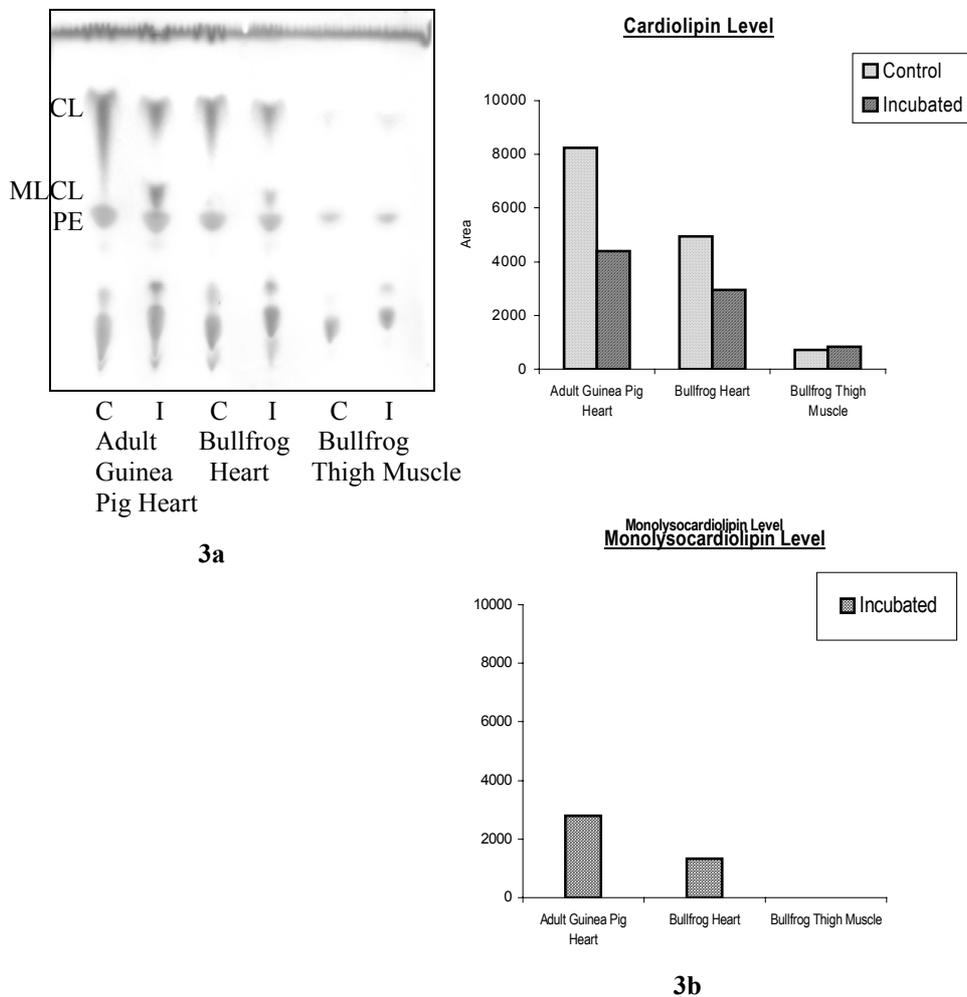
Fig. 3

(a) TLC chromatograms obtained from chloroform–methanol extracts of control (C lanes) and incubated (I lanes) samples of guinea-pig heart, bullfrog heart and bullfrog thigh muscle. Levels of CL are highest for guinea-pig heart, less for bullfrog heart, and bullfrog thigh muscle contains a minute amount. After in-vitro incubation, a noticeable high level of MLCL is produced in guinea-pig heart, much less in bullfrog heart, and none in bullfrog thigh muscle. MLCL formation is indicative of the action of endogenous PLA_1 and/or PLA_2 on endogenous CL in both guinea-pig heart and bullfrog heart. (b) Bar-chart presentation, using Excel software, revealing the contrast in the level of CL in guinea-pig heart, bullfrog heart, and bullfrog thigh muscle, both in control samples and after incubation (upper plots). During in-vitro incubation more MLCL is formed in guinea-pig heart than bullfrog heart and none is produced in bullfrog thigh muscle (lower plots)

DISCUSSION

This comparative, correlative TLC analysis of whole-tissue homogenates of control and in-vitro-incubated samples of mammalian myocardia and bullfrog cardiac muscle and skeletal thigh muscle revealed:
  (a) a unique, high level of CL in all the mammalian myocardia studied, which was preferentially deacylated during in-vitro incubation, producing monolysocardiolipin (MLCL);
  (b) bullfrog cardiac muscle contained much less CL and a minute amount of MLCL was formed during in-vitro incubation, compared with mammalian cardiac muscle; and
  (c) the level of CL was lowest in bullfrog skeletal thigh muscle and no MLCL was produced during incubation.

These data raise questions about the lipolytic properties of different tissues from different species of animal in relation to their CL content and its preferential deacylation, and further investigation is required.

We recently reported that the endogenous CL of some mammalian myocardia and of the pectoral muscles of some birds is preferentially deacylated by an endogenous phospholipase (PLA_1/PLA_2) during in-vitro incubation for 60 min at pH 7.4 and 38°C, producing MLCL and fatty acids, and with concurrent diminution of CL [12–14]. Use of thionine as spot test reagent [18] facilitated recognition of the deacylation products and CL. When the period of incubation was prolonged (3 h), *N*-acyl phosphatidylethanolamine (NAPE) was produced concurrently with the deacylation of CL, but in dog and cat cardiac muscle only, indicating species differences [12]. The deacylation of CL in mammalian myocardia reported here is indicative of the action of endogenous phospholipases (PLA_1 and/or PLA_2). In-vitro data obtained using endogenous and exogenous phospholipids as substrates has already shown that rat, guinea-pig, pig, and dog heart mitochondria contain PLA_1 and PLA_2; CL was not one of the substrates used in this work [19,20]. PLA_2 of rat liver mitochondria deacylates CL producing MLCL, and the latter is re-acylated via a transacylase [21]. These observations corroborate the preferential deacylation of CL by endogenous PLA_2 reported here in mammalian cardiac muscle.

Alteration of serum phospholipids in black bears during hibernation has been reported. Compared with black bears in the active state, during hibernation levels of phosphatidyl choline (PC), phosphatidyl serine (PS), phosphatidyl inositol (PI), and sphingomyelin (SM) increased whereas that of phosphatidyl ethanolamine (PE) decreased [22]. No information about

CL was included and it remains to be investigated whether levels of phospholipids, including CL, of other tissues, for example amphibian cardiac muscle and skeletal muscle, are affected during hibernation.

We have previously reported diminution of CL levels and the formation of lyso derivatives of CL, with concomitant mitochondrial swelling, in ischemic cardiac muscle of dog, cat, rabbit (experimentally produced), and man [5,6]. Myocardial ischemia selectively depleted CL in rabbit heart mitochondria with no change in the amounts of other phospholipids [7]. Other workers have reported that mitochondrial ischemia is associated with PLA_2 activation and accumulation of lysophospholipids [23,24]. Alkenyl phosphatidyl choline was the substrate in most of these studies [20,22–24]; CL was never used as substrate. The high level of essential fatty acids (linoleic and linolenic) unique to CL [25] seems significant. Cardiac muscle is highly dependent on oxidation of fatty acids as a major source of energy [21,24].

These data emphasize the importance of the deacylation of CL described here and seem to indicate that this deacylation is related to the energy requirement of the myocardium.

Several isoforms of cytosolic PLA_2 ($cPLA_2$) have been described, with preferential release of arachidonic acid from PC and PE species from different types of cell (canine myocardium, bovine brain, rat heart, rabbit kidney, rat testes) [26–30]. CL lacks arachidonic acid. Arachidonic acid-selective enzymes have been emphasized in work performed so far, because arachidonic acid is the rate-limiting precursor of eicosanoids, which are associated with the inflammatory response.

Although $cPLA_2$ isoforms have been the subject of several recent detailed reviews [31–33], the importance of $cPLA_2$ in control of cell function has not been clearly established, and many details still require further inquiry.

The proenzyme status of mammalian pancreatic PLA_2 provides in-situ protection against premature deacylation [34,35] and the cellular phospholipases of other tissues, including those described in this report, are under different control, which is yet to be elucidated. The discovery that pancreatic phospholipase A_2 occurs as a proenzyme activated by trypsin [35] has led to a search for other activators and inhibitors.

Overall, differing reports on properties such as Ca^{2+} requirements, pH optima, and temperature sensitivity [35,36] have made continuing investigation of this area necessary in a search for full understanding of the in-vivo function of all lipolytic enzymes endogenous to an organ, as de-

scribed here, and to provide an explanation of the need for such diversity of phosphoglycerides in different organs.

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