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Phospholipase D activity in subcellular membranes of rat ventricular myocardium

Vincenzo Panagia, Chuhua Ou, Yuji Taira, Jian Dai and Naranjan S. Dhalla

Division of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, Winnipeg (Canada) and Departments of Anatomy and Physiology, Faculty of Medicine, University of Manitoba, Winnipeg (Canada)

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The phospholipase D (PL D), which catalyzes the formation of phosphatidic acid (PA), was studied in rat myocardium using ^{14}C-labelled phosphatidylcholine (PC) as an exogenous substrate. Subcellular distribution experiments indicated the presence of PL D in particulate fractions only. Different procedures for the isolation of purified cardiac subcellular organelles showed the presence of PL D in sarcolemma (SL), sarcoplasmic reticulum (SR) and mitochondria with 14-, 11- and 5-fold enrichment when compared to the homogenate value, respectively. The activity of SL PL D was observed over a narrow acid pH range with an optimum at 6.5, and it showed a high specificity for PC while phosphatidylethanolamine and phosphatidylinositol showed a low rate of hydrolysis. Under optimal conditions, PA formation was linear for a 60-min period of incubation and the reaction rate was constant for 10 to 100 μg SL protein in the assay medium. The SR PL D displayed properties similar to those seen with the SL PL D. In membrane fractions PL D was also found to catalyze a transphosphatidylation reaction for the synthesis of phosphatidylglycerol. Assessment of the intramembranal levels of radioactive 1,2-diacylglycerol (DAG) in the absence or presence of KF suggested the presence of an active PA phosphohydrolase activity. This study indicates that a PC-specific PL D activity is localized in different membrane systems of the myocardium and may be associated with PA phosphohydrolase to act in a coordinated manner. The functional significance of PL D-dependent formation of PA in cardiac membranes is discussed.

Introduction

Phospholipase D (PL D, EC 3.1.4.4) is a phosphodiesterase which modifies the polar portion of phosphatidylcholine (PC) and/or other phospholipids by catalyzing the hydrolytic formation of phosphatidic acid (PA) with concomitant release of the non-phosphorylated base (for reviews, see Refs. 1 and 2). Choline, the aqueous fragment of PC hydrolysis, may be metabolized through the CDP-choline pathway to resynthesize PC [2] and may contribute to the acetylcholine homeostasis of cholinergic neurons [1,2]. Newly formed PA may enter the biosynthetic pathway of the anionic phospholipids [3,4] or may be cleaved to 1,2-diacylglycerol (DAG) by PA phosphohydrolase [4,5]. DAG is a common precursor for the synthesis of nitrogenous phospholipids and for the biosynthesis of triacylglycerol [4]. DAG also activates protein kinase C, which phosphorylates a number of target proteins [6], as well as yields arachidonate upon further hydrolysis by diacylglycerol and monoacylglycerol lipases leading to the synthesis of eicosanoids [7]. In addition to the hydrolytic reaction leading to PA formation, PL D catalyzes a transphosphatidylation reaction in which the phosphatidyl moiety of the substrate phospholipid is transferred to alcohols such as ethanol or glycerol producing phosphatidyl-ethanol or -glycerol, respectively [1,2]. Transphosphatidylation reaction is unique to PL D and has been used to ascertain the role of this enzyme in certain bioprocesses [1,8].

Although PL D has been studied in several tissues, the information on myocardium is very limited. PL D activity has been detected in the heart both in the hydrolytic [9] and in the transphosphatidylation [10] mode when crude microsomes from a variety of rat tissues were surveyed. It was also shown that perfusion

Correspondence: V. Panagia, Division of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, 351 Tache Avenue, Winnipeg, Manitoba, Canada R2H 2A6.

of the isolated rat hearts prelabeled with [^{3}H]choline with carbachol enhanced the efflux of choline [11] in parallel with the generation of PA [12]. The appearance either of free choline or PA indicates the presence of PL D activity [1]. Although the involvement of cardiac PL D in catalyzing PC hydrolysis after muscarinic receptor [11,12] or β-adrenoceptor [13] activation is likely [11,13], there is a lack of information regarding the presence of PL D activity in the cell membrane (sarcolemma) as well as other components of the cardiac cell. In this paper we provide biochemical evidence for the presence of PL D in different membrane systems of the rat myocardium and we report some of its properties.

Methods and Materials

Isolation of subcellular membranes. Male Sprague-Dawley rats weighing approx. 250–300 g were employed in this study. Animals were killed by decapitation, hearts were immediately excised and the ventricular tissue was then processed in pooled samples of three or more hearts for the isolation of various subcelular organelles. All procedures were carried out at 0–5°C.

Crude subcellular fractions were prepared by differential centrifugation as outlined by Sulakhe and Dhalla [14]. Sarcolemma-enriched membranes were obtained by the isolation procedures of Dhalla et al. [15], Frank et al. [16] and Pitts [17]. Membrane fraction enriched with the sarcoplasmic reticulum fragments was isolated according to the method of Harigaya and Schwartz [18] while mitochondria were isolated by the method of Sordahl et al. [19]. The activities of some marker enzymes such as ouabain-sensitive Na^+,K^+-ATPase [20], cytochrome-*c* oxidase [21] and rotenone-insensitive NADPH–cytochrome-*c* reductase [21] were measured to determine the purity of sarcolemma, mitochondria and sarcoplasmic reticulum, respectively.

Assay for phospholipase D activities. Phospholipase D hydrolytic activity was assayed by measuring the formation of labeled phosphatidic acid in the presence of [^{14}C]phosphatidylcholine essentially as described by Kobayashi et al. [22]. The exogenous phospholipid substrate was prepared by combining egg phosphatidylcholine and tracer [^{14}C]phosphatidylcholine; the solvents were evaporated under N_2 stream and an aqueous solution of sodium oleate was then added. This mixture was sonicated for 30 min with a Branson 1200 sonicator and appropriate aliquots were used for the assay. Unless otherwise indicated, the incubation of the membranes (50 μg protein) was carried out at 30°C for 90 min in a final volume of 120 μl containing 50 mM β,β-dimethylglutaric acid - 10 mM EDTA (pH 6.5), 25 mM KF, 5 mM sodium oleate and 2.5 mM [^{14}C]phosphatidylcholine (0.167 μCi/μmol). The reaction was terminated by the addition of 2 ml of chloroform/methanol (2:1, by vol.) followed by 0.5 ml of 0.1 M KCl. The tubes were shaken vigorously for 5 min and then centrifuged at 2000 × g for 10 min. After aspiration of the aqueous phase, the chloroform phase was washed according to Folch et al. [23] to remove nonlipid contaminants. Blanks were carried out under identical conditions except that heat-denatured membrane proteins (10 min, 90°C) [24] were added to the reaction mixture. The final chloroform phase was evaporated almost to dryness under N_2 stream. The residue was immediately dissolved in 30 μl of chloroform containing 30 μg of phosphatidic acid as a carrier and quantitatively applied to the silica gel 60 A F-254 thin-layer plates (0.25 mm thick) under a light N_2 stream. The chloroform-containing test tubes were washed twice with 30 μl of chloroform and each washing was again applied to the layer. The chromatogram was run at room temperature in a solvent system containing chloroform/methanol/acetone/acetic acid/water (50:15:15:10:5, by vol.). After the solvent front had migrated approximately 15 cm, the plates were air dried at room temperature. The lipid spots were visualized by exposure to iodine vapors, scraped after the disappearance of the iodine color, and radioactivity was counted in 5 ml Ecolume. Corrections were made for the quenching due to silica gel in all the data expressed. The lipid migration was monitored using authentic unlabelled lipid standards. The areas corresponding to PA and DAG (1,2-dioleoylglycerol) standards were visualized autoradiographically (Fig. 1). It should be noted that DAG standard comigrated with the solvent front (R_f = 1) which, in sample's runs, contained radioactive DAG derived from PA dephosphorylation [22]. Free fatty acid standard (oleic acid) migrated distinctly below the DAG spot with a R_f value of 0.94. The transphosphatidylation activity of phospholipase D was assayed by measuring the [^{3}H]phosphatidylglycerol formation as described by Chalifour and Kanfer [10]. Briefly, membranes (50 μg proteins) were incubated at 30°C for 15 min in 50 μl of medium containing 0.4 M [2-^{3}H]glycerol (2 Ci/mol), 50 mM β,β-dimethylglutaric acid - 10 mM EDTA, pH 6.5, and 5 mM sodium oleate. The reaction was terminated by adding 1.5 ml of chloroform/methanol (2·1, by vol.) and carrier phosphatidylglycerol (0.1 mg/ml). Thereafter, free glycerol was removed by partitioning once with 300 μl of 0.1 M KCl and seven times with 600 μl of chloroform/methanol/0.1 M KCl (3:47:48, by vol.). The final chloroform phase was evaporated almost to dryness under N_2 stream. The residue was immediately dissolved in 30 μl of chloroform, applied to silica gel 60 A F-254 thin-layer plates (0.25 mm thick) under a light N_2 stream, and developed at room temperature in a solvent system containing tetrahydrofuran/methylal/methanol/2 M ammonium hydroxide (50:40:10:5.5, by vol). Phosphatidylglycerol was located by iodine vapors in the presence of unlabelled lipid standard and its area

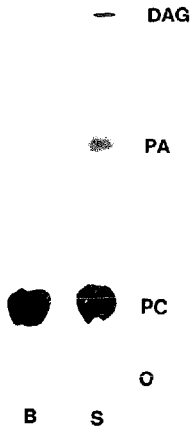

Fig. 1. Autoradiograph of ^{14}C-labelled lipids after thin-layer chromatography fractionation of a chloroform extract from phospholipase D assay. Phospholipase D hydrolytic activity was assayed as described in Methods and Materials. After thin-layer chromatography, the spots were visualized by autoradiography (Kodak X-OMAT AR) and identified by comperison with unlabelled lipid standards which were run in parallel and detected by iodine vapors. B = blank; S = sample; O = origin; PC = phosphatidylcholine; PA = phosphatidic acid; DAG = 1,2-diacylglycerol.

was removed for radioactivity counting. Blanks containing heat-denatured membrane proteins (10 min, 90°C) were carried out under identical conditions.

Protein was determined by the method of Lowry et al. [25] with bovine serum albumin (fraction V) as a standard. All experiments were repeated at least two or three times and each assay was done in triplicate.

Materials. L-α-1-Palmitoyl-2-[^{14}C]oleoylphosphatidylcholine (spec. act., 58.0 mCi/mmol), L-α-1-palmitoyl-2-[^{14}C]arachidonylphosphatidylethanolamine (52.3 mCi/mmol), L-α-1-stearoyl-2-[^{14}C]arachidonylphosphatidylinositol (38.5 mCi/mmol) and [2-^{3}H]glycerol (10 Ci/mmol) were purchased form New England Nuclear, Mississauga, Ont. (Canada). Phosphatidylcholine (egg), phosphatidic acid and sodium oleate were obtained from Serdary Research laboratories, London, Ont. (Canada). L-α-Phosphatidylcholine (bovine heart or bovine liver), L-α-phosphatidylethanolamine (bovine liver), L-α-phosphatidylinositol (soybean or bovine liver), 1,2-dioleoyl-*rac*-glycerol and alamethicin were from Sigma Chemical, St. Louis, MO (U.S.A.). Silica gel 60 A F-254 thin-layer chromatography plates were obtained from Whatman International Ltd., Maidston (U.K.). All other reagents were of analytical grade or of the highest grade available.

Results

Subcellular distribution of phospholipase D

The subcellular distribution of PL D activity was monitored in crude fractions obtained from heart homogenate by differential centrifugation (Table I). Although the specific activity of PL D in the 1000 × *g* fraction (which is believed to contain sarcolemma, myofibrils, nuclei and cell debris) was low, this fraction, due to very high protein yield, accounted for the largest amount (54%) of the total enzyme activity present in the homogenate. The 40 000 × *g* fraction (mainly containing sarcoplasmic reticulum) showed the highest specific activity but, due to the low protein yield, the total enzyme activity in this fraction was about 15%. About 17% of enzyme activity was associated with the 10 000 × *g* fraction (primarily containing mitochondria). The particulate nature of PL D in cardiac tissue is evident because the enzyme activity was not detected in the soluble fraction (105 000 × *g* supernatant).

Subcellular localization of phospholipase D

The biochemical localization of PL D in rat heart was studied in purified subcellular preparations. PL D

TABLE I

Distribution of phospholipase D activity in fractions obtained by differential centrifugation of the homogenate from rat heart ventricular tissue

Results are average of two separate experiments done in triplicate (variation <10%). Phospholipase D hydrolytic activity was assayed at 30°C as described in Methods and Materials. Recovery (%) indicates the percentage distribution of total activity in various fractions, relative to homogenate activity (100%). PA = phosphatidic acid.

Fraction	Protein yield (mg/g tissue)	Specific activity (nmol PA/mg per h)	Total activity (nmol PA/mg)	Recovery (%)
Homogenate	166.7	6.4	1066.9	100
1000 × *g* (10 min)	81.0	7.1	575.1	53.9
10 000 × *g* (20 min)	8.6	21.2	182.3	17.1
40 000 × *g* (45 min)	1.4	115.6	161.8	15.1
105 000 × *g* (60 min)	1.2	44.6	53.5	5.0
105 000 × *g* Supernatant	44.4	0.0	0.0	0.0

TABLE II

Phospholipase D-dependent transphosphatidylation and marker enzyme activities in cardiac subcellular membranes

Values are means ± SE of three to four different membrane preparations and are expressed as: (a) nmol phosphatidylglycerol formed per mg per h; (b) μmol P_i/mg per h; (c) nmol cytochrome *c*/mg per min; (d) nmol cytochrome *c* reduced per mg per min. Sarcolemma, mitochondria and sarcoplasmic reticulum fragments were isolated according to Pitts [17], Sordahl et al. [19], and Harigaya and Schwartz [18], respectively. Assays were performed as indicated in Methods and Materials. Ouabain-sensitive Na^+,K^+-ATPase activity was determined in the presence of alamethicin (0.6 mg/mg membrane protein) as described earlier [20]. Data in parentheses indicate the relative specific activity that is the specific activity in the fraction/the specific activity in the homogenate.

	Sarcolemma	Mitochondria	Sarcoplasmic reticulum
A. Transphosphatidylation (a)	7.3 ± 0.5 (14.6)	3.1 ± 0.2 (6.2)	4.8 ± 0.3 (9.6)
B. Marker enzymes			
Ouabain-sensitive Na^+,K^+-ATPase (b)	26.2 ± 2.1 (15.0)	1.4 ± 0.2 (0.8)	1.8 ± 0.2 (1.0)
Cytochrome-*c* oxidase (c)	59.1 ± 7.4 (0.6)	373.1 ± 32.2 (4.0)	13.6 ± 2.0 (0.15)
Rotenone-insensitive NADPH–cytochrome-*c* reductase (d)	2.1 ± 0.3 (0.4)	1.4 ± 0.1 (0.27)	41.4 ± 5.6 (8.08)

activity was monitored in the transphosphatidylation mode by quantitating the formation of phosphatidylglycerol, a well characterized product of this reaction [1], from intramembranal phospholipid substrate and exogenous [2-^{3}H] glycerol [3,10]. The SL membranes yielded by discontinuous sucrose density gradient [17] showed the highest transphosphatidylation activity; this enzyme activity was also present in mitochondria and SR (Table II). Because transphosphatidylation reaction is catalyzed only by PL D [8], it can be concluded that PL D is present in SL, SR and mitochondrial membranes of the rat cardiac cell. The above subcellular membranes were also examined for the marker enzyme activities to assess the possible extent of cross contamination (Table II). After alamethicin treatment [20], the SL marker ouabain-sensitive Na^+,K^+-ATPase showed a 15-fold increase in SL in comparison to the homogenate value; the low activity detected in both mitochondria and SR suggests a small degree of SL contamination in these fractions. The cytochrome-*c* oxidase (mitochondrial marker) and rotenone-insensitive NADPH–cytochrome-*c* reductase (sarcoplasmic reticular marker) activities in the SL fraction were 0.6- and 0.4-fold of the corresponding values in the heart homogenate, respectively. The relative specific activity of cytochrome-*c* oxidase and rotenone-insensitive NADPH–cytochrome-*c* reductase in mitochondrial and SR preparations was about 4.0- and 8.1-fold, respectively (Table II). These observations appear to indicate that the above fractions were relatively pure and had

TABLE III

Phospholipase D hydrolytic activity and 1,2-diacylglycerol level in subcellular fractions isolated by different procedures from rat heart ventricle

Values are means ± S.E. of four experiments done in triplicate. Assays were performed at 30°C as described in Methods and Materials. The relative specific activity is defined in the legend to Table II. Phospholipase D specific activity is expressed as nmol of phosphatidic acid formed per mg per h. SR = sarcoplasmic reticulum.

Methods and fractions	Phosphatidic acid		1,2-Diacylglycerol	
	nmol/mg per h	relative spec. act.	nmol/mg per h	relative spec. act.
Homogenate	5.8 ± 0.3	–	2.4 ± 0.1	-
A. Sarcolemma				
Dhalla et al. [15]	10.7 ± 0.4	1.8	5.7 ± 0.4	2.4
Frank et al. [16]	23.6 ± 1.7	4.0	15.0 ± 1.1	6.2
Pitts [17]	81.3 ± 4.3	14.0	27.0 ± 1.8	11.2
B. Mitochondria				
Sordahl et al. [19]	32.5 ± 1.5	5.6	6.9 ± 0.3	2.9
C. SR fragments				
Harigaya and [18] Schwartz	65.4 ± 2.9	11.3	11.8 ± 0.4	4.9

minimal cross contamination. When the hydrolytic activity of PL D was assessed in purified cardiac membranes, SL yielded by the method of Pitts [17] showed the highest specific activity while relatively low values were found in the other two SL preparations (Table III). The exact reasons for this finding are not clear at present but it is possible that varying degrees of activation or deactivation of the enzyme may have occurred during different isolation procedures. Such a view is consistent with our finding that the PL D activity in the purified SR fraction (Table III) was lower than that in the crude $40000 \times g$ fraction (Table I). It should be pointed out that the intramembranal accumulation of radioactive 1,2-diacylglycerol occurred in all the fractions examined (Table III). This may reflect the presence of an active phosphatidate phosphatase in cardiac subcellular membranes as described for other cell types [26,27].

Properties of the sarcolemmal phospholipase D

The possible involvement of PL D in catalyzing PC hydrolysis after muscarinic receptor or β-adrenoceptor activation of the heart [11,13] confers particular functional importance to the hydrolytic activity of the sarcolemmal enzyme. Therefore, the SL PL D was studied in the membrane fraction [17] which was found to be highly enriched in this enzyme activity (Table III). Because PA was hydrolyzed to DAG by a membrane phosphatidate phosphohydrolase [3,26,27] and because KF partially inhibits the PA phosphohydrolase [1,3], different concentrations of this fluoride salt were tested to minimize the DAG formation. High levels of radioactive DAG and reduced PA formation were found in the absence of KF (Fig. 2). Inhibition of DAG and increase of PA production were found to occur at 10 mM KF. The concentration of 25 mM KF, which was routinely

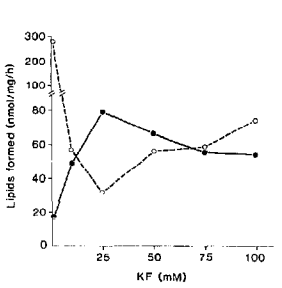

Fig. 2. Effect of KF on phospholipase D activity and 1,2-diacylglycerol level in heart sarcolemma. SL membranes [17] were assayed at 30°C. Data are from a typical experiment; each point represents the average of triplicate measurements which differed less than 10%. Phosphatidic acid (●); 1,2-diacylglycerol (○).

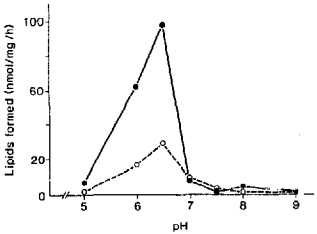

Fig. 3. Effect of pH on phospholipase D activity of rat heart sarcolemma. SL fraction was isolated according to Pitts [17]. The buffers were: 50 mM β,β-dimethylglutarate-NaOH for pH 5.0 to 7.5, and 50 mM glycylglycine-HCl for pH 8.0 and 9.0 [9]. Assays were carried out at 30°C as described in Methods and Materials. Data are from a typical experiment; each point represents the average of triplicate measurements which differed less than 10%. Phosphatidic acid (●); 1,2-diacylglycerol (○).

employed in the initial sets of experiments, proved to be optimal because the PA formation was 4.4-fold higher than the DAG formation. Further increase in KF concentrations resulted in the PA and DAG formation being progressively decreased and increased, respectively (Fig. 2). These data differ from those obtained with brain microsomes where intramembrane PA was undetectable in the absence of KF and DAG was higher than PA at all fluoride concentrations (25 to 250 mM) tested [3]. At any rate, the partial inhibition of radioactive DAG formation by KF indicates the presence of a substantial amount of PA phosphohydrolase activity in cardiac SL.

To determine the pH optimum for PL D, SL membranes were incubated at different pH values, from 5 to 9, using established buffer systems [9]. The enzyme activity was observed over a narrow acid pH range with an optimum at pH 6.5 (Fig. 3); this is in agreement with previous findings in other cell systems [3,9]. Unlike brain microsomes [3], however, the intrasarcolemmal level of PA formed was considerably higher than that of DAG (Fig. 3). The optimal temperature of incubation for the PL D was 30°C (Fig. 4) as reported for brain microsomes [10]. However, shifting the temperature from 25°C to 30°C, a limited increase in PA formation (1.12-fold) was noted while DAG formation increased 3.75-fold. The PA to DAG ratio diminished significantly ($P < 0.05$, $n = 4$) from 8.4 ± 1.3 at 25°C to 3.3 ± 0.2 at 30°C. Therefore, it was concluded that the temperature sensitivity of the enzymes responsible for the synthesis of PA (PL D) and DAG (likely PA phosphatase) was probably different, and that incubation at 25°C would be favorable for minimizing PA phosphatase activity with only marginal reduction of PL D activity. Accordingly, further studies were performed at

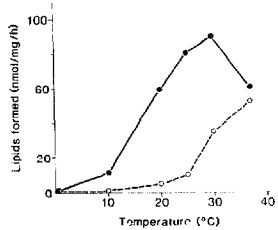

Fig. 4. Temperature dependence of sarcolemmal phospholipase D. SL membranes [17] were assayed at pH 6.5 and at the indicated temperatures. Data are from a typical experiment; each point represents the average of triplicate measurements which differed less than 10%. Phosphatidic acid (●); 1,2-diacylglycerol (○).

25°C. Under optimal assay conditions, PA formation was linear for a 90-min period of incubation (Fig. 5A) and the reaction rate was constant when 10 to 100 μg SL protein were employed for the assay (Fig. 5B). Different phospholipids, which have been found to be substrates for different forms of PL D in various cell types [1,8,24,27], were examined as possible substrates for the heart SL phospholipase D. Under our assay conditions, the enzyme showed a stringent specificity for PC; phosphatidylethanolamine (PE) and phosphatidylinositol (PI) from various sources were hydrolyzed at a very low rate (Table IV).

Properties of the sarcoplasmic reticular phospholipase D

A quite active intracellular PL D was found in the SR membrane and shared a number of similar properties such as optimal pH, temperature and KF concentration as well as substrate specificity with the SL enzyme (Table V). It should be noted that the change of incubation temperature from 25°C to 30°C induced a drop in the SR PA to DAG ratio which was of the same magnitude of that observed with the SL membranes. However, unlike SL, the intra SR PA and DAG formation did not change by increasing KF concentration from 25 to 50 mM (Table V).

TABLE IV

Substrate specificity of sarcolemmal phospholipase D

Values are means ± S.E. of three experiments done in triplicate. SL membrane (50 μg) [17] were assayed at pH 6.5, 25°C and for 90-min incubation periods. Various phospholipid substrates were prepared as indicated in Methods and Materials by combining: (A) phosphatidylcholine from egg, bovine heart or bovine liver and tracer [^{14}C]PC; (B) phosphatidylethanolamine from egg or bovine liver and tracer [^{14}C]PE; (C) phosphatidylinositol from soybean or bovine liver and tracer [^{14}C]PI. Relative activity is expressed as percent of the activity toward phosphatidylcholine from egg. PA = phosphatidic acid.

Phospholipid substrate and source	Phospholipase D	
	nmol PA/mg per h	relative activity
A. Phosphatidylcholine		
egg	77.3 ± 3.0	100
bovine heart	109.9 ± 3.5	142.1
bovine liver	79.0 ± 9.0	102.2
B. Phosphatidylethanolamine		
egg	5.2 ± 1.8	6.7
bovine liver	5.0 ± 1.2	6.5
C. Phosphatidylinositol		
soybean	2.2 ± 0.7	2.8
bovine liver	3.5 ± 1.0	4.5

Discussion

This study indicates that a phospholipase D catalyzing the hydrolytic formation of PA is localized in different membrane systems of the cardiac cell. Subcellular distribution showed that a major proportion (50 to 55%) of the total activity was recovered in the 1000 × *g* fraction containing sarcolemma, while no activity was

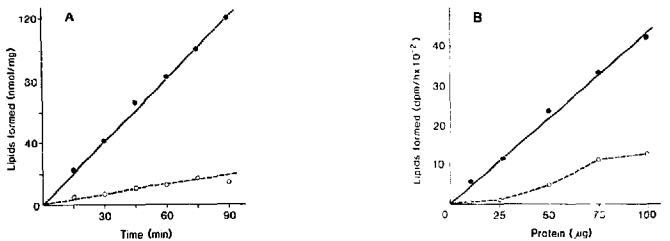

Fig. 5. Time course and protein dependence of sarcolemmal phospholipase D activity. In A, 50 μg SL protein [17] were assayed at pH 6.5, 25°C, and for the indicated times of incubation. In B, the phospholipase D activity of the indicated amounts of SL protein was estimated at pH 6.5, 25°C, and for a 90-min incubation period. Data are from typical experiments; each point represents the average of triplicate measurements (variation < 10%). Phosphatidic acid (●); 1,2-diacylglycerol (○).

TABLE V

Influence of various factors on the hydrolytic activity of sarcoplasmic reticular phospholipase D

Values are means ± S.E. of three experiments. SR fragments were isolated according to Harigaya and Schwartz [18]. Assays related to KF, pH, temperature and phospholipid substrate were performed as indicated in the legends of Figs. 2, 3, 4 and Table IV, respectively, and as described in Materials and Methods. PC = phosphatidylcholine (egg); PE = phosphatidylethanolamine (egg); PI = phosphatidylinositol (soybean).

Experimental conditions	Phosphatidic acid (nmol/mg per h)	1,2-Diacylglycerol (nmol/mg per h)
A. KF (mM)		
10	47.1 ± 4.3	26.2 ± 5.9
25	63.8 ± 1.5	15.0 ± 2.6
50	63.5 ± 0.7	17.2 ± 1.0
B. pH		
6.6	29.2 ± 3.8	5.5 ± 0.1
6.5	53.5 ± 1.3	14.0 ± 0.7
7.0	12.8 ± 1.2	13.6 ± 1.1
C. Temperature (°C)		
20	45.1 ± 3.2	5.3 ± 1.5
25	55.1 ± 2.1	5.6 ± 0.9
30	57.6 ± 3.3	18.2 ± 2.0
D. Phospholipid substrate		
PC	63.3 ± 2.8	8.9 ± 1.3
PE	1.8 ± 0.6	0.2 ± 0.1
PI	1.2 ± 0.5	0.4 ± 0.2

detected in the cytosolic fraction. Different procedures for the isolation of purified cardiac subcellular organelles revealed the presence of PL D in SL, SR and mitochondrial membranes with 14-, 11- and 5-fold enrichment factor, respectively. Although these results confirm that the surface membrane contains most of the PL D activity, the presence of PL D in SR and mitochondrial fractions suggests that a significant portion of the total cellular enzyme activity in the heart is localized intracellularly. Since the pattern for PL D activity purification in different fractions followed that seen with well known marker enzymes, the PL D activity detected in the subcellular membranes under study may not be due to cross contamination but in fact may be inherent to each membrane system. It is worth noting that, in this study, the lack of nucleotides in the assay medium of the hydrolytic reaction excludes any contribution of the phosphoinositide-specific phospholipase C/DAG kinase pathway for PA production [7]. This supports the view that the observed PA generation could be accounted for only by a PL D-dependent cleavage. Further, all the purified fractions exhibited transphosphatidylation activity, a unique feature of PL D [8], and this can be taken to suggest the association of PL D to the cardiac membranes. Apparently, this enzyme has no strict requirement for Ca^{2+} because in all membranes it displayed a substantial activity in the presence of 10 mM EDTA. It should be noted that most of the mammalian membrane-bound phospholipases D are Ca^{2+}-independent [1,27]. However, the existence in human serum of a Ca^{2+}-dependent PL D with stringent substrate specificity for glycan-PI and not for PI has been reported [28], while two PL D activities with diverse subcellular localization and biochemical properties have been found in human neutrophils [8]. Upon testing the SL and SR membranes with the major phospholipid classes that are hydrolyzed by PL D in other tissues [1,8,24], myocardial PL D was found to display a high specificity for PC as this was the only phospholipid hydrolyzed at significant rates. Therefore, in the heart a PC-specific PL D exists for the production of PA independently of the phosphoinositide-specific phospholipase C/DAG kinase pathway [7]. The PL D described here appears to be unrelated to the previously reported membrane-bound phosphodiesterase of the PL D type which hydrolyzes *N*-acyl-PE to *N*-acylethanolamine and PA while it does not hydrolyze to any extent PE or PC [29].

Intramembranal accumulation of the radioactive DAG was noted in cardiac subcellular organelles incubated with exogenous radioactive PC, as reported for different cell types [3,27,30]. Formation of DAG from PC may be due to either a PC-hydrolyzing phospholipase C (PC-PL C) activity or to the sequentially coupled PC-PL D and PA phosphohydrolase activities [30,31]. The latter pathway seems to be responsible for the DAG formation observed in this study for the following reasons: (a) the PA to DAG ratio resulted to be 0.06 and 2.50 in the absence and presence, respectively, of an optimal concentration of KF (25 mM, Fig. 2) which is known to partially inhibit PA phosphohydrolase [1,3]; (b) PA phosphohydrolase was found to be present in cardiac SL vesicles [32]; (c) endogenous PC can yield DAG upon PL D activation in various systems [3,30,31]; (d) a soluble form of PL C specific for PC and PE has been found in dog heart cytosol [33], but this cellular localization excludes any participation of PC-PL C in forming DAG under our experimental conditions. Therefore, it can be proposed that in cardiac membranes a DAG pool is derived from PC via a PC-specific PL D/PA phosphohydrolase pathway in addition to DAG formed by phosphoinositide hydrolysis [7,31].

The biochemical properties of the cell membrane PL D described here and the possibility that its activity is affected during cardiomyocyte stimulation [11,13] suggest that the role of this enzyme may be that of actively generating important lipid molecules for the signal transduction under receptor occupancy and for the final cell response to agonists. The functional significance of PL D-dependent increase of PA in cardiac SL membranes is indicated by recent studies. For example,

treatment with exogenous PL D increased the cardiac SL Na^+-Ca^{2+} exchange activity [32]. Ca^{2+} binding and force of contraction of the heart [34]. The observation that exogenous addition of PA and PL D generates Ca^{2+}-dependent slow action potentials in depolarized rat atrium supports a role for PA in mediating an increase of Ca^{2+} influx into the cardiac cells [35]. PA has also been reported to stimulate the SL Ca^{2+} pump [36] which is involved in the efflux of Ca^{2+} from the cell during relaxation of the myocardium [37]. We have shown that SL levels of PA are decreased in failing myocardium [38] and suggested that high levels of PA in the hypertrophied rabbit heart SL may be causally related to the increased activity of the Ca^{2+} pump [39]. These results suggest that SL PL D hydrolytic activity may be implicated in regulating the Ca^{2+} movements in myocardium thus affecting the contractile function. Further, a rapid formation of PA under agonist stimulation of the heart [12] and its potential action in enhancing the polyphosphoinositide hydrolysis [40] indicate that this phospholipid may have signalling functions.

The role of PL D-derived PA in SR and mitochondrial function is unclear at present; however, it is worth noting that PA has been reported to release Ca^{2+} from cardiac SR thus providing a new pathway for modulating SR Ca^{2+} transport [41]. It is also possible that the PC-hydrolyzing PL D activity alone or in association with PA phosphohydrolase may contribute to the synthesis of other phospholipids or triacylglycerol via PA or DAG intermediates [4]. In this regard, increased cytosolic amounts of choline, another product of PC degradation by PL D, may contribute to the final resynthesis of PC at the SR level via the CDP-choline pathway [42]. These newly formed PC molecules in the SR membrane may then become available for intermembrane translocation by phospholipid transfer proteins [43,44]. Such a process can be seen to restore the depleted PC level of the cardiac membranes. The possibility that free choline released by PL D-dependent PC breakdown may participate in the acetylcholine synthesis in the heart, as proposed for the brain [1,2], cannot be ruled out at present. Furthermore, the characteristics of the DAG pool, which seems to originate from PC in cardiac membranes, need to be defined with respect to the formation of DAG molecules during receptor stimulation and their role in cell signalling processes in terms of protein kinase C activation [31] and free fatty acid formation as well as ultimate synthesis of eicosanoids [7].

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