Subcellular localization of the phospholipases A of rat heart: evidence for a cytosolic phospholipase A_1

Gilles Nalbone[1] and Karl Y. Hostetler

Department of Medicine, University of California, San Diego, and the Veterans Administration Medical Center, San Diego, CA 92161

Abstract During myocardial ischemia increased levels of lysoglycerophospholipids have been reported which may be deleterious to myocardial function. Phospholipases are presumed to be important in the regulation of this process. To further quantify and characterize the activity of heart phospholipases, we carried out a systematic analysis of phospholipase A activity in rat heart subcellular fractions isolated by the method of Palmer et al. (J. Biol. Chem. 1972. **262:** 8731–8739). Neutral phospholipase A was recovered predominately in the cytosolic (soluble) fraction which represented 46% of recovered activity, while the microsomal and subsarcolemmal mitochondrial fractions represented 15% and 12% of the total recovered activity, respectively. Cytosolic phospholipase A differed from the two principal membrane-bound phospholipases A in its pH dependence and apparent K_m for substrate. The cytosolic enzyme had a K_m (apparent) for dioleoylphosphatidylcholine of 0.07 mM versus 0.28–0.33 mM for the membrane-associated phospholipases A. Acid phospholipase A activity had a subcellular distribution consistent with a lysosomal localization. Lysophospholipase was found principally in the cytosolic, microsomal, and the subsarcolemmal and interfibrillar mitochondrial fractions where it represented 46, 17, 6.3, and 6.9% of the recovered activity, respectively. The positional specificity of the respective phospholipases was assessed. This analysis was complicated by the fact that in heart, lysophospholipase has an observed V_{max} 3.6- to 4.5-fold greater than that of phospholipase A in the various subcellular fractions. Equations were derived to obtain corrected values for the activity of phospholipases A_1 and A_2. Using this method we found that the cytosolic and lysosomal fractions contained phospholipase A_1, while the mitochondrial fractions contained primarily phospholipase A_2. In heart microsomes, the positional specificity of phospholipase A could not be determined because lysophospholipase activity was very high and lysophosphatidylcholine did not accumulate. —**Nalbone, G., and K. Y. Hostetler.** Subcellular localization of the phospholipases A of rat heart: evidence for a cytosolic phospholipase A_1. *J. Lipid Res.* 1985. **26:** 104–114.

Supplementary key words lysophospholipase • phospholipase A_2

The phospholipid bilayer constitutes the permeability barrier of biological membranes and provides the environment in which transbilayer and peripheral membrane proteins are oriented (1). In myocardial ischemia, alterations in membrane phospholipid metabolism are thought to be of great importance since phospholipid degradation products, fatty acids and lysophospholipids, are potentially cytotoxic and may cause irregular heart rhythms (2–6). In addition, lysophosphatidylcholine may contribute to ischemic injury by promoting calcium entry as reported by Sedlis et al. (7). Finally, depletion of phospholipids in heart membranes (8–10) may ultimately result in loss of the permeability barrier needed to support the normal biological functions of these membranes.

In view of the potential importance of phospholipids and lysophospholipids, detailed information about the enzymes acting on these compounds is needed to allow a better understanding of membrane events in myocardial ischemia. Phospholipase A, which hydrolyzes either the *sn*-1 or *sn*-2 acyl ester of phospholipids, has been described previously in several different subcellular membrane fractions of the heart. Weglicki et al. (11) found phospholipase A activities in heart mitochondrial and microsomal fractions, but Gross and Sobel (12) did not demonstrate significant phospholipase A activity in heart microsomes. Palmer et al. (13) isolated two populations of heart mitochondria, subsarcolemmal and interfibrillar mitochondria. Using endogenous phospholipids as substrate, they reported the presence of both phospholipases A_1 and A_2 in these two populations of mitochondria. Acid phospholipase A has been reported previously in the lysosomal fraction of heart (14).

Lysophospholipase hydrolyzes the remaining acyl ester of the lysoglycerophospholipid molecule. Since high levels of lysophospholipids might have deleterious effects during ischemia, it might be proposed that a function of lysophospholipase is to prevent membrane damage by maintaining low levels of these compounds.

Abbreviations: FA, fatty acid; LPC, lysophosphatidylcholine.

[1]Present address: INSERM U130, 10 Avenue Viton, 13009 Marseille, France.

Recently, Gross and Sobel (12, 15) reported lysophospholipase activity in microsomal and cytosolic fractions from rat heart. However, in a previous report, it was indicated that the mitochondrial fraction from heart does not exhibit significant lysophospholipase activity (11).

It is difficult to gain a quantitative view of the contribution of various subcellular fractions to phospholipase A and lysophospholipase activity in heart because previous studies did not employ complete subcellular fractionation sequences and different conditions and substrates were used to assay for phospholipase A and lysophospholipase. We have now systematically examined the activity of phospholipase A and lysophospholipase in rat heart subcellular fractions. Our results indicate that the cytosolic fraction is the major intracellular site of neutral phospholipase A activity in rat heart. Microsomes and subsarcolemmal mitochondria also contain substantial neutral phospholipase A activity. Acid phospholipase A is principally enriched in the light mitochondrial fraction and its subcellular distribution is consistent with a lysosomal localization. Lysophospholipase is found in the cytosolic, microsomal and subsarcolemmal and interfibrillar mitochondrial fractions. Properties of the respective enzymes are reported.

MATERIALS AND METHODS

Preparation of subcellular fractions

Male rats of the Fischer 344 strain (Charles River, Boston, MA) weighing between 200 and 300 g were used. For each experiment ten rats were fasted overnight, killed by cervical fracture, and the hearts were removed quickly and placed in buffer A (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, pH 7.4) at 4°C. After removal of the nonventricular tissues, the hearts were rinsed several times with buffer A, blotted dry, and weighed. Approximately 6 g of heart was cut into small pieces and divided into two equal aliquots. Buffer A containing 2 mM EGTA was added to a final concentration of 10 g tissue/100 ml buffer. Each aliquot was homogenized with a Polytron tissue processor (PT-10 generator) for 3 sec at a rheostat setting of 6.5 as described by Palmer, Tandler, and Hoppel (16). Centrifugations and other operations were done at 4°C. Subsarcolemmal and interfibrillar mitochondria, light mitochondria, microsomes, and cytosolic fractions were obtained as noted below. The homogenate was centrifuged at 500 *g* for 10 min. The pellet was resuspended in buffer A with a Potter-Elvehjem homogenizer and centrifuged at 500 *g*. The low speed pellet was reserved at 4°C. The supernatants were combined and centrifuged at 3000 *g* for 10 min to obtain the subsarcolemmal mitochondria (M_s) which was washed twice with buffer A containing 2 mM EGTA. The postmitochondrial supernatants were combined and centrifuged at 17,000 *g* for 10 min to obtain the light mitochondrial pellet (L) which was washed twice with the same buffer. The resulting supernatants were combined and centrifuged at 100,000 *g* for 1 hr to obtain the microsomal pellet (P) and the cytosolic fraction. The low speed pellet was resuspended in buffer B (100 mM KCl, 50 mM MOPS, pH 7.4, and 2 mM EGTA) with a Potter-Elvehjem homogenizer and subjected to Nagarse protease action (5 mg per g of heart) for 30 sec followed by centrifugation at 5,000 *g* for 5 min (16). The resulting supernatant was called S_1. The resuspended pellet was centrifuged at 500 *g* to obtain the nuclei and debris pellet which was washed twice with buffer B containing 2 mM EGTA. The combined supernatants were centrifuged at 3,000 *g* for 10 min, and the resulting interfibrillar mitochondria pellet (M_i) was washed twice with buffer B containing 2 mM EGTA. The postmitochondrial supernatants were combined and reserved (S_2). All protein fractions were resuspended in buffer A containing 0.5 mM EGTA and stored at −60°C until use.

All experiments with the cytosolic fraction, except those shown in Fig. 1, were done with a 5-fold concentrated form. The cytosolic fraction was concentrated at 4°C under nitrogen (65 psi) with a 50-ml Amicon ultrafiltration cell containing a YM-10 diaflo membrane (Amicon Corporation, Lexington, MA).

Protein, phosphorus, and marker enzyme assays

Protein was measured by the dye-binding method of Bradford (17) using rabbit gamma globulin as standard (protein assay kit, Bio-Rad, Richmond, CA). Lipid phosphorus was determined according to the method of Rouser, Fleischer, and Yamamoto (18). Succinate INT dehydrogenase was determined by the method of Pennington (19). The N-acetylglucosaminidase was assayed according to Koldovsky and Palmieri (20) and NADPH-cytochrome c reductase was determined in the presence of rotenone according to Sottocasa et al (21).

Phospholipase A assays

Phospholipase A was assayed as previously described (22). Incubation mixtures contained 0.2 mM [1-^{14}C]-dioleoylphosphatidylcholine (Amersham, Arlington Heights, IL), sp act, 0.91 to 1.36 mCi/mmol and 50 mM Tris-HCl (pH 8.4, unless otherwise indicated) in a final volume of 0.2 ml. Lysosomal phospholipase A was analyzed with 50 mM sodium acetate buffer (pH 4.8). Phosphatidylcholine was dispersed by sonication in water for 5 min under nitrogen. Unless otherwise indicated, assays of phospholipase (and lysophospholipase) were carried out with protein fractions that has been

stored at −70 °C for periods of time varying from 1 day to 3 weeks. The reaction was started by addition of 0.15 to 0.90 mg of the respective proteins; 6 mM Ca^{2+} as the chloride was added last and the mixtures were incubated at 37 °C for 30 min. The reaction was stopped and the [1-^{14}C]oleic acid was isolated by the Dole extraction as modified by van den Bosch and Aarsman (23). One ml of the heptane phase was passed over a small silica gel column and the [1-^{14}C]oleic acid in the eluate was counted by liquid scintillation spectrometry as previously described (22). The results have been corrected for a control incubated without protein.

The studies on contribution of lysophospholipase to fatty acid release and the positional specificity of the phospholipases A were done with [1-^{14}C]dioleoylphosphatidylcholine and 1-palmitoyl-2-[1-^{14}C]oleoylphosphatidylcholine, respectively, (Amersham) adjusted by addition of unlabeled phosphatidylcholine to a specific activity of 1.36 mCi/mmol. The incubation mixture contained 0.2 mM sonicated substrate and 50 mM buffer at the indicated pH. The reaction was started by addition of protein followed by 6 mM Ca^{2+}; incubations were done at 37 °C for 30 min. The reaction was stopped by addition of 4.0 ml of chloroform–methanol 2:1 (v/v) and the lipids were extracted according to the method of Folch, Lees, and Sloane Stanley (24). The aqueous phase contained 0.01 N HCl to minimize losses of lysophospholipids. The chloroform extracts were reduced in volume under nitrogen and subjected to thin-layer chromatography on 10 × 10 cm plates of silica gel (HPTLC plates, EM Reagents, Elmsford, NY). The plates were first developed to 5 cm above the origin with chloroform–methanol–water 65:35:5 (v/v/v), dried for 20 min in a nitrogen atmosphere and developed to the top with heptane–diethylether–formic acid 90:60:4 (v/v/v). Spots corresponding to lysophosphatidylcholine, phosphatidylcholine, monoglyceride, diglyceride, and free fatty acid were located by development with iodine vapors and scraped into scintillation vials. Radioactive lipids were counted as previously described (22). The results were corrected for a control incubated without protein.

Lysophospholipase assay

The lysophospholipase assay medium contained 0.2 mM 1-[1-^{14}C]palmitoyllysophosphatidylcholine, (New England Nuclear, Boston, MA) sp act 0.41 to 0.68 mCi/mmol, 50 mM Tris HCl, pH 8.1, in a final volume of 0.2 ml. The respective protein fractions (0.15 to 0.9 mg) were added and the reaction was carried out at 37 °C for 30 min. Release of [1-^{14}C]oleic acid was measured as noted above, except that 1 ml of the heptane phase was used directly for liquid scintillation counting (22). All results were corrected for a control incubated without protein.

Chemicals

Dioleoylphosphatidylcholine, egg phosphatidylcholine, and palmitoyllysophosphatidylcholine (99% purity) were obtained from Sigma Corp., St. Louis, MO. Other chemicals were of analytical reagent grade. Chloroform and methanol were redistilled before use.

Derivation of equation to estimate the contribution of phospholipase A and lysophospholipase to fatty acid release from [1-^{14}C]dioleoylphosphatidylcholine

When FA_d and LPC_d are the observed nmol fatty acid or lysophosphatidylcholine released during incubation of [1-^{14}C]dioleoylphosphatidylcholine, P represents the fraction of the total fatty acid released from [1-^{14}C]dioleoylphosphatidylcholine by the action of phospholipase A (either A_1 or A_2) and L represents the fraction of total fatty acid due to the action of lysophospholipase on [1-^{14}C]oleoyllysophosphatidylcholine. The following equation describes this relationship:

$$\frac{FA_d}{LPC_d} = \frac{P + L}{P - L} \quad . \qquad Eq.\ 1$$

It is asumed that phospholipase A hydrolyzes only one acyl group from phosphatidylcholine (either the *sn*-1 or *sn*-2 ester) and that lysophospholipase does not hydrolyze diacylphosphoglycerides and is equally active against *sn*-1 or *sn*-2 acyl lysophosphoglycerides. L can approach, but can never exceed, P with the result that the maximal value for L is 0.50. L and P can be determined from equation 1 since L + P = 1. Finally, it is assumed that other types of reactions such as transacylation, phospholipase C, and diglyceride lipase are negligible.

Derivation of an equation describing the effect of variable contributions of lysophospholipase on the measurement of phospholipases A_1 and A_2 using 1-palmitoyl-2-[1-^{14}C]oleoylphosphatidylcholine as substrate

With 2-[1-^{14}C]oleoylphosphatidylcholine as substrate, radioactive fatty acid is produced directly by the action of phospholipase A_2 and the other product, lysophosphatidylcholine, is unlabeled. Phospholipase A_1 action produces [^{14}C]lysophosphatidylcholine and unlabeled fatty acid. Lysophospholipase action on this product results in the production of ^{14}C-labeled fatty acid. These relationships are described by equation 2 below:

$$\frac{FA_s}{LPC_s} = \frac{A_2 + (A_1)(L)}{A_1 - (A_1)(L)} \quad . \qquad Eq.\ 2$$

FA_s and LPC_s represent the observed production of ^{14}C-labeled fatty acid and lysophosphatidylcholine from phosphatidylcholine specifically labeled with [^{14}C]oleic acid in the *sn*-2 position. A_1 and A_2 represent

the fraction of phosphatidylcholine hydrolyzed to lysophosphatidylcholine by phospholipases A_1 or A_2, respectively, and L represents the fraction of fatty acid release due to the action of lysophospholipase as determined in equation 1 above using [1-^{14}C]dioleoylphosphatidylcholine as substrate. The numerator represents radioactive fatty acid formed by phospholipase A_2 plus that formed by the action of lysophospholipase on the product of phospholipase A_1 action. The denominator represents radioactive lysophosphatidylcholine produced by phospholipase A_1 minus that hydrolyzed to fatty acid by lysophospholipase. Finally, it is also assumed that the respective enzymatic reaction rates do not differ with [1-^{14}C]dioleoylphosphatidylcholine or 1-palmitoyl-2-[1-^{14}C]oleoylphosphatidylcholine as substrate.

RESULTS

Subcellular distribution of phospholipases A and lysophospholipase

Neutral phospholipase A activity was found principally in the cytosolic, microsomal and subsarcolemmal fractions as shown in **Fig. 1.** The results represent the average of four separate experiments. The percentage of the total phospholipase A activity recovered in these fractions represented 45.8, 17.4, and 12.2%, respectively. Cytosolic phospholipase A activity has not previously been reported in heart. The observed specific activity of the subsarcolemmal mitochondria fraction was 8-fold greater than that of the interfibrillar mitochondria fraction which contained only 2.4% of the total recovered phospholipase A activity. However, this difference may have been overestimated, as the interfibrillar mitochondrial phospholipase A activity was not strictly linear with time and protein in agreement with the observations of Palmer et al. (13). All other subcellular fractions, including the subsarcolemmal mitochondria, exhibited linearity with time and protein under the assay conditions used. This problem has been studied in more detail and is discussed below (Table 2). Based on the subcellular distribution of marker enzymes, it appears that most of the phospholipase A activity measured in the light mitochondria and nuclei and debris fractions represented contamination with microsomes and mitochondria. As noted above, the cytosolic fraction which results from Polytron treatment alone contained 46% of the recovered phospholipase A activity. This cannot be explained solely by a solubilization of enzymes from mitochondria and microsomes based on marker enzyme results (below).

Acid phospholipase A activity exhibited an 8.3-fold enrichment in the light mitochondrial fraction. This

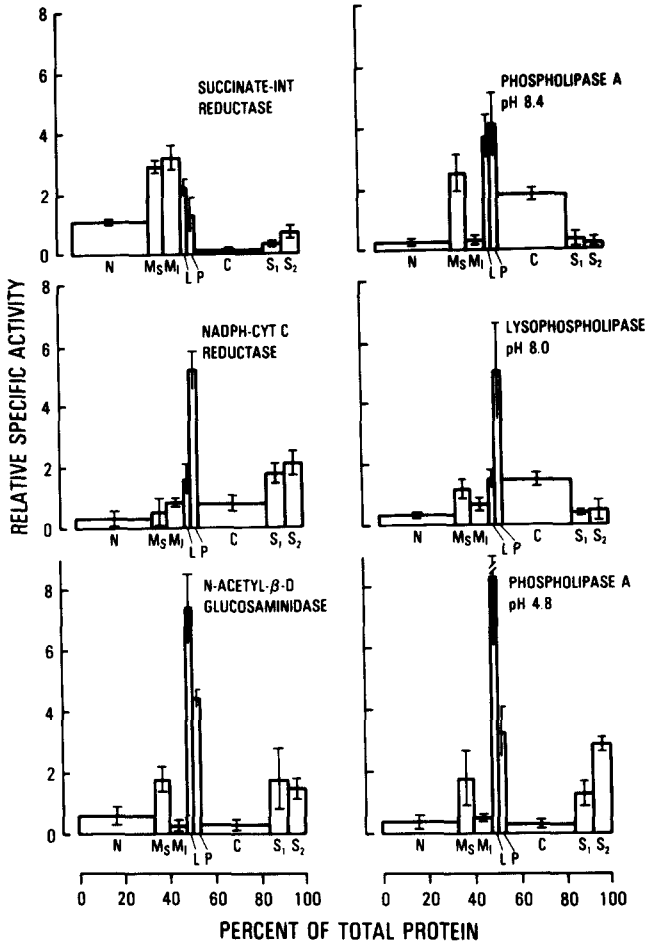

Fig. 1 Distribution of marker enzymes (left panels), phospholipases and lysophospholipase (right panels) in rat heart subcellular fractions. Each fraction is represented on the ordinate scale by its relative specific activity (percentage of total recovered activity/percentage of total protein). On the abcissa scale each fraction is represented (cumulatively from left to right) by its protein content expressed as percentage of total recovered protein. Homogenate specific activities (nmol $hr^{-1}mg^{-1}$): succinate INT reductase, 2050 ± 263; NADPH-CytC reductase, 24.1 ± 8.3; N-acetyl-β-D glucosaminidase, 89 ± 43.3; phospholipase A, pH 8.4, 1.72 ± 0.2; lysophospholipase, pH 8.0, 11.9 ± 3.5; phospholipase A, pH 4.8, 0.26 ± 0.12. Recoveries (%): protein, 116 ± 14; succinate INT reductase, 117 ± 33; NADPH-CytC reductase, 188 ± 99; N-acetyl-β-D glucosaminidase, 71.2 ± 9.5; phospholipase A, pH 8.4, 60 ± 17.0; lysophospholipase, pH 8.0, 66.7 ± 23; phospholipase A, pH 4.8, 150 ± 8.0. The above values represent averages of four separate preparations ± standard deviation, except for NADPH-CytC reductase and phospholipase A, pH 4.8, which represent three preparations. Abbreviations: N, nuclei and debris; M_s, subsarcolemmal mitochondria; M_I, interfibrillar mitochondria; L, light mitochondria; P, microsomes; C, cytosol or soluble fraction; S_1, post-5,000 *g* Nagarse supernatant; S_2, post-3,000 *g* M_I supernatant.

fraction contained 12.8% of the total recovered activity while the subsarcolemmal mitochondria, interfibrillar mitochondria, microsomal and cytosolic fractions contained 9.7, 4.0, 11.6, and 11.3 percent of the recovered activity, respectively. However, since the subcellular distribution of N-acetyl-β-D-glucosaminidase, a lysosomal marker enzyme, was identical to that of acid

phospholipase A, the latter enzyme appeared to be strictly of lysosomal origin.

The subcellular distribution of the lysophospholipase activity measured at pH 8.0 showed a pattern generally similar to that observed with phospholipase A except that the activity of lysophospholipase was greater in the interfibrillar mitochondria fraction. The subsarcolemmal mitochondria, interfibrillar mitochondria, microsomal and cytosolic fractions contained the bulk of the lysophospholipase activity representing 6.9, 6.3, 17.2, and 45.7% of the total recovered activity, respectively.

Subcellular distribution of protein and marker enzymes

As shown in Fig. 1 and **Table 1**, the nuclei and debris fraction contained 34.4% of the total recovered protein, while the subsarcolemmal mitochondria and interfibrillar mitochondria fractions contained 5.6 and 8.5% of the protein, respectively. The percentage of protein in the two populations of mitochondria was in accordance with previous reports (16). The light mitochondrial fraction contained only 1.8% of the protein while the microsomal fraction and the cytosolic fractions contained 3.4 and 30.1% of the total recovered protein, respectively.

The subcellular distribution of succinate INT reductase, the mitochondrial marker enzyme, is also shown in Fig. 1. The two populations of mitochrondria showed the greatest enrichment with succinate INT reductase representing approximately 3-fold that of the homogenate. The subsarcolemmal mitochondria and interfibrillar mitochondria fractions accounted for 16.5 and 27.5% of the total recovered marker enzyme, respectively. About 3% of the total mitochondrial marker was present in the microsomal and soluble fractions with a specific activity representing 35.2 and 3.6% of that observed in the mitochondrial fractions, respectively.

The microsomal marker enzyme, NADPH cytochrome C reductase, showed a 5.2-fold enrichment in the microsomal fraction (Fig. 1 and Table 1). This fraction contained 17.6% of the total recovered enzyme marker. The soluble fraction contained 22.9% of the total marker enzyme, but the enrichment of this enzyme was only 0.8-fold of the homogenate and its specific activity represented only 14.7% of that measured in the microsomal fraction. The subsarcolemmal mitochondria and interfibrillar mitochondria contained 3.3 and 7.4% of the total recovered NADPH cytochrome C reductase, respectively, and the specific activity represented 8.8 and 17.8% of that of the microsomal fraction, respectively.

The lysosomal marker enzyme, N-acetyl-β-D-glucosaminidase, showed a 7.6-fold enrichment in the light mitochondria fraction. This fraction contained 13.7% of the total recovered activity while the subsarcolemmal mitochondria, interfibrillar mitochondria, microsomal and soluble fractions contained 10.0, 2.3, 13.1, and 10.7% of this recovered activity, respectively.

Finally, these three marker enzymes were present in significant quantities in the nuclei and debris, S_1 and S_2 fractions, presumably because it was not possible to obtain a complete release of all of the organelles entrapped in the small particles of heart tissue that remained after the brief Polytron treatment (nuclei and debris and S_1). S_2 represents the postmitochondrial supernatant containing Nagarse.

Effect of Nagarse on mitochondrial phospholipase A activity

As noted above, the specific activity of neutral phospholipase A was about 8 times greater in the subsarcolemmal mitochondria fraction than that of the interfibrillar mitochondria fraction. However, the protease treatment required for isolation of the latter fraction might have been responsible for its diminished phospholipase A activity. Therefore, we treated the subsarcolemmal mitochondria fraction with Nagarse under the same conditions used to obtain interfibrillar mitochondria. Subsarcolemmal mitochondria were reisolated and assayed for phospholipase A. Both subsarco-

TABLE 1. Percentage of proteins and enzymes marker activities expressed as the percentage of the total recovered activity

Fraction	Protein	NADPH Cyt C Reductase	Succinate INT-Reductase	N-Acetyl-β-D-Glucosaminidase	Acid P'Lipase A	Neutral P'Lipase A	Lyso-P'Lipase
Nuclei and debris	34.3 ± 4.4	12.8 ± 1.3	37.1 ± 5.4	22.2 ± 13.0	15.2 ± 8.6	8.7 ± 5.2	12.8 ± 2.9
M_s	5.6 ± 0.6	3.3 ± 2.8	16.5 ± 2.4	10.0 ± 2.4	9.7 ± 4.9	12.2 ± 6.7	6.9 ± 1.6
M_I	8.5 ± 0.6	7.4 ± 0.2	27.5 ± 4.9	2.3 ± 1.6	4.0 ± 0.7	2.4 ± 1.2	6.3 ± 1.9
Light mitochondria	1.8 ± 0.3	3.0 ± 1.0	4.2 ± 1.1	13.7 ± 1.04	12.8 ± 2.0	8.5 ± 3.0	2.7 ± 0.4
Microsomes	3.4 ± 0.2	17.6 ± 2.9	3.2 ± 0.7	13.1 ± 5.0	11.6 ± 2.1	17.4 ± 8.0	17.2 ± 6.2
Cytosol	30.1 ± 3.1	22.9 ± 7.7	3.1 ± 2.5	10.7 ± 5.9	11.3 ± 2.6	45.8 ± 14.1	45.7 ± 2.6
S_1	8.4 ± 0.9	14.8 ± 2.3	2.9 ± 0.8	15.8 ± 9.7	11.6 ± 5.2	2.8 ± 3.3	3.6 ± 0.4
S_2	7.6 ± 1.8	17.1 ± 4.6	5.6 ± 3.0	11.8 ± 4.2	23.5 ± 5.5	2.6 ± 2.9	3.3 ± 2.2

The above values represent the average of four separate preparations ± standard deviation except for NADPH-Cyt C reductase and phospholipase A, pH 4.8, which represent three preparations. Abbreviations: M_s, subsarcolemmal mitochondria; M_I, interfibrillar mitochondria; S_1, post-5,000 *g* Nagarse supernatant; S_2, post-3,000 *g* M_I supernatant; P'Lipase, phospholipase.

lemmal and interfibrillar mitochondria were assayed at much lower enzyme protein levels, 0.05 to 0.1 mg/tube, in order to obtain reaction rates that were more nearly linear. As shown in **Table 2**, after Nagarse treatment the phospholipase A activity in the reisolated subsarcolemmal mitochondria fraction fell from 6.34 to 4.09 nmol H^{-1} mg^{-1}, representing a decrease of 30%. Mitochondrial fractions which had each been treated once with Nagarse were compared; subsarcolemmal mitochondria were 1.5-fold more active than interfibrillar mitochondria under these assay conditions. Further treatment of interfibrillar mitochondria with Nagarse resulted in a further decline in activity from 2.71 to 1.40 nmol $mg^{-1}h^{-1}$. Although it is difficult to rigorously compare the two mitochondrial phospholipase A activities, we conclude that interfibrillar mitochondria had somewhat less phospholipase A activity than that of subsarcolemmal mitochondria after comparable Nagarse treatment.

pH Dependence of phospholipases A and lysophospholipases

The pH dependence of phospholipase A activity in the respective subcellular fractions of rat heart was determined. All fractions, except the light mitochondria fraction, exhibited a broad pH optimum centered near pH 8.4 (**Fig. 2**). The activity of cytosolic phospholipase A did not decline sharply between pH 8.0 and 7.0 (−15%), compared with the activity of the mitochondrial and microsomal phospholipases A_1 which decreased by nearly 45%. Phospholipase A activity in the light mitochondrial fraction exhibited a somewhat sharper optimum at pH 4.8 (**Fig. 3**) but some phospholipase A activity was also present in the neutral and alkaline pH range (data not shown), indicating contamination with microsomal and mitochondrial protein as would be expected based on the results of the marker enzyme studies shown in Fig. 1 and Table 1.

TABLE 2. Effect of Nagarse treatment on the phospholipase A activity of subsarcolemmal and interfibrillar mitochondria

	Phospholipase A_1 nmol $mg^{-1}hr^{-1}$		
Fraction	Control	One Nargarse Treatment	Two Nargarse Treatments
M_s	6.34 ± 1.37	4.09 ± 0.63	
M_I		2.71 ± 0.53	1.40 ± 1.36

The subsarcolemmal mitochondria fraction (M_s) was obtained as described in Materials and Methods and consisted of 33 mg of protein. The resuspended M_s fraction was divided into two aliquots, control and Nargarse-treated. The treated aliquot was incubated with 0.23 mg of Nargarse under conditions identical to those used to obtain the interfibrillar mitochondria (M_I) as described in Methods. Interfibrillar mitochondria were also divided into two aliquots and one was treated with Nargarse a second time as described above and reisolated. All assays were carried out using freshly isolated fractions which had not been frozen. The results represent a single preparation measured in triplicate. The respective pellets obtained after 3000 g centrifugation for 10 min were assayed for phospholipase A activity using 0.05–0.10 mg of enzyme protein per tube as described in Methods. Results are given as mean ± standard deviation.

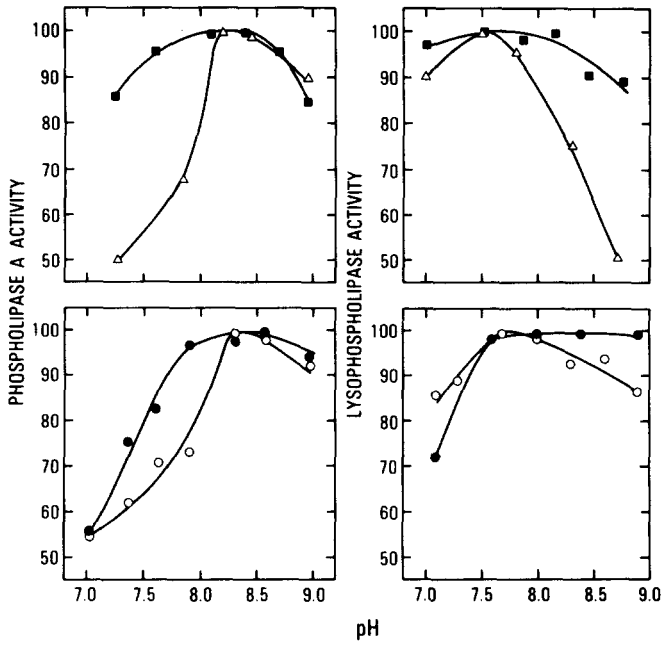

Fig. 2 Effect of pH on the activities of phospholipase A (left panels) and lysophospholipase (right panels) in: cytosol (■) and microsomes (△) (upper panels), subsarcolemmal mitochondria (●), and interfibrillar mitochondria (○) (lower panels). Experimental conditions are described in the Material and Methods section. Enzymes activities are expressed as a percentage of the maximal activity.

Lysophospholipase exhibited a broad pH dependence in all fractions with an optimum at pH 7.6 for microsomes and interfibrillar mitochondria, and at pH 8.0 for the subsarcolemmal mitochondria and soluble fractions (Fig. 2). In general the soluble lysophospholipase exhibited a broader pH dependence than that of the other fractions. In the acid pH range, 3.0 to 5.6, lysophospholipase activity was very low in the light mitochondrial (lysosomal) fraction, and appeared to be due to residual neutral lysophospholipase activity. For this reason, lysophospholipase was not further investigated in the acid pH range.

Effect of calcium and EDTA

The dependence of the phospholipase A activities on calcium is shown in **Table 3**. While the phospholipase A activities were stimulated to different degrees in the respective fractions, 6 mM Ca^{2+} was sufficient to produce full activity in all of the fractions studied. EDTA (10 mM) did not produce complete inhibition of the phospholipase A activities, suggesting the presence of phospholipases A which were not calcium-dependent. Acid phospholipase A of the light mitochondrial (lyso-

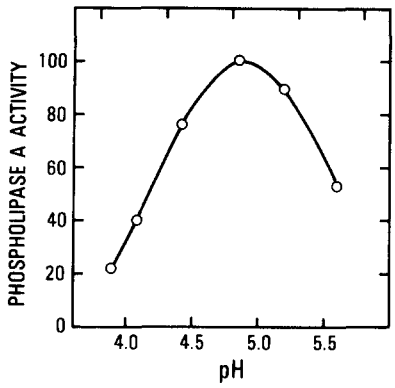

Fig. 3 Effect of pH on the activity of phospholipase A in the light mitochondria (lysosomal) fraction of rat heart. Experimental conditions are described in the Material and Methods section. Enzyme activity is expressed as a percentage of the maximal activity.

somal) fraction did not require calcium and was not inhibited by EDTA.

Kinetic studies of the phospholipases A and lysophospholipases

The respective subcellular fractions were incubated with increasing concentrations of either [1-^{14}C]dioleoylphosphatidylcholine or 1[1-^{14}C]palmitoyllysophosphatidylcholine. The apparent K_m and V_{max} were determined from the intercepts of Lineweaver-Burk plots and the results are shown in **Table 4.** The apparent K_m of phospholipase A for dioleoylphosphatidylcholine was similar in the subsarcolemmal mitochondrial fraction and in microsomes, ranging from 0.28 to 0.33 mM. Similarly, the apparent K_m of lysophospholipase for lysophosphatidylcholine did not differ greatly in these fractions, ranging from 0.24 to 0.28 mM. In contrast, the apparent K_m values of cytosolic phospholipase A and lysophospholipase were 0.07 and 0.02 mM, respectively, much lower than the values obtained for the respective mitochondrial and microsomal enzymes.

The apparent V_{max} of neutral phospholipase A was highest in the microsomal fraction, 17.9 nmol hr^{-1}mg^{-1}, followed by 4.66 nmol hr^{-1}mg^{-1} for subsarcolemmal mitochondria and 3.04 nmol hr^{-1}mg^{-1} for cytosol, respectively. The apparent V_{max} of lysophospholipase was greatest in the microsomal fraction, 83 nmol hr^{-1}mg^{-1} followed by subsarcolemmal mitochondria, cytosol, and interfibrillar mitochondria at 18.8, 10.8, and 10.2 nmol hr^{-1}mg^{-1}, respectively. Interestingly, the apparent V_{max} for lysophospholipase was 3.6 to 4.5 times greater than that of phospholipase A in the respective subcellular fractions. Acid phospholipase A had an apparent K_m of 0.66 mM and an apparent V_{max} of 16.8 nmol hr^{-1}mg^{-1}.

Contribution of lysophospholipase to release of [1-^{14}C]-oleic acid from [1-^{14}C]dioleoylphosphatidylcholine

Since the apparent V_{max} for lysophospholipase was much greater than that of phospholipase A as shown in Table 4, it seemed possible that phospholipase A activity measured with [1-^{14}C]dioleoylphosphatidylcholine might be overestimated. Therefore we carried out more detailed studies of this reaction in which all of the possible products were analyzed by thin-layer chromatography. The results are shown in **Table 5.** If only phospholipase A was active, one would find stoichiometric production of fatty acid and lysophosphatidylcholine with a product ratio of 1.0. However, when an active lysophospholipase is also present, [^{14}C]lysophosphatidylcholine may be converted to ^{14}C-labeled fatty acid (and unlabeled glycerophosphocholine) and the ratio of fatty acid to lysophosphatidylcholine (FA/LPC) will exceed 1.0. This was found to be the case in each of the heart subcellular fractions studied. In the light mitochondria (lysosomal) fraction, the FA/LPC ratio was low, 1.1, corresponding to a fractional lysophospholipase contribution to FA release of 0.05. FA/LPC ratios in the subsarcolemmal mitochondria and cytosol fractions were intermediate, 1.6 and 2.2, representing lysophospholipase fractional contributions of 0.18 and 0.27, respectively. However, in the microsomal fraction, lysophosphatidylcholine could not be detected. In this situation (FA/LPC = ∞), the fractional contribution of lysophospholipase to fatty acid release is 0.50. In the various fractions, monoglyceride and diglyceride, products of phospholipase C and/or transacylation reactions, were present in small amounts varying from 2.8 to 4.9% of total products. After correction of the V_{max} values of Table 4 for the fractional contribution of lysophospholipase, the following rates were obtained for phospholipase A (nmol mg^{-1}hr^{-1}): subsarcolemmal

TABLE 3. Effect of EDTA and Ca^{2+} on the activity of the intracellular phospholipases A of rat heart

Fraction	Phospholipase A (nmol hr^{-1}mg^{-1})			
	EDTA 10 mM	Ca^{2+} 1.5 mM	Ca^{2+} 3 mM	Ca^{2+} 6 mM
Subsarcolemmal mitochondria	0.64	0.88	1.44	2.00
Light mitochondria, pH 4.8	1.60	n.d.	n.d.	1.60
Microsomes	3.00	3.84	4.16	4.44
Cytosol	1.60	1.64	2.04	2.16

The phospholipase A assays with Ca^{2+} or EDTA were done as described in Materials and Methods. In the experiments with EDTA, the proteins were first incubated with EDTA at 4°C for 10 min. Unless otherwise indicated, incubations were done at pH 8.4; n.d., not determined. Abbreviations as in Table 1.

TABLE 4. Kinetic properties of rat heart phospholipases A and lysophospholipases

Fraction	K_m (apparent)		V_{max} (apparent)		n
	PLA	LPL	PLA	LPL	
Subsarcolemmal mitochondria	0.31 ± 0.14	0.24 ± 0.18	4.64 ± 1.88	18.8 ± 5.6	4
Microsome	0.33 ± 1.0	0.25 ± 0.06	17.9 ± 9.20	83.0 ± 14.0	3
Cytosol	0.073 ± 0.01	0.023 ± 0.004	3.04 ± 0.32	10.8 ± 2.1	3
Light mitochondria, pH 4.8	0.66 ± 0.12	n.d.	16.8 ± 10.8	n.d.	3

Sonicated [1-^{14}C]dioleoylphosphatidylcholine was the substrate for phospholipase A (PLA) studies. Incubations were carried out as described in Methods. Each determination of V_{max} and K_m was obtained with six different concentrations of substrate which varied from 0.08 mM to 0.4 mM. Unless otherwise indicated, the incubations were done at pH 8.4 in the presence of 6 mM Ca^{2+}. Each determination has been corrected for a control incubation without protein. For lysophospholipase studies (LPL), incubations with [1-^{14}C]palmitoyllysophosphatidylcholine were done as noted in Methods. Each determination of V_{max} and K_m was obtained with six different concentrations of substrate which varied from 0.04 mM up to 0.2 mM. For each substrate concentration the value obtained with a control incubated without protein has been subtracted. Three or four separate preparations were studied. Abbreviations as in Table 1. K_m app (mM) and V_{max} app (nmol $mg^{-1}hr^{-1}$) were determined by linear regression analysis of the double reciprocal plots. R values were 0.99 or greater in each instance; n, number of separate determinations; n.d., not determined.

mitochondria, 3.80; microsomes, 8.95; cytosol, 2.22; and light mitochondria (pH 4.8), 16.0.

We attempted to find incubation conditions that would be unfavorable for microsomal neutral lysophospholipase so that lysophosphatidylcholine would accumulate and the positional specificity of phospholipase A could be determined. Since detergents have been reported to inhibit other lysophospholipases (25), we carried out incubations of heart microsomes with [1-^{14}C]dioleoylphosphatidylcholine in the presence of 1 mg/ml sodium deoxycholate or Triton X-100. However, as shown in **Table 6**, significant quantities of lysophosphatidylcholine still could not be detected although the overall rate of fatty acid release was greatly reduced. Similarly, incubation for a shorter time (10 min), incubation at a higher pH (9.0), or in the presence of a large sink of unlabeled lysophosphatidylcholine did not result in accumulation of [1-^{14}C]lysophosphatidylcholine; FA/LPC ratios ranging from 26 to infinity were obtained indicating a fractional contribution of lysophospholipase to fatty acid release of 0.48 to 0.50.

Positional specificity of phospholipases A

Rat heart subcellular fractions were incubated with 1-palmitoyl-2-[1-^{14}C]oleoylphosphatidylcholine and the products were analyzed by thin-layer chromatography. The results are shown in **Table 7.** Subsarcolemmal mitochondria principally catalyzed the release of [1-^{14}C]oleic acid, which represented 68% of the products. However, with cytosol and light mitochondria, [1-^{14}C]lysophosphatidylcholine was released, representing 55 and 80% of the products, respectively. After correction for the action of lysophospholipase as described in equation 2 (Methods), the phospholipase A_1 values for cytosol and light mitochondria were 81 and 86%, respectively, while rate heart subsarcolemmal mitochondria had primarily phospholipase A_2 (67%). Interfibrillar mitochondria had only phospholipase A_2 activity (data not shown). As noted above, the positional specificity of microsomal phospholipase A could not be determined with certainty due to the extremely active lysophospholipase (c.f. Tables 5 and 6).

TABLE 5. Hydrolysis of [1-^{14}C]dioleoylphosphatidylcholine by rat heart: contribution of lysophospholipase to fatty acid release

Product	Subcellular Fraction			
	Subsarcolemmal Mitochondria	Microsomes	Cytosol	Light Mitochondria, pH 4.8
FA, %	57.8	97.2	67.0	49.8
LPC, %	37.1	0	30.1	46.4
MG + DG, %	4.9	2.8	2.9	3.7
FA_d/LPC_d	1.6	∞	2.2	1.1
L	0.18	0.50	0.27	0.05

Incubations were carried out as described in Methods at pH 8.4 unless otherwise indicated. The reactions were stopped by the addition of 4.0 ml of chloroform–methanol 2:1 and analyzed by thin-layer chromatography as described in Methods. FA, fatty acid; LPC, lysophosphatidylcholine; MG + DG, monoglyceride + diglyceride; L, fraction of fatty acid release due to action of lysophospholipase.

TABLE 6. Effect of short incubation time, increased pH, unlabeled lysophosphatidylcholine, and detergents on the hydrolysis of [1-^{14}C]dioleoylphosphatidylcholine by the rat heart microsomal fraction

	Incubation Condition					
Product	pH 8.4	10 min Incubation	pH 9.0	0.4 mM LPC	1 mg/ml DOC	1 mg/ml Triton X-100
FA	6.11	6.05	5.49	1.68	2.14	1.04
LPC	0.038	0	0.21	0	0	0.03
FA_d/LPC_d	161	∞	26	∞	∞	39
L	0.50	0.50	0.48	0.50	0.50	0.49

Incubations were carried out as described in Methods; unless otherwise indicated, incubations were for 30 min at 37°. Results given as nmol of product $mg^{-1}hr^{-1}$. Abbreviations: FA, fatty acid; LPC, lysophosphatidylcholine; DOC, sodium deoxycholate; L, fraction of fatty acid release due to action of lysophospholipase.

DISCUSSION

Our studies show that the cytosolic fraction is the major intracellular site of neutral phospholipase A in rat heart representing 46% of the total recovered phospholipase A activity. The cytosolic enzyme differs from the microsomal and mitochondrial phospholipases A in a number of ways. It has an apparent K_m for dioleoylphosphatidylcholine of 0.07 mM versus 0.33 and 0.31 mM with the microsomal and subsarcolemmal mitochondria phospholipase A, respectively. The pH dependence of cytosolic phospholipase A is broader than that of the subsarcolemmal mitochondria and microsomal fractions; and finally, the cytosolic enzyme is a phospholipase A_1 while the mitochondria contain principally phospholipase A_2. General solubilization of membrane-bound enzymes cannot account for the presence of a neutral phospholipase A in the cytosol (46% of recovered activity) since the total activity of the microsomal and mitochondrial marker enzymes in the cytosolic fraction represents only 24 and 3.1% of recovered activity, respectively. Thus, the enzyme is probably cytosolic. However, we cannot completely exclude the possibility that the cytosolic phospholipase A is loosely associated with one of the membrane fractions (such as the microsomal fraction) and is subsequently released by Polytron treatment.

Cytosolic phospholipases A_1 have recently been reported in rat brain (26) and liver (27) but have not been previously reported in heart. Our findings do not agree with the data of Gross and Sobel (12) who could not detect phospholipase A in the soluble fraction. However, these authors used sonicated [^{14}C]dipalmitoylphosphatidylcholine, an extraordinarily poor substrate for measuring phospholipase A in many cases. In our prior studies of purified liver lysosomal phospholipase A_1 (22), we found that sonicated [^{14}C]dipalmitoylphosphatidylcholine was not hydrolyzed by the purified enzymes (unpublished observation); at low Triton X-100 levels, the activity of the two major lysosomal phospholipase A_1 isoenzymes was only 3 to 9% of the maximal rates obtained with optimal levels of Triton X-100. When we incubated rat heart homogenate with sonicated [^{14}C]dipalmitoylphosphatidylcholine without Triton X-100, the observed phospholipase A activity was only 15% of that obtained with sonicated [1-^{14}C]dioleoylphosphatidylcholine (DOPC) (data not shown).

The relative specific activity of neutral phospholipase A was highest in the microsomal fraction, representing an enrichment of 4-fold over that of the homogenate. This fraction contained 15.3% of total recovered activity. Our results with microsomes agree in general with those of Weglicki et al. (11) but are contrary to the findings of Gross and Sobel (12) who could not demonstrate phospholipase A in heart microsomes. However, as noted above, these authors used sonicated [^{14}C]dipalmitoylphosphatidylcholine, a very poor substrate for phospholipases A from heart or liver (22).

TABLE 7. Hydrolysis of 2-[1-^{14}C]oleoylphosphatidylcholine by rat heart subcellular fractions: positional specificity of phospholipases A

	Subcellular Fraction		
Product	Subsarcolemmal Mitochondria	Light Mitochondria (pH 4.8)	Cytosol
FA, %	68	18	38
LPC, %	25	80	55
MG + DG, %	7	2	7
FA_s/LPC_s	2.7	0.23	0.69
Corr. % PLA_1	33	86	81
Corr. % PLA_2	67	14	19

Incubations were carried out as described in Methods. Unless otherwise indicated, incubations were done at pH 8.4. Results are the average of two determinations and are expressed as % of recovered product. Corrected values for phospholipase A_1 and A_2 were obtained using equations 1 and 2 in the Methods. Abbreviations as in Table 5. PLA_1, phospholipase A_1; PLA_2, phospholipase A_2.

The light mitochondrial fraction exhibited acid phospholipase A activity consistent with previous findings in the heart (14). Our studies show that the myocardial acid phospholipase A is a phospholipase A_1 which does not require calcium. These findings are similar to results reported previously for purified liver and kidney lysosomal phospholipase A_1 (22, 28).

Heart cytosol also contained 46% of the recovered lysophospholipase activity. Cytosolic phospholipase A_1 could be completely separated from lysophospholipase by gel filtration by virtue of the fact that it behaves as a very high molecular weight complex, appearing in the void volume of a Sephadex G-150 column (data not shown). Interestingly, the apparent K_m of cytosolic lysophospholipase was 0.023 mM which was only one-eleventh of the apparent K_m of the membrane-associated enzymes, which ranged from 0.24 to 0.28 mM (Table 3). The pH dependence and apparent K_m of the cytosolic lysophospholipase in this report are similar to the results obtained by others with a purified preparation of cytosolic lysophospholipase (15). Neutral lysophospholipase is also present in the two populations of mitochondria. These findings are not in agreement with the report of Weglicki et al. (11), who assumed that there was no lysophospholipase activity in the mitochondrial fraction on the basis of a FA/LPC ratio that was near unity. Gross and Sobel (12) found very low levels of lysophospholipase activity in the mitochondrial fraction of rat heart, 1.8 nmol $mg^{-1}hr^{-1}$. We could not detect acid lysophospholipase activity in rat heart fractions including the light mitochondrial (lysosomal) fraction. In this regard, rat heart appears to be different from rat liver where a very active acid lysophospholipase of lysosomal origin has been partially purified (22).

Lysophosphatidylcholine has been implicated in electrophysiological disorders which occur during myocardial ischemia (3–6). Therefore, it is of particular interest to note that lysophospholipase appears to be much more active than phospholipase A in heart; the ratio of apparent V_{max} of lysophospholipase/phospholipase A ranges from 3.6 to 4.5 in the respective fractions (a minimum estimate since the phospholipase A values are not corrected for the contribution of lysophospholipase to fatty acid production). Since the apparent K_m in the respective fractions for phosphatidylcholine and lysophosphatidylcholine is grossly similar, the action of lysophospholipase during myocardial ischemia would tend to minimize the accumulation of lysoglycerophospholipids.

Future studies on the regulation of phospholipase A activity in myocardial ischemia may be of considerable physiological interest inasmuch as lysophospholipids appear to be involved in the pathogenesis of electrophysiological disorders during ischemia. Our studies show that the cytosolic fraction contains the bulk of the myocardial phospholipase A activity while considerable activity is also present in heart microsomes and subsarcolemmal mitochondria. Phospholipase A appears to be the rate-limiting step in the degradation of phospholipid in heart subcellular fractions and may be a factor of primary importance during myocardial ischemia.

This work was supported in part by NIH grant GM-24979, the Specialized Center of Research on Ischemic Heart Disease grant HL-17682, the Merck Institute for Therapeutic Research, Rahway, NJ, and the Research Service of the San Diego Veterans Administration Medical Center. G. N. wishes to acknowledge the support of The Philippe Foundation, New York, NY and La Fondation pour la Recherche Médicale.

Manuscript received 11 April 1984.

REFERENCES

1. Singer, J., and G. N. Nicholson. 1972. The fluid mosaic model of the structure of cell membranes. *Science.* **175:** 720–722.
2. Oliver, M. F., and P. A. Yates. 1971. Induction of ventricular arrhythmias by elevation of arterial free fatty acids in experimental myocardial infarction. *Cardiology.* **56:** 359–364.
3. Sobel, B. E., P. B. Corr, A. K. Robison, R. A. Goldstein, F. X. Witkowski, and M. S. Klein. 1978. Accumulation of lysophosphoglycerides with arrhythmogenic properties in ischemic myocardium. *J. Clin. Invest.* **62:** 546–553.
4. Snyder, D. W., W. A. Crafford, Jr., J. L. Glashow, O. Rankin, and B. E. Sobel. 1981. Lysophosphoglycerides in ischemia myocardium effluents and potentiation of their arrhythrogenic effects. *Am. J. Physiol.* **241:** H700–707.
5. Man, R. Y. K., and P. C. Choy. 1982. Lysophosphatidylcholine causes cardiac arrhythmia. *J. Mol. Cell. Cardiol.* **14:** 173–175.
6. Man, R. Y. K., T. Wong, and P. C. Choy. 1983. Effects of lysophosphoglycerides on cardiac arrhythmias. *Life Sci.* **32:** 1325–1330.
7. Sedlis, S. P., P. B. Corr, B. E. Sobel, and G. G. Ahumada. 1983. Lysophosphatidylcholine potentiates Ca^{2+} accumulation in rat cardiac myocytes. *Am. J. Physiol.* **244:** H32–38.
8. Vasdev, S. C., G. P. Biro, R. Narbaitz, and K. J. Kako. 1980. Membrane changes induced by early myocardial ischemia in the dog. *Can. J. Biochem.* **58:** 1112–1119.
9. Shaikh, N. A., and E. Downar. 1981. Time course of changes in porcine myocardial phospholipid levels during ischemia. *Circ. Res.* **49:** 316–325.
10. Chien, K. R., J. P. Reeves, M. Buja, F. Bonte, R. W. Parkey and J. T. Willerson. 1981. Phospholipid alterations in canine ischemic myocardium: temporal and topographic correlation with Tc-99m-PPi accumulation and an in vitro sarcolemmal Ca^{2+} permeability defect. *Circ. Res.* **48:** 711–719.
11. Weglicki, W. B., M. Waite, P. Sisson, and S. B. Shoet. 1971. Myocardial phospholipase A of microsomal and mitochondrial fractions. *Biochim. Biophys. Acta.* **231:** 512–519.

12. Gross, R. W., and B. E. Sobel. 1982. Lysophosphatidylcholine metabolism in rabbit heart: characterization of metabolic pathways and partial purification of myocardial lysophospholipase-transacylase. *J. Biol. Chem.* **257:** 6702–6708.
13. Palmer, J. W., P. C. Schmid, D. R. Pfeiffer, and H. H. O. Schmid. 1981. Lipids and lipolytic enzyme activities of rat heart mitochondria. *Arch. Biochem. Biophys.* **211:** 674–682.
14. Franson, R. F., M. Waite, and W. B. Weglicki. 1972. Phospholipase A activity of lysosomes of rat myocardial tissue. *Biochemistry.* **11:** 472–476.
15. Gross, R. W., and B. E. Sobel. 1983. Rabbit myocardial cytosolic lysophospholipase. Purification, characterization and competitive inhibition by L-palmitoylcarnitine. *J. Biol. Chem.* **258:** 5221–5226.
16. Palmer, J. W., B. Tandler, and C. L. Hoppel. 1977. Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat heart. *J. Biol. Chem.* **252:** 8731–8739.
17. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* **72:** 248–254.
18. Rouser, G. S., S. Fleischer, and A. Yamamoto. 1969. Two-dimensional thin-layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* **5:** 494–496.
19. Pennington, R. J. 1961. Biochemistry of dystrophic muscle: mitochondrial succinate-tetrazolium reductase and adenosine triphosphatase. *Biochem. J.* **80:** 649–654.
20. Koldovsky, O., and M. Palmieri. 1971. Cortisone-evoked decrease of acid β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase and arylsulfatase in the ileum of rats. *Biochem. J.* **125:** 697–701.
21. Sottocasa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. An electron transport system associated with the outer membrane of liver mitochondria. *J. Cell Biol.* **32:** 415–438.
22. Hostetler, K. Y., P. J. Yazaki, and H. van den Bosch. 1982. Purification of lysosomal phospholipase A: evidence for multiple isoenzymes. *J. Biol. Chem.* **257:** 13367–13373.
23. van den Bosch, H., and A. J. Aarsman. 1979. A review on methods of phospholipase determination. *Agents Actions.* **9:** 382–389.
24. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple procedure for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
25. van den Bosch, H. 1982. Phospholipases. *In* Phospholipids, New Comprehensive Biochemistry, Vol. 4. J. N. Hawthorne and G. B. Ansell, editors. Elsevier Biomedical Press, Amsterdam. 313–358.
26. Doherty, F. J., and C. E. Rowe. 1980. The intracellular location of a Ca^{2+}-stimulated phospholipase A_1 in rat brain. *Brain Res.*, **197:** 113–122.
27. Dawson, R. M. C., R. F. Irvine, N. L. Hemington, and H. Kirasawa. 1983. The alkaline phospholipase A_1 of rat liver cytosol. *Biochem. J.* **209:** 865–873.
28. Wolgast, D. M., and K. Y. Hostetler. 1982. Aminoglycoside nephrotoxicity and kidney lysosomal phospholipase A. *Clin. Res.* **31:** 77A.