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PHOSPHATIDIC ACID PHOSPHATASE AND PHOSPHOLIPASE A ACTIVITIES IN PLASMA MEMBRANES FROM FUSING MUSCLE CELLS

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SUMMARY

Plasma membranes from fusing embryonic muscle cells were assayed for phospholipase A activity to determine if this enzyme plays a role in cell fusion. The membranes were assayed under a variety of conditions with phosphatidylcholine as the substrate and no phospholipase A activity was found. The plasma membranes did contain a phosphatidic acid phosphatase which was optimally active in the presence of Triton X-100 and glycerol. The enzyme activity was constant from pH 5.2 to 7.0, and did not require divalent cations. Over 97 % of the phosphatidic acid phosphatase activity was in the particulate fraction. The subcellular distribution of the phosphatidic acid phosphatase was the same as the distributions of the plasma membrane markers, $(Na^+ + K^+)$-ATPase and the acetylcholine receptor, which indicates that this phosphatase is located exclusively in the plasma membranes. There was no detectable difference in the phosphatidic acid phosphatase activities of plasma membranes from fusing and non-fusing cells.

INTRODUCTION

Differentiating embryonic muscle in cell culture is a system that is well suited for studying the role of the cell surface membrane during the growth and maturation of embryonic cells. The muscle cell plasma membrane is the center of the development of several muscle-specific activities [1], including fusion of the mononucleated muscle cells to form multinucleated myotubes [2]. Because purified plasma membranes from cultured muscle cells can be isolated by a relatively simple procedure [3], the muscle cell surface membrane proves to be a useful subject for the characterization of embryonic surface membranes in general and for the study of membrane fusion in particular.

The possibility that phospholipase A is involved in membrane fusion was suggested by Lucy and coworkers after they found that lysophosphatidylcholine, a

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product of phospholipase A activity on phosphatidylcholine, could stimulate fusion of hen erythrocytes and fibroblasts [4]. It was suggested that an endogenous phospholipase A might be active during naturally-occurring cell fusion and that the lysophospholipid product would serve to disrupt the membrane bilayer structure and allow membrane fusion to occur. Plasma membranes isolated from fusing and nonfusing cultured muscle cells show no significant differences in the composition of membrane lipids, including lysolipids [5], although this does not necessarily rule out the possibility of the involvement of phospholipase A in muscle cell fusion [4]. We therefore began to assay the plasma membranes from fusing muscle cells for phospholipase A activity under a variety of conditions. We found no phospholipase A activity in the plasma membranes from fusing muscle cells. While this work was in progress, however, we discovered that the plasma membranes contain a phosphatidic acid phosphatase, an enzyme that is important in the biosynthesis of phosphatidylcholine, phosphatidylethanolamine, and triacylglycerols [6]. The phosphatidic acid phosphatase in the muscle cells is unusual in that it is located only on the surface membranes of these cells. This paper describes some properties of the phosphatidic acid phosphatase in the plasma membrane fractions from fusing and nonfusing muscle cells.

METHODS

Materials. Sodium [^{3}H]acetate and $^{32}P_i$ were purchased from New England Nuclear, phospholipase D, snake venom phospholipase A_2, and neutral lipid standards from Sigma, phospholipid standards from Serdary Research Laboratories, Triton X-100 from Packard, Cutscum from Fisher, and 3a70 from Research Products International.

Cell cultures. Procedures and media for the culture of embryonic chick muscle cells and fibroblasts have been described previously [3, 5]. Chang liver cells were supplied by Alfred W. Alberts of this laboratory.

Membrane isolation. Plasma membrane fractions were isolated as described [3, 5] with the following modification suggested by Dr. James P. Quigley: after the initial centrifugation at $1700 \times g_{av}$, the pellet was suspended and centrifuged at $650 \times g_{av}$. The supernates were combined and centrifuged at $33\,000 \times g_{av}$ as described. The purity of the plasma membrane fraction was unaffected by this modification but the yield was substantially increased.

Radioactive lipid substrates

[^{3}H]Phosphatidylcholine. Chang liver cells (4–6 75 mm T-flasks) were cultured in serum-containing medium and transferred to serum-free medium plus insulin as described [7]. After 48 h in the absence of serum, 20–30 mCi of sodium [^{3}H]acetate, 100 Ci/mol, were added to the cells, which were harvested [7] 30–35 h later. Radioactive lipids were extracted from the cells by the method of Bligh and Dyer [8] and were separated on columns of silicic acid/silicate according to Rouser et al. [9]. Phosphatidylcholine obtained in this manner was 96–98 % pure. Contamination by phosphatidic acid could be kept to less than 0.2 % by eluting the column first with solutions of chloroform containing less than 20 % methanol before eluting with the chloroform/methanol/water solutions; otherwise phosphatidic acid accounted for 1–

1.5 % of the total radioactivity. The distribution of label in the phosphatidylcholine was determined by treatment with snake venom phospholipase A_2 and thin-layer chromatography of the products; 51 % of the label was in the 2-position and 49 % in in the 1-position.

[^{32}P]*Phosphatidylcholine.* Chang liver cells were prepared in the same manner as above except that the P_i content of the serum-free medium was reduced to 20 % of its usual level. 20 mCi of carrier-free $^{32}P_i$ were added to the medium to label the lipids.

[^{32}P]*Phosphatidic acid.* [^{32}P]phosphatidylcholine was treated with phospholipase D and the phosphatidic acid was isolated as the calcium salt according to Kates and Sastry [10]. The phosphatidic acid prepared in this manner was 92–99 % pure.

Enzymatic assays

Phospholipase A. Plasma membranes were assayed at 37 °C under various conditions in a final volume of 30–50 µl. [^{3}H]phosphatidylcholine was used as the substrate and was added as a solution in ethanol or as a suspension in a detergent. The reaction was terminated and the products were extracted into heptane as described [11].

Phosphatidic acid phosphatase. Usual assay conditions included the following: 0.3 mM [^{32}P]phosphatidic acid, about 2000 cpm/nmol; Triton X-100, 1 mg/ml; 0.05 M triethanolamine · HCl, pH 7.0; 38 % glycerol; bovine serum albumin, 2 mg/ml; in a final volume of 30 µl. The reaction was started by the addition of 1 µl of phosphatidic acid suspended in Triton X-100, 30 mg/ml, and was carried out for 20 min at 37 °C. The reaction was terminated by the addition of 0.2 ml chloroform/methanol (1 : 2) and the water-soluble reaction product was extracted by the method of Bligh and Dyer [8] using appropriately reduced volumes. The water-soluble phase was removed and added to 0.5 ml water plus 10 ml 3a70 scintillation cocktail to determine radioactivity.

Analytical procedures. The purity of the lipid substrates was monitored by two-dimensional, thin-layer chromatography [12]. The heptane-extractable reaction product was analyzed by thin-layer chromatography on silica gel G in petroleum ether/diethyl ether/acetic acid (80 : 75 : 1.5). The heptane-extractable reaction product was also evaporated to dryness with N_2 and hydrolyzed by treating with 10 % NaOH in methanol at 75 °C for 3 h. The mixture was then acidified and extracted three times with ether. The ether extracts were combined, evaporated, and chromatographed as described above.

The water-soluble reaction products were chromatographed on Whatman No. 1 paper in the descending manner in solvent system I: methanol/formic acid/water (80 : 13 : 7) [13] or II: phenol saturated with water/methanol/formic acid/water (40 : 40 : 13 : 3). The paper chromatograms were scanned in a Vanguard model 880 Automatic Chromatogram Scanner. Excess glycerol was removed from the water-soluble fraction before chromatography by running the fraction over a small amount of DE52 ion-exchange resin, acetate form, in a cotton-plugged Pasteur pipette. The DE52 was then washed with water to remove the glycerol and the reaction product was eluted with 0.5 M ammonium acetate. The eluate was evaporated to dryness and chromatographed as described above. The $(Na^+ + K^+)$ATPase assay, [^{125}I]iodo-α-bungarotoxin binding and other miscellaneous procedures were described [3, 5].

RESULTS

To determine if there is a phospholipase A involved in muscle cell fusion, purified plasma membranes from fusing muscle cells were tested for phospholipase A activity. The substrate was phosphatidylcholine isolated from cultured Chang liver cells grown in the presence of sodium [^{3}H]acetate to label the fatty acyl chains. The fatty acids liberated by the enzyme could then be selectively extracted from the assay mixture into heptane. The plasma membranes from fusing cells were assayed for phospholipase A activity under a wide variety of conditions, including various divalent cations, chelating agents, salt concentrations, buffers, pH ranges, and a large number of nonionic, cationic, and anionic detergents. No phospholipase A activity was found using any of these conditions.

In the course of these experiments it was noted that some preparations of phosphatidylcholine were slightly contaminated with phosphatidic acid and when these preparations were used as the substrate, a radioactive, heptane-soluble product was detected. The labeled product co-chromatographed with authentic 1,2-diacylglycerol (Fig. 1); when the product was subjected to alkaline conditions sufficient to hydrolyze carboxylic esters, all of the radioactivity chromatographed with free fatty acid (Fig. 1), which is a further indication that the original product was diacylglycerol. To determine if this formation of diacylglycerol was due to the activity of a phosphatidic acid phosphatase associated with the plasma membranes, [^{32}P]phosphatidylcholine and [^{32}P]phosphatidic acid were prepared. The enzyme was assayed by measuring the release of ^{32}P-labelled product into the water-soluble phase of a conventional chloroform/methanol/water partitioning, where the ^{32}P-labelled substrates remained in the chloroform-soluble phase. The water-soluble product in solvent system I was a single peak which co-chromatographed with carrier P_i (Fig. 2A). The product was shown to be distinct from phosphorylcholine by chromatography in solvent system II (Fig. 2B–D). The P_i carrier smeared in the latter solvent

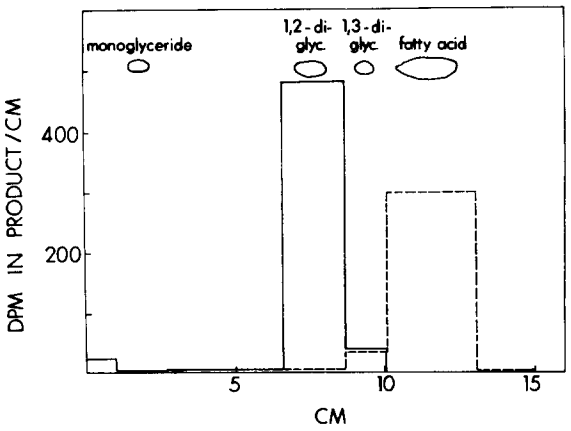

Fig. 1. Heptane-extractable product of phosphatase. Phosphatidic acid phosphatase was assayed as described in Methods except that the substrate was the mixture of phosphatidylcholine and phosphatidic acid. ——, heptane-extractable reaction product; – – – –, the same product after alkaline hydrolysis.

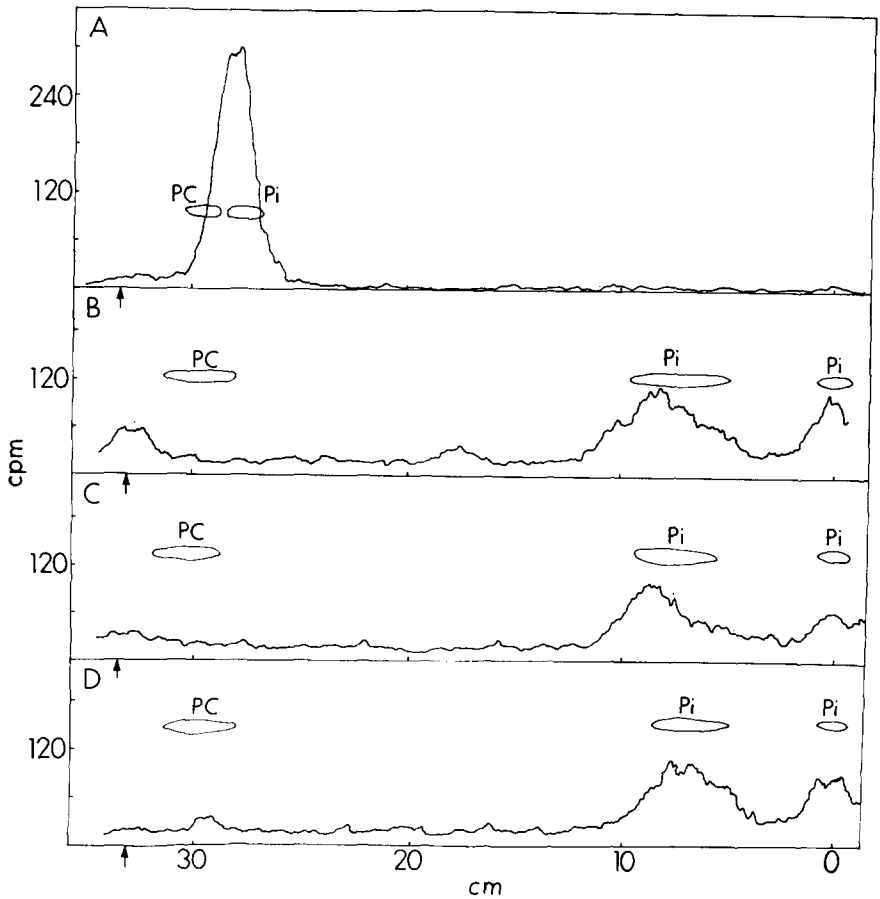

Fig. 2. Water-soluble product of phosphatase. Phosphatidic acid phosphatase was assayed as described in Methods. The substrate was phosphatidic acid in B and phosphatidylcholine plus phosphatidic acid in A and C. In D, 2000 cpm $^{32}P_i$ were added to the reaction mixture instead of a substrate. About 0.1 mmol each of P_i and phosphorylcholine (PC) were added to each sample before chromatography. The arrow designates the solvent front.

system and was in part left at the origin, but since the labelled products of reactions using either pure [^{32}P]phosphatidic acid (B) or a mixture of [^{32}P]phosphatidic acid and [^{32}P]phosphatidylcholine (C) showed the same chromatographic profile as $^{32}P_i$ (D), it can be concluded that the water-soluble reaction product is P_i and that the enzyme is a phosphatidic acid phosphatase.

Some properties of the phosphatidic acid phosphatase are shown in Figs. 3 and 4. The phosphatase activity is proportional to enzyme concentration up to 0.3 μg of plasma membrane protein (Fig. 3A), and to the time of incubation for at least 20 min (Fig. 3B). The activity remains constant from pH 5.2 to 7.0 (Fig. 3C). The dependence of the phosphatase activity on phosphatidic acid concentration is shown in Fig. 3D. According to these data the apparent K_m for the muscle cell enzyme is $6 \cdot 10^{-5}$ M, but this value has been found to vary with different suspensions of phos-

phatidic acid. High concentrations of glycerol stimulated the phosphatase activity; the optimum glycerol concentration was 38 % (Fig. 4A). Triton X-100 was required for enzymatic activity in the presence of phosphatidylcholine (Fig. 4B). A number of

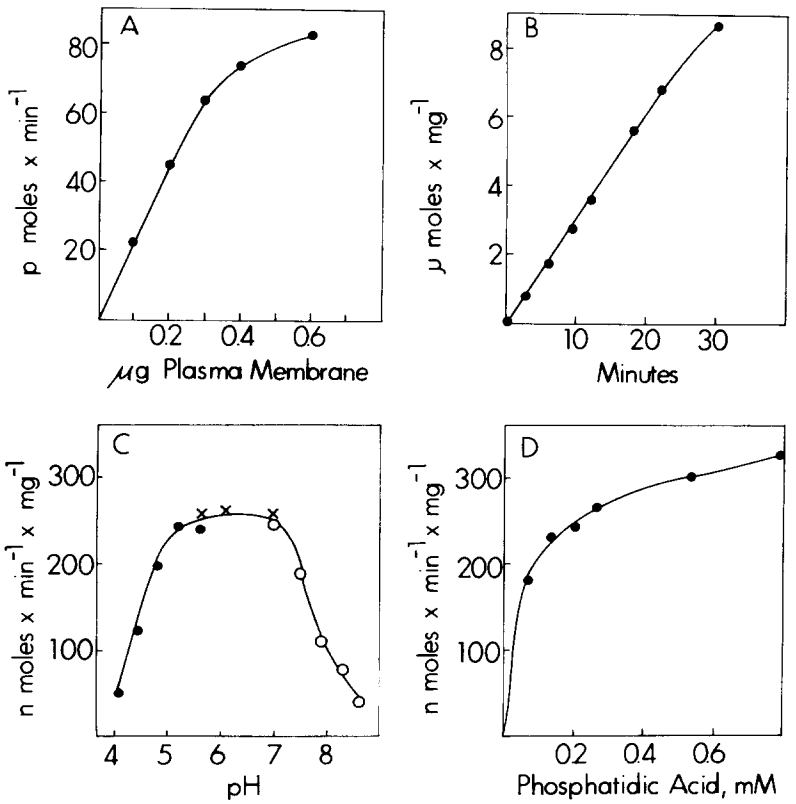

Fig. 3. Properties of phosphatidic acid phosphatase. The phosphatase was assayed as described in Methods.

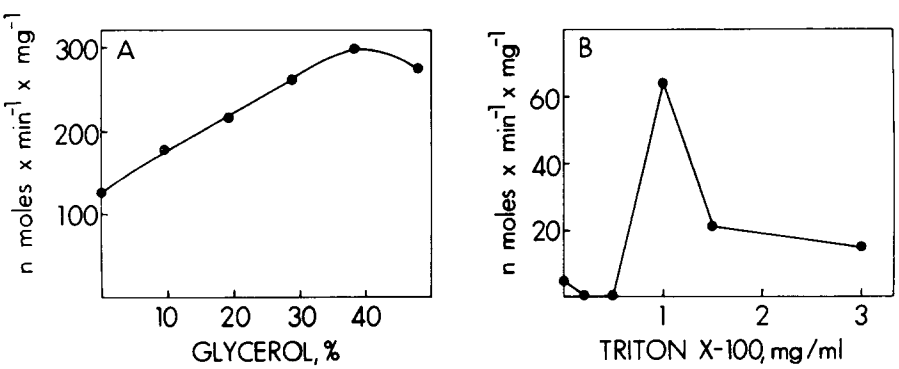

Fig. 4. Glycerol and Triton X-100 stimulation of phosphatidic acid phosphatase. Conditions were the same as for Fig. 3 except that phosphatidylcholine plus phosphatidic acid was the substrate in B.

nonionic, cationic and anionic detergents were tested for their ability to support enzymatic activity, but the only one that could be substituted for Triton X-100 was Cutscum. The dependence of activity on the Triton X-100 concentration was not tested with pure phosphatidic acid as substrate because high concentrations of the detergent were necessary to suspend the small volumes of phosphatidic acid used in these assays. Neither Ca^{2+} nor Mg^{2+} stimulated the phosphatase, and EDTA did not inhibit it. To determine if the phosphatidic acid phosphatase is simply a non-specific phosphatase the plasma membranes were assayed for activity using *p*-nitrophenylphosphate as substrate. Under optimal conditions for the phosphatidic acid phosphatase the *p*-nitrophenylphosphatase activity was less than 10 % of the phosphatidic acid phosphatase activity, which indicates that the latter is not a non-specific phosphatase.

Previous reports have indicated that phosphatidic acid phosphatase is distributed throughout a variety of subcellular organelles as well as the particle-free supernate [14–20]. When the muscle cell phosphatidic acid phosphatase was assayed in the supernatant and particulate fractions of a crude homogenate, over 97 % of the total activity was in the particulate fraction. The subcellular distribution of the phosphatidic acid phosphatase was determined by measuring its activity in the various subcellular fractions produced during the plasma membrane isolation. These subcellular fractions are the particulate fraction of the crude homogenate, a low-speed pellet containing the nuclei, mitochondria, and some of the plasma membrane, and three sucrose-gradient fractions where I+II designates the fraction in which the plasma membranes were most enriched [3]. The phosphatase activity was compared to the activity of the plasma membrane marker, either $(Na^+ + K^+)$-ATPase or

TABLE I

SUBCELLULAR DISTRIBUTION OF PHOSPHATIDIC ACID PHOSPHATASE

Enzyme activities are expressed as nmol · min^{-1} · mg^{-1}, [^{125}I]iodo-α-bungaratoxin binding as cpm · mg plasma membrane $protein^{-1}$. The numbers in parentheses refer to the relative enrichment with respect to the crude particulate fraction. In experiment A phosphatidic acid was used as substrate and in experiment B, phosphatidylcholine plus phosphatidic acid.

A. Fraction	$(Na^+ + K^+)$-ATPase	Phosphatidic acid phosphatase
Crude particulate	13 (1.0)	107 (1.0)
Low-speed pellet	12 (0.9)	112 (1.0)
Sucrose gradient:		
I+II	70 (5.4)	455 (4.3)
III	37 (2.8)	329 (3.1)
IV+V	9 (0.7)	105 (1.0)
B. Fraction	**Bound α-bungarotoxin**	**Phosphatidic acid phosphatase**
Crude particulate	12 (1.0)	7 (1.0)
Low-speed pellet	13 (1.1)	7 (1.0)
Sucrose gradient:		
I+II	75 (6.2)	42 (5.9)
III	32 (2.7)	16 (2.2)
IV+V	8 (0.7)	4 (0.6)

TABLE II

PHOSPHATIDIC ACID PHOSPHATASE ACTIVITY IN PLASMA MEMBRANES FROM FUSING AND NON-FUSED CELLS

"Low Ca^{2+}" cultures were those grown in low Ca^{2+} medium for 3 days to prevent fusion [5]. In some of these cultures the Ca^{2+} concentration was increased and fusion was allowed to proceed for 2 h. "High Ca^{2+}" cultures were those grown in high Ca^{2+} medium for 2 days, when no significant fusion had occurred, or for 3 days, when extensive fusion was taking place. Enzyme activity is expressed as nmol · min^{-1} · mg^{-1}. A, B, and C were separate experiments.

	Culture conditions		Phosphatidic acid phosphatase
A.	Low Ca^{2+}	non-fused	280
	Low Ca^{2+}	fusing	220
B.	Low Ca^{2+}	fusing	103
	High Ca^{2+}	non-fusing	112
	High Ca^{2+}	fusing	122
C.	Low Ca^{2+}	fusing	58
	Fibroblasts	—	38

[^{125}I]iodo-α-bungarotoxin bound to the acetylcholine receptors. The plasma membrane markers and the phosphatase showed the same subcellular distribution (Table I), indicating that the phosphatidic acid phosphatase measured under these conditions is located exclusively on the plasma membranes in the cultured muscle cells.

The reactions catalyzed by phosphatidic acid phosphatase and phospholipase C are analogous in that the lipid-soluble reaction product of both enzymes is 1,2-diacylglycerol. Because it has been reported that treatment of muscle cell cultures with phospholipase C inhibits cell fusion [21], it was of interest to determine if there is a difference in phosphatidic acid phosphatase activity in the plasma membranes from fusing and non-fused cells. Table II lists several experiments comparing activities in the plasma membranes from cells isolated under different culture conditions, and in any single experiment there is no significant difference in activity between plasma membranes from fusing and non-fused cells. In fact, the phosphatidic acid phosphatase is found in the plasma membranes from cultured chick embryo fibroblasts which indicates that the primary role of this enzyme is not concerned with muscle cell fusion.

DISCUSSION

The absence of detectable phospholipase A activity in the plasma membranes of fusing muscle cells does not necessarily rule out the possibility of a phospholipase A-mediated mechanism for muscle cell fusion. Perhaps the proper conditions that support enzymatic activity were not among those tested, or there may be a phospholipase A-type enzyme that has a specific substrate requirement and will not work on phosphatidylcholine. It would also be desirable to assay for phospholipase A activity using endogenous labeled phospholipids. It is, however, technically difficult to label the fatty acid moieties of these lipids to a high specific activity since the cells require serum and embryo extract for growth, both of which contain high levels of

lipid. We have detected no degradation of any ^{32}P-labeled plasma membrane phospholipids during fusion, although this does not rule out phospholipase activity since the lysophospholipid product could be quickly re-acylated. On the other hand, the hypothesis that membrane fusion occurs via a phospholipase A-induced rearrangement of membrane structure has had little supporting evidence. This hypothesis was originally suggested by Lucy and coworkers [4] when they found that lysophosphatidylcholine, a product of phospholipase A activity on phosphatidylcholine, can induce fusion of fibroblasts and hen erythrocytes, although a large variety of other substances have since been found also to induce fusion [22–26]. Indeed, Lucy and coworkers recently stated that the involvement of lipases in membrane fusion "is debatable" and are now emphasizing a different model for membrane fusion [6]. It is possible that lysophosphatidylcholine is only one of many compounds that can reproduce the structural changes that take place in naturally-fusing membranes.

The properties of the phosphatidic acid phosphatase from muscle cell plasma membranes are not unlike the properties of this enzyme in other subcellular fractions from various tissues [14–20]. The phosphatidic acid phosphatases generally do not require divalent cations. Mg^{2+} inhibits the microsomal enzymes from chicken liver [14] and cat intestinal mucosa [19], but this may be due to the formation of an insoluble magnesium-phosphatidic acid precipitate [14]. The reported Michaelis constants vary considerably, but this probably reflects the insoluble nature of the substrate. The phosphatidic acid phosphatases are active at either a broad pH range from pH 5 to 7 or 8 [14] as reported here for the muscle cell enzyme, or they show optimal activity between pH 6 and 7 [15, 17, 19]. Ionic detergents are inhibitory [15, 17], and only nonionic detergents have been found to support activity [17].

Phosphatidic acid phosphatase has been found in a variety of tissues [14, 15]. In previous reports the enzyme is fairly widely distributed among subcellular fractions, including mitochondria, lysosomes, "microsomes", and particle-free supernatant fractions [14–19]. Coleman [20] was the first to compare the subcellular distribution of the phosphatidic acid phosphatase from rat liver with a plasma membrane marker. While the phosphatase did show a wide subcellular distribution in Coleman's studies, a substantial amount (up to 37 %) of the enzyme was found in the plasma membrane fraction. On the other hand, the phosphatidic acid phosphatase from cultured muscle cells as measured under the conditions reported in this paper is exclusively located in the cell surface membrane since the distributions of the phosphatase activity and the plasma membrane marker are the same. This, of course, does not exclude the existence in other subcellular fractions of phosphatidic acid phosphatases that may be active under different conditions or at a later stage of embryonic development. The enzyme in the muscle cell plasma membranes is assayed in the presence of Triton X-100 while the previously reported phosphatases have been assayed in the absence of detergent, although the rat liver mitochondrial enzyme is also active in the presence of Triton X-100 [17]. The present report demonstrates that the wide distribution of phosphatidic acid phosphatase activity in subcellular fractions may be due to the presence of a number of enzymes with different properties and, perhaps, different functions, each specific to a certain subcellular organelle.

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