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PULMONARY PHOSPHATIDIC ACID PHOSPHATASE: EVIDENCE FOR A MEMBRANE-BOUND PHOSPHATIDIC ACID-DEPENDENT ACTIVITY ASSOCIATED WITH THE HIGH SPEED SUPERNATANT OF RAT LUNG

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SUMMARY: The 104,000 x *g* supernatant fraction from rat lung contains a greater proportion of the phosphatidic acid phosphatase activity toward membrane-bound phosphatidic acid than the microsomal fraction. The microsomal fraction is more effective in hydrolyzing aqueously dispersed phosphatidic acid. The effects of various ions and chelators, particularly Mg^{2+} and EDTA, suggest that these two activities are distinct. These results indicate that the supernatant fraction of rat lung contains a phosphatidic acid phosphatase activity which may play an important role in pulmonary glycerolipid synthesis.

A number of studies in different tissues have suggested that phosphatidic acid phosphatase (EC 3.1.3.4) plays a role in the control of neutral glyceride and phosphoglyceride synthesis (1,2). The importance of phosphatidic acid phosphatase in lung tissue has been suggested by recent experiments which have demonstrated that the level of this enzyme is increased in rabbit fetal lung both during late gestation (3) and after glucocorticoid administration to induce pulmonary maturation (4,5). It has been suggested that these increases in the activity of phosphatidic acid phosphatase may be responsible for the increased levels of 1,2-dipalmitoyl-*sn*-glycerol-3-phosphorylcholine (3,5), the principal component of the pulmonary surfactant. It has been observed in a number of tissues, including lung (6), that the major enzyme activity is associated with the particulate fractions, predominantly in the microsomal fraction. In these investigations (3-6), aqueously dispersed PA[1] was used as the substrate. However, when phosphatidic acid phosphatase activities

[1] The abbreviations used are: EGTA, ethylene glycol-bis (β-aminoethyl ether) N,N[1]-tetraacetic acid; G3P, glycerol-3-phosphate; TLC, thin layer chromatography; PA, phosphatidic acid; tricine, N-tris-(hydroxymethyl)methyl-glycine.

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were measured in liver (7-9), intestine (10) and adipose tissue (11), using PA formed biosynthetically on microsomal membranes, the major activity was localized in the cytosol, little activity being found in the particulate fractions. It has been suggested that the cytosol activity is the only activity involved in glycerolipid synthesis (7-10). This communication reports that rat lung supernatant contains a phosphatidic acid phosphatase activity directed towards membrane-bound PA and this activity appears to be more significant with membrane-bound PA than the microsomal activity.

EXPERIMENTAL PROCEDURES: Most of the methods and the sources of the materials have been previously reported (4,12,13). Membrane-bound PA was prepared enzymatically from [^{14}C]G3P or [^{32}P]G3P with rat liver microsomes essentially as previously described (13), but on a larger scale and with slight modifications in the time (30 min), KF concentration (40 mM), G3P concentration (1.0 mM), and fatty acids used (0.05 mM palmitic acid and 0.05 mM oleic acid as the potassium salts). After 15 min, 2 μmoles of ATP were added and the reaction was continued for another 15 min. The microsomes were isolated by centrifugation at 104,000 x *g* for 1 h, redispersed, recentrifuged, resuspended in the original volume of the tissue and heat-inactivated for 5 min at 100°C. This preparation, which was normally used as the membrane-bound PA substrate, contained no residual phosphatidic acid phosphatase activity.

Membrane-bound phosphatidic acid phosphatase was routinely assayed in a standard system containing (final vol. 100 μl): 2.4-3.0 nmoles [^{32}P]PA, 0.4-0.9 mg cytosol protein and 50 mM tricine buffer (pH 7.4). The reaction was terminated by the addition of 200 μl of cold 0.4 M perchloric acid. After centrifugation, an aliquot was counted in Aquasol (4). Under the standard assay conditions, the release of ^{32}Pi was proportional to the amount of protein up to 0.9 mg and time to 30 min. This activity, which was stable to freezing and thawing, had a broad pH optimum from pH 7.4-8.0. Concomitant experiments with membrane-bound [^{14}C]PA demonstrated that the release of ^{32}Pi closely paralleled the formation of [^{14}C] neutral glycerides. ^{14}C-Labelled lipids were extracted, washed and separated by TLC on silica gel G plates impregnated with 0.35 N oxalate using petroleum ether:acetone:formic acid (76:24:0.25) as previously described (13).

Microsomal phosphatidic acid phosphatase was determined using aqueously dispersed PA as previously described (4), except that tris-maleate buffer (pH 7.4) was used. The release of Pi was proportional to the amount of microsomal protein up to 1.0 mg and with time to 120 min. The supernatant fraction demonstrated only a slight activity with aqueously dispersed PA.

RESULTS: Esterification of [^{14}C]G3P by rat lung microsomes in the presence of fatty acids, ATP, CoA and Mg^{2+}, results in an accumulation of radioactivity in PA (74%) (Fig. 1a). When these microsomes were reisolated and incubated to measure endogenous phosphatidic acid phosphatase activity, the percent of the total radioactivity migrating with the diacylglycerol fraction only increased from 12 to 16%. However, when lung supernatant was added, the

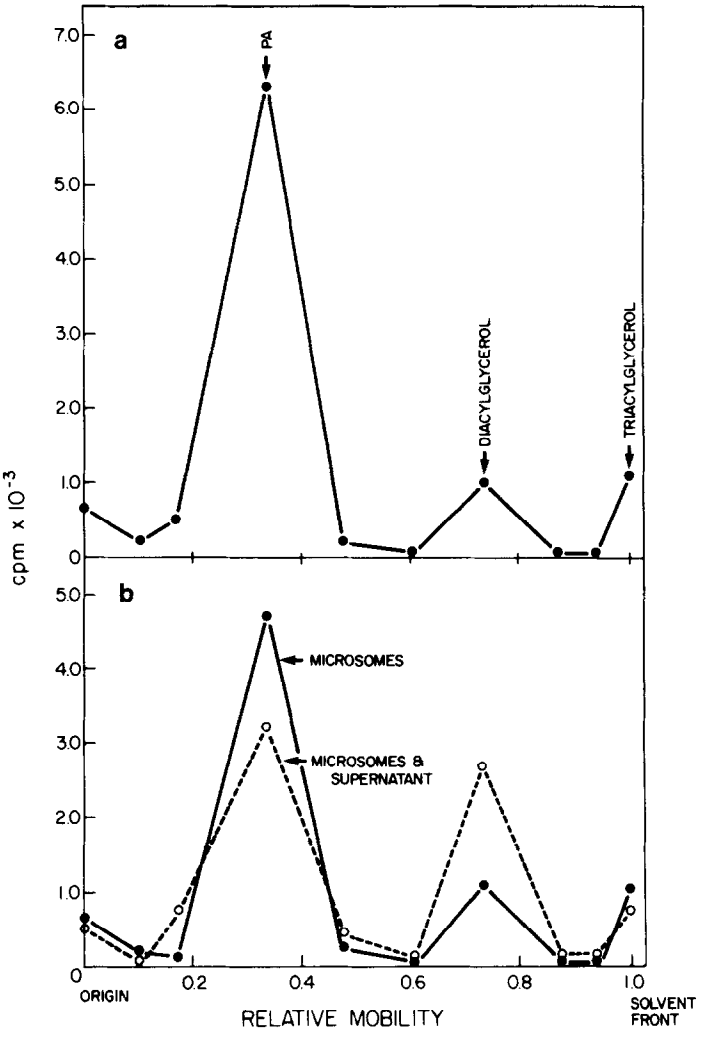

FIGURE 1: (a) TLC separation of the products of the esterification of [^{14}C]G3P by lung microsomes. The incubation conditions were the same as the G3P esterification system for preparation of membrane-bound PA described in the Experimental Procedures, except KF was eliminated and 1.5 ml of a lung microsomal suspension (3.66 mg protein) were added to the incubation medium (final volume, 2.5 ml). Microsomes were reisolated and resuspended to their original volume. The lipids were separated by TLC as described in the Experimental Procedures. (b) Phosphatidic acid phosphatase activity of rat lung microsomes and the effect of addition of supernatant. Aliquots (500 µl) of the resuspended lung microsomes described above in (a) (0.825 mg microsomal protein) were incubated alone (●——●) or in the presence of supernatant (○——○). The incubation medium contained in a 1.0 ml final volume: 2.67 mg lung supernatant protein and/or 0.825 mg microsomal protein, 50 mM tricine buffer (pH 7.4). After incubation for 40 min at 37°C, the radioactivity in the lipids was determined as above.

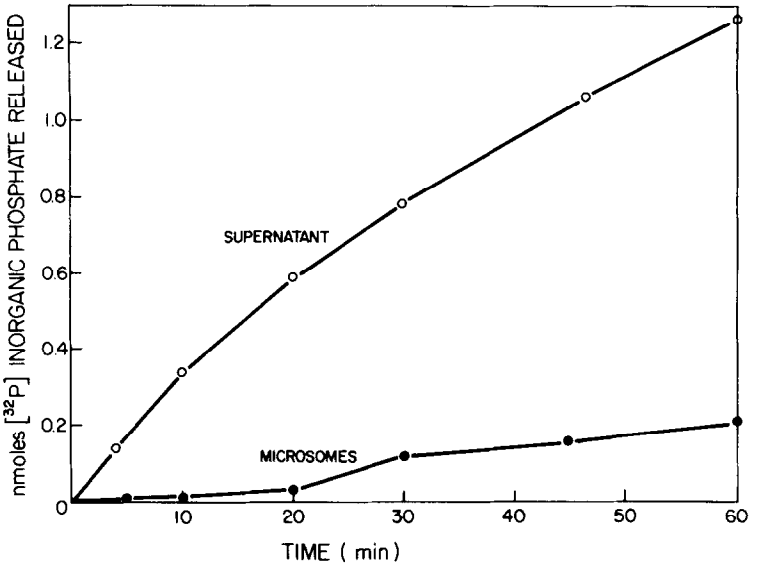

FIGURE 2: Membrane-bound phosphatidic acid substrate utilization by rat lung supernatant (O—O) and microsomal (●—●) fractions. Aliquots of the respective subcellular fractions corresponding to 60 µl of a 20% rat lung homogenate were incubated in the standard assay system using membrane-bound [^{32}P]PA described under Experimental Procedures.

radioactivity in diaclyglycerol increased to 40% of total. There was a corresponding decrease in the radioactivity in PA (Fig. 1b).

For comparative purposes, aliquots of the microsomal and cytosol fractions corresponding to 60 µl of a 20% (w/v) whole lung homogenate were incubated with membrane-bound [^{32}P]PA. Under these conditions the major phosphatidic acid phosphatase activity was associated with the cytosol (Fig. 2). By incubating the membrane-bound substrate with larger amounts of supernatant, up to 90% of the total ^{32}P-label could be released (data not shown).

Table 1 summarizes the effects of various ions and chelators on the supernatant (membrane-bound PA) and the microsomal (aqueously dispersed PA) activities. The properties of these two activities share some common features. Both activities exhibited an inhibition to a varying extent with all the cations and anions tested except Mg^{2+}, which showed a slight stimulation at low concentrations. Generally the supernatant activity was more sensi-

TABLE 1. The effect of various ions and chelators on the supernatant and microsomal phosphatidic acid phosphatase activities from rat lung

Addition (mM)	Relative Activities (% control)					
	Microsomal			Supernatant		
	2.5	5	10	2.5	5	10
Mn^{2+}	82.0	32.0	27.0	38.7	16.6	5.8
Ca^{2+}	78.0	58.5	58.5	67.2	43.4	11.6
Mg^{2+}	104.3	64.0	36.3	115.1	117.0	105.7
F^-	81.0	84.0	43.0	93.1	81.3	70.8
EDTA	98.0	93.7	110.6	0.0	0.0	0.0
EGTA	91.0	83.0	91.0	107.0	98.7	83.1

The specific activities with the standard assays were: supernatant (membrane-bound [^{32}P]PA) activity, 6.0 ± 0.5 pmoles ^{32}Pi released/min/mg protein; and microsomal (aqueously dispersed PA) activity, 18.7 ± 1.2 nmoles Pi released/min/mg protein. Average values from 3 separate experiments are presented.

tive to Ca^{2+} and Mn^{2+}, whereas the microsomal activity was more sensitive to F^- and Mg^{2+}. The most striking differences between the two activities were found with Mg^{2+}. While both activities were slightly stimulated at 2.5 mM Mg^{2+}, the microsomal activity was markedly inhibited at higher concentrations. The Mg^{2+} dependence of the supernatant activity was illustrated by the complete inhibition of PA hydrolysis in the presence of low concentrations of EDTA. (Only a slight effect was noted with EGTA at these same concentrations.) In contrast, the microsomal activity was essentially unaffected up to 10 mM EDTA. The inhibitory effect of EDTA treatment of the supernatant activity could be reversed by addition of Mg^{2+} (data not shown). A requirement for Mg^{2+} only in the presence of EDTA has been noted previously and explained by the presence of endogenous Mg^{2+} in the microsomal fraction (8).

DISCUSSION: In agreement with investigations conducted in other tissues

(7-11,13), these studies demonstrate that the esterification of G3P by lung microsomes in the presence of fatty acids, CoA, and Mg^{2+} leads to an accumulation of PA (Fig. 1a). This suggests that under optimal conditions, the microsomal phosphatidic acid phosphatase may be rate-limiting for glycerolipid synthesis. At least one reason for the accumulation of PA appears to be the absence from the system of the phosphatidic acid phosphatase activity associated with the cytosol. These results suggest that the enzyme activity associated with the supernatant fraction is the major effector of diacylglycerol synthesis. The relevance of this observation to pulmonary metabolism is related to the fact that diacylglycerol is the direct precursor of phosphatidyl choline, the principal component of the pulmonary surfactant.

Interpretation of the various ion and chelator effects in terms of defining two separate activities must necessarily be guarded. The most striking differences between these two activities can be found in their Mg^{2+} dependencies (Table 1); the soluble activity exhibits a distinct requirement for Mg^{2+}, whereas the microsomal activity is Mg^{2+}-independent. On the basis of Mg^{2+} dependencies, Jamdar and Fallon (11) concluded that there were at least two phosphatidic acid phosphatases in adipose tissue. However, it should be noted that the properties of a soluble protein could be drastically altered when bound to a biological membrane. Only purification of the activities from both sources will distinguish whether they are different forms of the same enzyme or are different proteins.

Phosphatidic acid phosphatase activities have been detected in many subcellular fractions of lung tissue (3-6), but their relation to phosphatidyl choline synthesis is not clear. With the recent suggestion that lamellar bodies in lung tissue do not have the capacity to synthesize phosphatidyl choline *de novo* (14,15), the importance and role of the phosphatidic acid phosphatase activity previously demonstrated in these inclusion bodies (16,17) must be questioned. The present evidence supports the general conclusion that the bulk of phosphatidyl choline is synthesized in the endo-

plasmic reticulum and transported from there to the lamellar bodies (18,19). Because lung microsomes are rate-limiting with regard to phosphatidic acid phosphatase activity, however, the problem remains to account for the hydrolysis of PA formed *in vivo* as a membrane-bound biosynthetic intermediate by the endoplasmic reticulum. This communication has shown that the cytosol has a high activity relative to the microsomal fraction when membrane-bound PA is used as substrate. We suggest this activity could be important for the hydrolysis of PA formed on the endoplasmic reticulum and therefore eventual surfactant synthesis in the lung.

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REFERENCES

1. Hübscher, G. (1970) in Lipid Metabolism (Wakil, S.J., ed.), Academic Press, New York, pp. 279-370.
2. Schacht, J. and Agranoff, B. (1973) Biochem. Biophys. Res. Comm. 50, 934-941.
3. Schultz, F.M., Jimenez, J.M., MacDonald, P.C. and Johnston, J.M. (1974) Gynecol. Invest. 5, 222-229.
4. Possmayer, F., Duwe, G., Metcalfe, R., Stewart-DeHaan, P.J., Wong, C., Las Heras, J. and Harding, P.G.R. (1977) Biochem. J. 166, 485-494.
5. Brehier, A., Benson, B.J., Williams, M.C., Mason, R.J. and Ballard, P.L. (1977) Biochem. Biophys. Res. Comm. 77, 883-890.
6. Mavis, R.D., Finkelstein, J.N. and Hall, B.P. (1977) ABSTRACT Fed. Proc. 36, 790.
7. Smith, M.E., Sedgwick, B., Brindley, D.N. and Hübscher, G. (1967) Eur. J. Biochem. 3, 70-77.
8. Mitchell, M.P., Brindley, D.N. and Hübscher, G. (1971) Eur. J. Biochem. 18, 214-220.
9. Lamb, R.G. and Fallon, H.J. (1974) Biochim. Biophys. Acta 348, 166-178.
10. Johnston, J.M., Rao, G.A., Lowe, P.A. and Schwarz, G.E. (1967) Lipids 2, 14-20.
11. Jamdar, S.C. and Fallon, H.J. (1973) J. Lipid Res. 14, 517-524.
12. Possmayer, F. and Strickland, K.P. (1967) Can. J. Biochem. 45, 53-61.
13. Hendry, A.T. and Possmayer, F. (1974) Biochim. Biophys. Acta 369, 156-172.
14. Tsao, F.H.C. and Zachman, R.D. (1977) Pediat. Res. 11, 849-857.
15. Barańska, J. and Van Golde, L.M.G. (1977) Biochim. Biophys. Acta 488, 285-293.
16. Spitzer, H.L., Rice, J.M., MacDonald, P.C. and Johnston, J.M. (1975) Biochem. Biophys. Res. Comm. 66, 17-23.
17. Meban, C. (1972) J. Cell Biol. 53, 249-252.
18. Chevalier, G. and Collet, A.J. (1972) Anat. Rec. 174, 289-310.
19. Rooney, S.A., Page-Roberts, B.A. and Motoyama, E.K. (1975) J. Lipid Res. 16, 418-425.