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MICROSOMAL PHOSPHOLIPASE D OF RAT BRAIN AND LUNG TISSUES

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Summary: Phospholipase D of rat brain and lung tissue is enriched in the microsomal fraction. A comparative study of the activity of this enzyme from various rat tissues revealed that significantly greater levels were present in brain and lung particles than in other tissues assayed. The enzyme was found to have an absolute requirement for detergent and a pH optimum of 6.5. This is the first report of the detection of membrane bound phospholipase D activity.

Introduction: Only a few reports have appeared dealing with phospholipase D of mammalian tissues (1-3). This enzyme hydrolyzes phosphatidylcholine and phosphatidylethanolamine (2), the two most abundant phosphoglycerides of mammalian membranes, to phosphatidic acid, a key intermediate in the metabolism of triacylglycerols and phosphoglycerides. Phospholipase D therefore is potentially important for lipid and membrane metabolism.

This enzyme had been previously believed absent from mammalian tissues (4) largely due to the inability of detecting its activity using various particulate fractions. This activity has only unequivically been detected in detergent solubilized rat brain particulate fractions (1). We wish to describe conditions for the *in vitro* assay of the membrane bound activity in mammalian tissues.

Materials and Methods:

Materials: [9,10-^{3}H] Oleic acid was purchased from Amersham Corporation and [2-^{3}H] glycerol was purchased from New England Nuclear. Phosphatidylcholine (egg) was obtained from Pierce Chemical Co and 1-acyl-lysophosphatidy choline (beef brain) was from Serdary Research Laboratories, London, Ont. Sodium taurodeoxycholate (A Grade) was from Calbiochem, San Diego, Cal. HPTLC silica gel Merck 60 thin layer chromatography plates were purchased from Terochem Laboratories, Edmonton, Alt.

Methods: Preparation of [^{3}H] Phosphatidylcholine Substrate

1-Acyl-2-[9,10-^{3}H] oleoyl-sn-glycero-3-phosphorylcholine was synthesized by the procedure of Webster and Cooper (5). A suspension of the [^{3}H] phosphatidylcholine and egg phosphatidylcholine was sonicated in 5 mM β,β-

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dimethylglutarate buffer, pH 6.5, at 50 watts, under nitrogen for 30 minutes on ice, using a Heat Systems-Ultrasonics Inc. Model W185 probe type sonicator. The solution was then centrifuged at 100,000 x g for 30 minutes and the resulting supernatant employed as the substrate in these studies.

Assay of Phospholipase D

Phospholipase D activity was assayed in the presence of 50 mM β,β-dimethylglutarate-NaOH buffer, pH 6.5, 5 mM EDTA, 50 mM KF, 6 mM sodium taurodeoxycholate and 0.5 μmole of the [^{3}H]-phosphatidylcholine microdispersion, (1.94 Ci/mole). The reaction was initiated by adding up to 200 μg of protein and the final mixture, 100 μl total volume, was incubated at 30°C for 15 minutes. The reaction was terminated by adding 1.5 ml of chloroform-methanol (1:1, v/v) containing phosphatidic acid carrier and the mixture then extracted according to Bligh and Dyer (6). The lower chloroform phase was dried and applied to thin layer chromatography plates which were developed twice in the same direction. Solvent mixture I consisted of diethyl ether-acetic acid (100:1, v/v) and solvent mixture II consisted of chloroform-acetone acetic acid-methanol-water (50:20:10:10:5, v/v). After visualizing the lipids with iodine, the silica corresponding to phosphatidic acid was scraped into a scintillation vial containing Scintiverse counting mixture (Fisher Sci. Co.) with 0.5 ml water and then counted in a Searle Mark III scintillation counter.

Preparation of Subcellular Fractions

The preparation of subcellular fractions was according to the procedure of Cotman and Matthews (7). The initial homogenate (H) was centrifuged 160 x g for 5 minutes to remove unbroken cells. The crude nuclear fraction (P1), and the crude mitochondrial fraction (P2) were both washed by resuspending in 0.32 M sucrose and recentrifuging. The mitochondrial supernatant was centrifuged 17,000 x g for 10 minutes to pellet the P2-B fraction. The P2-B supernatant was centrifuged at 100,000 x g for 90 minutes to yield the microsomal fraction (P3) and the cytosolic fraction (S). The microsomes utilized for the tissue survey was the particulate fraction obtained by centrifuging 17,000 x g for 10 minutes and then 100,000 x g for 90 minutes.

NADPH-cytochrome C reductase was assayed according to the procedure of Sottocasa et al. (8). Protein was determined by a modification of the Lowry method (9) and phosphorous was determined by the method of Bartlett (10).

Results: The distribution of phospholipase D activity in the primary subcellular fractions prepared from rat brain and lung tissues are seen in Table 1. Enrichments of 2.1 and 3.9 fold over the starting homogenates were found in the brain and lung P3 fractions respectively, suggesting a microsomal location for phospholipase D. This conclusion was supported by a similar enrichment of the microsomal marker enzyme NADPH-cytochrome C reductase in this particulate fraction.

The relative specific activity of phospholipase D in the microsomes from various rat tissues is seen in Fig. 1. Although the enzyme was detected in all tissues surveyed, there was a wide range of specific activity values. Brain and lung had the highest specific activities compared to the other tissues examined.

Table I. Subcellular Localization of Phospholipase D in Rat Brain and Lung Tissues

	BRAIN					LUNG				
Tissue Fraction	Protein mg	Specific activity nmoles/mg/hr	Enrich-ment	Percent of total activity	Enrich-ment of NADPH-cytochrome C reductase activity	Protein mg	Specific activity nmoles/mg/hr	Enrich-ment	Percent of total activity	Enrich-ment of NADPH-cytochrome C reductase activity
H, Homogenate	720	26.0	1.00	100.0	1.00	561	16.6	1.00	100.0	1.00
P1, Crude nuclei	56	15.6	0.60	4.7	0.58	121	7.6	0.46	9.9	0.53
P2, Crude mitochondria	216	23.1	0.88	26.6	0.73	57	47.0	2.83	28.8	1.20
P2-B, microsomal wash	48	41.2	1.58	10.5	0.86	13	54.8	3.30	7.6	2.73
P3, microsomes	126	54.8	2.11	36.9	2.06	70	64.3	3.87	48.0	4.34
S, cytosol	132	0.6	0.02	0.4	0.21	152	2.7	0.16	4.4	0.35

Subcellular fractions were prepared as described under methods. The standard assay was used in the presence of 150 µg protein of each fraction.

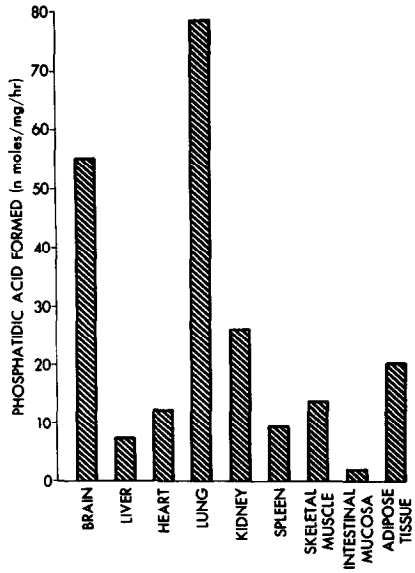

Fig. (1) The Specific Activity of Phospholipase D in Microsomes from Various Rat Tissues. The assay was as described in the text using 150 µg of microsomal protein.

The reaction was linear for 15 minutes at 30°C and was linear with up to 200 µg protein. Metal ions were not required. The addition of 5 mM EDTA did not inhibit the activity, but resulted in an approximate 40% stimulation while 5 mM $CaCl_2$ was found to be slightly inhibitory.

The effect of varying the pH on the enzyme activity is seen in Fig. 2A. Both the brain and lung enzymes respond almost identically with an optimum at pH 6.5. The effect of varying substrate concentration on phospholipase D activity is similar with both particles as shown in Fig. 2B. Substrate inhibition occurs above the optimal concentration of 5 mM. However, this inhibition is less pronounced with the lung enzyme which displays a 24% inhibition compared to 76% for the brain enzyme at 12.5 mM phosphatidylcholine.

The effect of detergent on the activity is shown in Fig. 2C. Phospholipase D was undetectable in the absence of added taurodeoxycholate with the microsomes from both sources and above the optimal 6 mM concentration inhibition occured. The activation curve for phospholipase D shows some differences in the responses of the brain and lung enzymes. Taurodeoxycholate at 2 mM

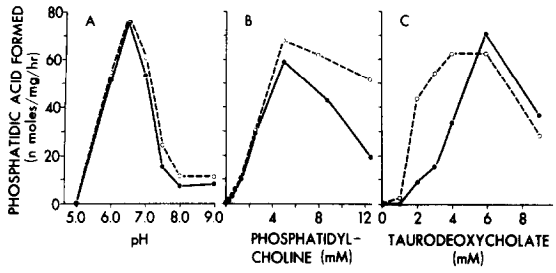

Fig. (2) a) The Effect of pH in Rat Brain and Lung Microsomal Phospholipase D. The assay as described in the text with 50 mM β,β-dimethylglutarate-NaOH buffer for the range of pH 5.0 to 7.5 and 50 mM glycylglycine-HCL buffer was used for pH 8.0 and 9.0

b) The Effect of Phosphatidylcholine Concentration on Rat Brain and Lung Phospholipase D. The standard assay system was used in the presence of varying amounts of the [^{3}H] phosphatidylcholine microdispersion.

c) The Effect of Varying Taurodeoxycholate Concentration on Rat Brain and Lung Phospholipase D. The standard assay system was used while varying the amount of taurodeoxycholate as indicated.

concentration resulted in a 71% of maximal stimulation of the lung microsomal activity, while only producing 13% of maximal activity with the brain microsomes.

Discussion: Phospholipase D has been known to exist in plants since 1947 (11), however, it was not until 1975, nearly 3 decades later, that its presence was shown in mammalian tissue (1). This previous inability to demonstrate its existence presumably was the result of inadequate conditions employed for its assay. As presented in Fig. 2C, this activity is undetectable in the absence of taurodeoxycholate. It is also evident that the appropriate concentration of this detergent must be added since inhibition occurs at higher concentrations. Previously, mammalian phospholipase D was only detectable in detergent solubilized rat brain membranes (1) and in human eosinophiles (3).

The results of subcellular fractionation suggest that the activity is associated with the microsomes of both lung and brain tissues. These two tissues also have greater enzyme activities than the other tissues surveyed. The presence of the enzyme in all tissues examined, but with dissimilar activities may be a result of its physiological role.

The ability of bile salts and other detergents to activate certain membrane bound enzymes (12-15) is a phenomenon of which mammalian phospholipase D appears to be a rather striking example. The mechanism by which taurodeoxycholate activates this enzyme and whether or not this reflects some in vivo regulatory process are unknown at this time.

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