MAGNESIUM-DEPENDENT SPHINGOMYELINASE

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SUMMARY: An enzyme which requires divalent metals and hydrolyses sphingomyelin to ceramide and phosphorylcholine is present in rat and human brain and practically absent from other organs. The greatest activity is associated with the microsomal fraction. It had an optimal pH at about 7.4, required magnesium or manganese ions and was completely inhibited by EDTA. Triton X-100 was required for optimal activity and this detergent could also be used to partly solubilize the enzyme from rat brain microsomes. Lecithin was hydrolyzed at only 2% of the corresponding rate of hydrolysis of sphingomyelin.

Sphingomyelinase (Sphingomyelin choline phosphohydrolase, EC 3.1.4.12) is a hydrolase of the phospholipase C family (1). In animal tissues it is of lysosomal origin and has an optimal activity at about pH5. In animal tissues, it has been isolated and partially purified from brain (2), liver (3,4) and spleen (5). Recently it has been suggested that there may be several isoenzymes (6). The above preparations did not require metal ions for activity and were strictly specific for sphingomyelin; lecithin or phosphatidylethanolamine were not hydrolyzed. Other enzymes with phospholipase C activity in animal tissues have been reported. An enzyme which splits of the phosphoinositol moiety of the phosphoinositides has long been known (1,7,8) and a phospholipase C which splits off the phosphorylethanolamine portion of phosphatidylethanolamine has recently been described; the activity of the latter enzyme was increased by the addition of EDTA (9). Roitman et al. have reported the presence of a phospholipase C, with an optimal pH at about 5 in rat brain (10), but this was retracted in a subsequent paper (11). In a recent communication, Quinn, while describing

a phosphodiesterase in brain which acts on glycerophosphorylcholine (12) did not find evidence for phospholipase C activity on lecithin.

This paper describes a sphingomyelinase, in rat and human brain which depends on divalent ions, has an optimal pH between 7-8 and catalyzes only little hydrolysis of lecithin. Schneider and Kennedy have suggested the presence of a similar enzyme in spleen of patients with Niemann-Pick's disease (5) and Hirshfeld and Loyter et al. have described a magnesium-dependent sphingomyelinase in the stroma of avian erythrocytes (13). Hydrolysis of glycerophosphatides by the latter two enzymes has not been tested.

MATERIALS AND METHODS: Substrates Two samples of tritium-labelled sphingomyelin were used. The first was labelled in the ceramide moiety by catalytic hydrogenation with tritium gas in the presence of palladium on charcoal (14). The second was labelled in the choline moiety by condensing $^{3}H_3$-methyliodide (Amersham) with ceramide phosphoryldimethylethanolamine (demethylated sphingomyelin, a generous gift of Prof. W. Stoffel) according to the method of Stoffel (15). Choline-labelled lecithin was a gift of Drs. Greenzaid and Heller, it was prepared according to ref. 16. 2-[^{3}H] oleyl lecithin and 1-[^{3}H] palmitoyl phosphatidylethanolamine were prepared according to reference 17. Triton X-100, sodium dodecylsulfate and EDTA were purchased from BDH; sodium taurocholate and taurodeoxycholate from CalBiochem; EGTA and Cetavlon from Sigma.

Preparation of brain microsomes and subcellular fractions. Brains of 14 day old rats were homogenized and subcellular fractions were prepared according to ref. 18. The microsomal fraction of adult (about 120g) rat brain or of human brain were prepared as follows: Brain was homogenized in 9 vols. of 0.25M sucrose. Debris was removed for 10 min. at 700xg, the mitochondrial fraction was sedimented for 10 min. at 25,000xg and microsomes for 1 hr. at 100,000xg. The microsomes were suspended in 0.25M sucrose (1ml per grm equivalent of brain tissue) and stored at -20°.

Assay of the enzymatic reaction: Incubation mixtures in volumes of 0.2ml contained 20 μmoles of Tris, pH7.4, 0.05 μmoles of sphingomyelin, 0.4mg of Triton X-100, 0.2 μmole of magnesium chloride and enzyme. After 1 hr. at 37° the reaction was terminated and the reaction products were isolated as follows: 1. Using sphingomyelin labelled in the ceramide moiety the reaction was terminated with Dole's reagent (2) and the ceramide released was isolated and counted using the procedure employed in the hydrolysis of lecithin (17). 2. Using choline-labelled sphingomyelin or lecithin: The reaction was terminated by the addition of 0.8ml of a mixture of chloroform and methanol, 2:1 (v/v). An aliquot of the upper phase was mixed with 3.5ml of Instagel (Packard) and counted in a liquid scintillation spectrometer.

Identification of the products of the reaction: 1. Identification of ceramide: sphingomyelin, labelled with tritium in the ceramide moiety was used, the reaction was terminated with Dole's reagent (2), and the heptane phase was

evaporated with nitrogen. The residue was dissolved in chloroform-methanol, 2:1 and spotted on thin layer plates of silica gel. The plates were developed in the following three, separate solvent systems: a. Chloroform-methanol-acetic acid, 94:4:2 (19) b. Chloroform-methanol-ammonia, 95:5:0.8 (20) and c. Chloroform-methanol-acetone-acetic acid-water, 80:2.5:15:2.5:2 (20). N-stearoyl-DL-dihydrosphingomyelin was used as reference compound in each case. The radioactivity on the plate was located with a Berthold radio-scanner and further verified by scraping the silica gel, mixing well with 1ml of Triton X-100-absolute ethanol, 1:1 and adding 10ml of scintillation fluid. The ceramide was located in duplicate lanes by spraying the plate with 10% sulfuric acid in methanol, followed by charring at 180°. 2. Identification of phosphorylcholine: The reaction mixture, containing ^{3}H-choline-labelled sphingomyelin was terminated with 4 volumes of chloroform-methanol, 2:1. The upper phase was evaporated, dissolved in methanol and applied to a thin layer plate of silica gel which was developed in chloroform-methanol-3M trichloroacetic acid-water, 40:60:20:12.5 (21). Phosphorylcholine, choline and glycerophosphorylcholine were used as reference compounds. The radio-activity on the plate was located using a Berthold radioscanner and the spots were visualized by spraying according to Doizaki et al. (21) with a mixture of 70% $HClO_4$-5N HCl-5%-ammonium molybdate-3M TCA-H_2O, 2:4:8:5:21. After drying at 100° the plates were further sprayed with a mixture of Dragendor's reagent - $Bi(NO_3)_3.5H_2O$ (1.7g in 100ml of 20% acetic acid) - 40% KI (w/v)-H_2O, 4:1:20.

Identification of diglyceride: Incubation mixtures containing 2-^{3}H-oleyl-phosphatidylcholine were terminated with Dole's reagent (2), the heptane was evaporated and the residue was spotted on thin layer plates of silica gel. The plates were developed in petroleum ether (40-60°)-ether-acetic acid (22), 60:40:1 (22) or in chloroform-methanol-acetic acid, 98:2:1 (22).

RESULTS: Distribution in rat organs and subcellular fractions. Rat brain, liver, kidney and spleen were homogenized in 0.25M sucrose and debris was

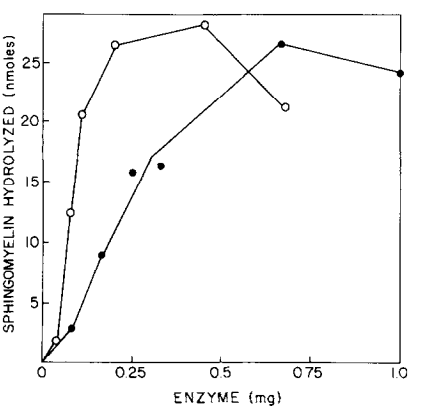

Fig. 1. Effect of the concentrations of the homogenate minus debris and microsomes on the rate of hydrolysis of sphingomyelin. Standard assay conditions were used.

removed for 10 min. at 700xg. The activity of brain homogenates exceeded those of the other tissues manyfold. The v versus E curves deviated from linearity (see Fig. 1). Therefore only the ascending portions of the curves were used to calculate the relative activities of the enzymes in the various tissues. The quantitative values were obtained by subtracting a small activity of control tubes to which EDTA was added; this represents the residual activity, at pH7.4 of the lysosomal enzyme. The approximate relative activities of the homogenates minus debris, expressed as nmoles of substrate hydrolyzed in an hour by a gram equivalent of tissue were: Brain 4400, liver 100, kidney 200, spleen 25. A subcellular fractionation of rat brain homogenate showed that most of the activity resided in the microsomal pellet, the relative activities (calculated for one gram equivalent) in the microsomal, mitochondrial, synaptosomal and lysosomal fractions were 100:42:26:7. Therefore, further studies were done using the 100,000xg sediment of rat brain. Again, deviation from linearity was observed at low levels of microsomal protein (Fig. 1) and a plateau was reached (or some inhibition was obtained) at higher concentrations. The controls without magnesium but with EDTA were only about 2-4% of the activity obtained in the absence of EDTA.

Properties of the enzyme: Triton X-100 (2-3mg/ml) was always added to the reaction mixture, only negligible activity was observed in the absence of this detergent. Taurocholate, Cetavlon, sodium dodecylsulfate or Miranol H2M could not replace the Triton. 0.2% Triton X-100 could also be used to solubilize part of the enzymatic activity. The microsomal preparation showed only a small dependence on magnesium ions. However, treatment with EDTA or EGTA, followed by dialysis against Tris buffer resulted in a preparation which required divalent ions. In a typical experiment EDTA-treated microsomes hydrolyzed 5nmoles of sphingomyelin in the absence of

Mg, 32 in the presence of 1mM Mg or Mn and 12 at 1mM Ca. The optimal pH of the reaction was between 7 and 8. The products were ceramide (identified in three separate solvents, see Methods) and phosphorylcholine. Free choline was not detected. Lecithin was hydrolyzed at only about 2% of the rate of hydrolysis of sphingomyelin. That this is indeed a residual phospholipase-C activity was verified by using ^{3}H-oleyl lecithin; thin layer chromatography showed the presence of diglyceride. Phosphatidylethanolamine was not hydrolyzed under the standard assay conditions. Microsomes could be stored for at least 5 months at -20^{o} with only little loss of activity; but preparations which had been treated with EDTA lost activity after 2 weeks at -20^{o}. Similar to the findings with rat organs, human liver, kidney and spleen had only little activity at pH 7.4. The activities of human brain were 6700 and 1800 nmoles hr^{-1} per gm equivalent of homogenates minus debris of a newborn and senile brain and 2500 and 250 for their respective microsomal fractions. The respective specific activities were 156 and 345 nmole mg^{-1} hr^{-1} for the homogenate and microsomes of the young human brain and 66 and 68 for the senile brain.

DISCUSSION: Animal tissues abound in enzymes which split off fatty acid residues of the glycerophosphatides (phospholipase A). However, the presence of phospholipase C has not been firmly established. The enzyme which hydrolyzes sphingomyelin belongs to the phospholipase C group. It utilizes both sphingomyelin and lecithin in bacteria (1) but not in animal tissues. Except for the very brief mentioning by Schneider and Kennedy (5) and the chicken erythrocyte enzyme (13) all sphingomyelinases of animal tissues are of lysosomal origin, i.e., they have an optimal pH at about 5 and do not require metal ions for activity (1). This paper reports the presence, in brain of a divalent metal-dependent sphingomyelinase with a slightly alkaline

optimal pH. The rate of hydrolysis by homogenates (less debris) of rat brain at pH 7.4, with magnesium was about 5 μmoles x hr^{-1} per gram equivalent. This value is greater than the corresponding activity of the lysosomal enzyme, as assayed at pH 5 (10). The microsomal sphingomyelinase probably has no general phospholipase C activity. Lecithin was hydrolyzed at about one fiftieth of the rate of hydrolysis of sphingomyelin. Phosphatidylethanolamine was not hydrolyzed even at this low rate. The enzyme here described differs from the recently-described phospholipase C type enzyme which hydrolyses phosphatidylethanolamine and is also localized in the microsomes, since the latter is stimulated by EDTA (9). The relation of the metal-dependent sphingomyelinase to the phosphatidylinositide splitting enzymes cannot be assessed to date. While the sphingomyelinase is membranal, the latter enzymes are present partly in microsomes but even moreso in the soluble fraction and require calcium for their activity. Experiments are planned to solubilize the microsomal sphingomyelinase, purify it and then determine its substrate specificity.

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