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113

Purified Rat Brain Microvessels Exhibit Both Acid and Neutral Sphingomyelinase Activities

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Abstract: Purified rat brain microvessels have been shown to hydrolyze radiolabeled sphingomyelin by means of two different enzyme systems. Enzymatic activity was detected at pH 7.4 and was strongly stimulated by magnesium or manganese and inhibited by calcium. Activity at pH 5.1 could also be found and was not dependent on any of these cations. At neutral pH and in the presence of magnesium, the rate of sphingomyelin hydrolysis did not exhibit a linear relationship with protein concentration. In contrast, increasing the protein concentration from 0.05 to 0.5 mg/ml resulted in a constant increase of sphingomyelin hydrolysis at pH 5.1. Kinetic parameters of both neutral and acid activities have been determined and were similar in magnitude to values reported previously for neural sphingomyelinases. This work demonstrates the occurrence of a neutral sphingomyelinase activity in purified rat brain microvessels, an observation raising the question of its role at the level of the blood–brain interface. **Key Words:** Sphingomyelinase—Magnesium—Neutral sphingomyelinase—Acidic sphingomyelinase—Brain—Microvessels. Carré J.-B. et al. Purified rat brain microvessels exhibit both acid and neutral sphingomyelinase activities. *J. Neurochem.* **52,** 1294–1299 (1989).

The blood–brain barrier consists of a continuous layer of endothelial cells joined together by tight junctions, which do not allow intercellular solute diffusion (Oldendorf, 1977). Hexoses, amino acids, purine compounds, and monocarboxylic acids, as well as several other physiological compounds, are transported across the blood–brain barrier with the aid of carrier-mediated mechanisms (Pardridge and Oldendorf, 1977). More recently, several studies were designed to investigate the relationships between synthesis and degradation of brain microvessel lipids and the functional role of this metabolism. Substantial lipoprotein lipase and acid lipase activities were measured in rabbit brain microvessels, observations suggesting that lipoproteins are metabolized within the cerebral microvasculature (Brecher and Kuan, 1979). Rat brain microvessels exhibit diacylglycerol lipase and kinase activities (Hee-Cheong et al., 1985). Both fatty acid oxidation and glycolysis supply energy to the endothelial cells (Goldstein, 1979; Betz and Goldstein, 1981). In rat brain microvessels, fatty acids might also be diverted to prostaglandin and leukotriene synthesis (Gerritsen et al., 1980; Baba et al., 1985). A polyunsaturated fatty acid-specific acyl-CoA synthetase and a nonspecific acyl-CoA synthetase were shown in rat brain microvessels (Morand et al., 1987). In addition, radioactive lipids are synthesized by rat brain microvessels incubated with [1-^{14}C]acetate (Homayoun et al., 1985).

Numerous publications on sphingomyelin hydrolysis activities in animal tissues have described two different sphingomyelinase enzymes. The first one shows maximal activity at ~pH 5.0, has no requirement for divalent cations, and has been found in animal tissues (Kanfer et al., 1966; Callahan et al., 1974). It is assumed that this lysosomal acid enzyme is responsible for sphingomyelin hydrolysis within phagocytic vesicles (Fowler and DeDuve, 1969). The second one exhibits an optimal pH of 7.4, has an absolute requirement for magnesium or manganese, and is not ubiquitous (Gatt, 1976; Rao and Spence, 1976). In the brain, neutral sphingomyelinase is located within the gray matter and, in particular, in the striatum (Spence et al., 1978). It has also been characterized and localized in bovine adrenal medulla (Bartolf and Franson, 1986) as well as in cultured neuroblastoma cells (Spence et al., 1982) and hen erythrocyte membranes (Rousseau et al., 1986). The neutral magnesium-dependent enzyme is thought to be almost exclusively present at the level of

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the plasma membrane. As a consequence of this activity, altering the phosphatidylcholine/sphingomyelin ratio (Shinitzky and Barenholz, 1974) or the cholesterol content of the plasma membrane (Demel et al., 1977) may strongly affect membrane fluidity and certainly more than one of its functions. It has been proposed that the neutral sphingomyelinase might be involved in the exocytotic release of catecholamines by perturbing the membrane phospholipid composition (Bartolf and Franson, 1986). Spence et al. (1982) suggested that the high concentration of the neutral sphingomyelinase in cells of neural derivation and its localization in plasma membrane would reflect its putative role in cell–cell and/or cell–ligand interactions peculiar to these cells. These hypotheses must retain some consideration with regard to the transport functions of brain microvessels, which are known to metabolize several neurotransmitters (Lai et al., 1975; Hardebo et al., 1980; Pardridge and Mietus, 1981). Therefore, a study was undertaken to assess the occurrence of sphingomyelinase activities in purified rat brain microvessels. It shows that both neutral and acid enzymes are present in this particular substructure of the brain and that they exhibit several characteristics similar to the sphingomyelinases of the gray matter or brain homogenates.

EXPERIMENTAL PROCEDURES

Materials

Dextran (MW = 70,000) was obtained from Pharmacia, the nylon mesh (pore size = 118 μm) from Desjobert (Paris, France), and glass beads from B. Braun (Mensugen, F.R.G.). [*choline-methyl*-^{14}C]Sphingomyelin (50 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.), and ACS-II scintillation liquid was from Amersham (Bucks, U.K.). EDTA, Triton X-100, brain sphingomyelin, and albumin (bovine, fatty acid free, fraction V) were purchased from Sigma.

Preparation of purified rat brain microvessels

Purified rat brain microvessels were prepared according to the method described by Goldstein et al. (1975) and slightly modified as follows. For one preparation, 15 Sprague–Dawley rats (2 months old) were killed by decapitation. The brains were immediately removed and placed in ice-cold buffer, made of oxygen-saturated Ringer solution containing 1.2 m*M* $MgCl_2$, 15 m*M* HEPES (pH 7.4), and 1% (wt/vol) albumin. The brainstem, cerebellum, and meninges were discarded. Cortical hemispheres free of choroid plexus and ependyma were minced with scissors in buffer. The tissue was homogenized in a glass homogenizer with a Teflon pestle (0.25 mm clearance) at 390 rpm (20 strokes). The homogenate was centrifuged at 1,000 *g* for 10 min. The pellet was resuspended in ice-cold buffer containing 17.5% (wt/vol) dextran to a concentration of 1 g of fresh tissue/13 ml of the dextran solution and centrifuged at 4,000 *g* for 15 min. The new pellet, which consisted of free nuclei, debris, and microvessels, was resuspended in buffer and then passed through nylon mesh, having a pore size of 118 μm under gentle vacuum. The microvessels were separated from nuclei by passing the suspension through a 1.2 × 1.5-cm column containing 0.25-mm-diameter glass beads. Nuclei and debris were removed by washing with buffer, and microvessels remained attached to the beads. After resuspension in buffer, microvessels were collected by gentle agitation and subsequent sedimentation of the beads. The microvessel suspension was centrifuged at 500 *g* for 5 min. Finally, to remove all traces of albumin, the pellet was washed twice with a solution made of 155 m*M* KCl in sodium phosphate buffer, pH 7.4. The purified brain microvessels were stored in a small volume of KCl buffer at −20°C for no more than 4 weeks or were immediately incubated and assayed for sphingomyelinase activity as described below.

The purity of the microvessel preparation was estimated first by its appearance in phase microscopy. Next, γ-glutamyl transpeptidase, known to be specifically associated with brain microvessels (Orlowski et al., 1974), has been used as a positive marker: its activity was assayed in brain homogenates as well as in purified brain microvessels to determine the degree of enrichment of the preparation (Orlowski and Meister, 1963). The presence or absence of neuronal and glial contaminants was assessed by immunohistofluorescence using marker antibodies directed against glial fibrillary acidic protein for astrocytes (Jacque et al., 1986), myelin basic protein (Jacque et al., 1985) and β-galactocerebrosides (Zalc et al., 1981) for oligodendrocytes and myelin, and neurofilaments (using a procedure similar to that used for glial fibrillary acidic protein; C. Jacque, unpublished data) for neurons.

Sphingomyelinase assay

Pellets of purified rat brain microvessels were resuspended in ice-cold water and homogenized in a glass homogenizer with a Teflon pestle driven at 400 rpm (20 strokes). Rat forebrains were homogenized in ice-cold water. The protein content of the homogenized preparation was determined by means of a fluorescence procedure adapted from that of Böhlen et al. (1973). Homogenates were diluted to a given protein concentration before final dilution to initiate incubation. [*choline-methyl*-^{14}C]Sphingomyelin was diluted with unlabeled sphingomyelin to a specific radioactivity of 0.2 mCi/mmol and mixed with Triton X-100 in chloroform/methanol (2:1 vol/vol). The mixture was evaporated to dryness under nitrogen, solubilized in concentrated buffer (272 m*M* acetate or Tris-HCl buffer), heated at 50°C for 1 min, and immediately cooled down to obtain a clear solution (Barenholz et al., 1966). The incubation was initiated by mixing 90 μl of the microvessel homogenate or the forebrain homogenate with 110 μl of the above radioactive sphingomyelin solution (Rao and Spence, 1976). Final conditions were as follows: 0.02–0.4 mg of protein/ml, 25–500 μ*M* radiolabeled sphingomyelin, and 0.1% (wt/vol) Triton X-100 in a final volume of 0.2 ml. The final buffer concentrations were either 150 m*M* sodium acetate (pH 5.1) or 150 m*M* Tris-HCl and 6 m*M* $MgCl_2$ (pH 7.4). Under standard conditions, sphingomyelinase was assayed for 20 min at pH 7.4 and for 60 min at pH 5.1. To stop the reaction, each sample was cooled down in ice-cold water and received 0.1 ml of 10% albumin in water (wt/vol). Then, 0.1 ml of 100% (wt/vol) trichloroacetic acid was added, followed by 0.8 ml of water (Sloan, 1972). Each tube was vortex-mixed and centrifuged for 4 min at 10,000 rpm in a Beckman Microfuge. Intact sphingomyelin coprecipitated with albumin in the pellet, whereas the supernatant retained radiolabeled phosphorylcholine released on hydrolysis of [*choline-methyl*-^{14}C]sphingomyelin. The acid enzyme is referred to as sphingomyelin phosphodiesterase (EC 3.1.4.12). Finally, 0.8 ml of the supernatant (total volume of 1.2 ml) was counted for radioactivity in 10 ml of ACS-II scintillation liquid. All enzyme activity values were calculated

from two duplicate experiments, i.e., four determinations; SE values varied within a range of 5–10% of each mean value.

RESULTS

The assay of γ-glutamyl transpeptidase, an enzyme associated with endothelial cells, showed that this enzyme activity was enriched 40-fold in the purified brain microvessels when compared with brain homogenate. Using a similar procedure for brain microvessel purification, Goldstein et al. (1975) reported a 20-fold enrichment. Specific marker antibodies showed that purified microvessels also contained residual debris of processes of glial (glial fibrillary acidic protein, myelin basic protein, and β-galactocerebroside markers) and neuronal (neurofilament marker) origin, although the contaminants did not exceed 10% of the preparation. These observations indicated that the microvessel fraction was but little contaminated with other membranes and suggested that neural tissue enzymes did not contribute significantly to the sphingomyelinase activities described below.

Microvessel homogenates (0.15 mg of protein/ml) were incubated in the presence of 200 μM [*choline-methyl*-^{14}C]sphingomyelin under standard conditions, and the effect of time on its hydrolysis was determined. At pH 5.1, sphingomyelin hydrolysis was linear for at least 120 min; at pH 7.4 and in the presence of magnesium, the rate of hydrolysis of sphingomyelin was constant during the first 20 min, followed by a much slower rate (data not shown). Accordingly, all subsequent incubations were performed for 60 min at pH

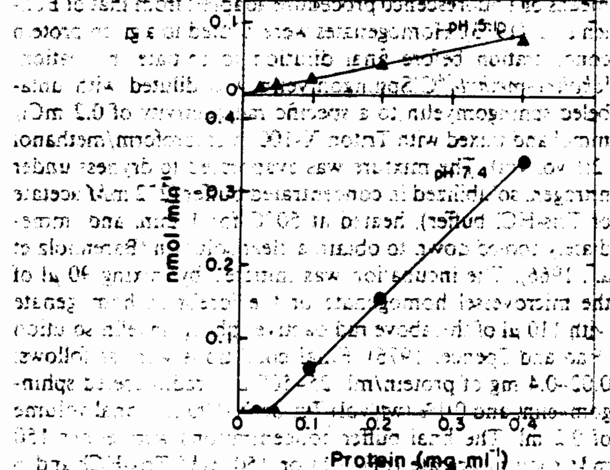

FIG. 1. Effect of increasing the protein concentration on sphingomyelin hydrolysis by purified rat brain microvessel homogenates. Increasing concentrations of purified rat brain microvessel homogenates were incubated at 37°C under the following conditions: 200 μM radiolabelled sphingomyelin, 0.1% (wt/vol) Triton X-100, and either 150 mM Tris-HCl and 6 mM $MgCl_2$ at pH 7.4 for 20 min (bottom panel) or 150 mM sodium acetate at pH 5.1 for 60 min (top panel). On incubation, the reaction was stopped, and sphingomyelinase activity was determined as described in Experimental Procedures and expressed as nmol of sphingomyelin hydrolyzed/min.

TABLE 1. *Divalent cation requirement for sphingomyelinase activities by purified rat brain microvessel homogenates*

	Relative activity (%)	
	pH 7.4	pH 5.1
$+MgCl_2$ (6 mM)	100[a]	128
$-MgCl_2$	51	100[b]
$-MgCl_2$ + EDTA (10 mM)	5	75
$-MgCl_2$ + $CaCl_2$ (6 mM)	29	75
$+MgCl_2$ (6 mM) + $CaCl_2$ (6 mM)	83	109
$-MgCl_2$ + $MnCl_2$ (6 mM)	121	140

Purified rat brain microvessel homogenates (0.3 mg of protein/ml) were incubated at 37°C with 200 μM radiolabeled sphingomyelin and 0.1% (wt/vol) Triton X-100, in 150 mM Tris-HCl at pH 7.4 for 20 min or 150 mM sodium acetate at pH 5.1 for 60 min and in the presence or absence of different divalent cations.

Sphingomyelinase activities were further determined as described in Experimental Procedures and expressed as percentages of the control values, i.e., [a]in the presence only of 6 mM $MgCl_2$ at pH 7.4 (2.5 nmol of sphingomyelin hydrolyzed/mg of protein/min) and [b]with no cation added at pH 5.1 (0.5 nmol of sphingomyelin hydrolyzed/mg of protein/min).

5.1 and for 20 min at pH 7.4. Incubating at pH 5.1 with increasing the microvessel concentration from 0.02 to 0.4 mg of protein/ml in the presence of 200 μM [*choline-methyl*-^{14}C]sphingomyelin resulted in an increase in the amount of hydrolysis product occurring at a constant rate (Fig. 1). On the other hand, hydrolysis activity at pH 7.4 and in the presence of magnesium was not linear in this range of protein concentration: activity was low at 0.02 and 0.05 mg of protein/ml and was drastically elevated further to reach a value of 0.33 nmol/min at 0.4 mg of protein/ml. However, the curve exhibited linearity between 0.05 and 0.4 mg of protein/ml.

The requirements for several divalent cations have been tested in an attempt to characterize further sphingomyelinase activities at pH 7.4 and 5.1 (Table 1). The pH 7.4 activity was slowed down in the absence of magnesium and was drastically reduced by EDTA. Substituting calcium for magnesium reduced the pH 7.4 activity to 29% of the control value. When magnesium was replaced by manganese, the pH 7.4 activity was increased. Adding magnesium or manganese to the incubation medium somewhat increased the pH 5.1 activity, whereas adding EDTA or calcium slightly reduced this activity. Adding both magnesium and calcium together had little effect on the pH 5.1 activity.

Kinetic parameters for both pH 7.4 and pH 5.1 sphingomyelinase activities in purified rat brain microvessel homogenates were further determined. Although the rate of hydrolysis at pH 7.4 and in the presence of magnesium as a function of protein concentration was not fully linear, sphingomyelinase activity was determined on increasing the sphingomyelin concentration in the presence of 0.4 mg of protein/ml. A Michaelis–Menten-type hyperbolic relationship was

observed between sphingomyelin concentration and phosphorylcholine formation (Fig. 2). Replotting sphingomyelinase activity at pH 7.4 in the double-reciprocal Lineweaver–Burk presentation permitted the calculation of an apparent K_m of 83 μM and an apparent V_{max} of 5.9 nmol/mg of protein/min. Furthermore, increasing concentrations of sphingomyelin were added to microvessel homogenate (0.1 mg of protein/ml), and sphingomyelinase activity was measured at pH 5.1 and in the absence of magnesium. Similarly, a Michaelis–Menten-type hyperbolic relationship was observed between sphingomyelin concentration and phosphorylcholine formation (Fig. 3). An apparent K_m of 114 μM and an apparent V_{max} of 0.8 nmol/mg of protein/min were determined for the pH 5.1 sphingomyelinase activity.

Sphingomyelinase activities were also determined in rat forebrain homogenate. At pH 7.4 and in the presence of magnesium, forebrain homogenate sphingomyelinase exhibited an apparent K_m of 61 μM and an apparent V_{max} of 7.2 nmol/mg of protein/min (0.4 mg of protein/ml). The latter calculation was performed, although no linear relationship was observed between protein concentration and the pH 7.4 sphingomyelinase activity. At pH 5.1 and in the absence of magnesium, forebrain homogenate sphingomyelinase exhibited an apparent K_m of 190 μM and an apparent V_{max} of 1.7 nmol/mg of protein/min.

DISCUSSION

The data presented in this article provide a description of two separate sphingomyelinase activities in purified rat brain microvessels. These two activities bear

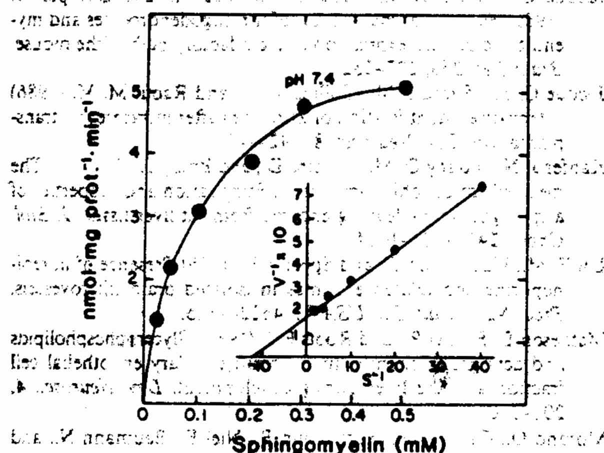

FIG. 2. Effect of radiolabeled sphingomyelin concentration on sphingomyelinase activity at pH 7.4 in purified rat brain microvessel homogenates. Purified rat brain microvessel homogenates (0.4 mg of protein/ml) were incubated for 20 min at 37°C in the presence of 25–500 μM radiolabeled sphingomyelin, 0.1% (wt/vol) Triton X-100, 150 mM Tris-HCl, and 6 mM $MgCl_2$ at pH 7.4. On incubation, the reaction was stopped, and sphingomyelinase activity was determined as described in Experimental Procedures and expressed as nmol of sphingomyelin hydrolyzed/mg of protein/min. Inset: Replotting in the double-reciprocal Lineweaver–Burk presentation.

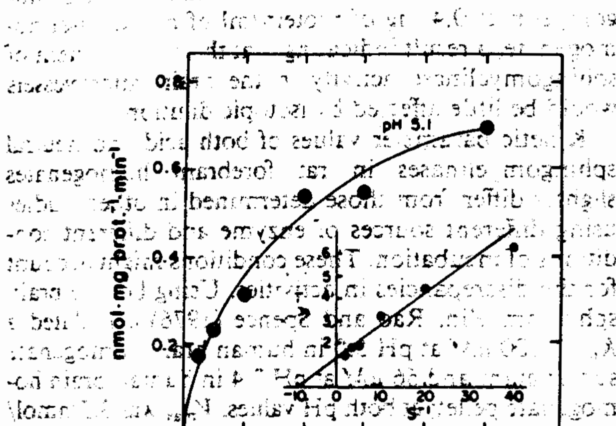

FIG. 3. Effect of radiolabeled sphingomyelin concentration on sphingomyelinase activity at pH 5.1 in purified rat brain microvessel homogenates. Purified rat brain microvessel homogenates (0.1 mg of protein/ml) were incubated for 60 min at 37°C in the presence of 25–500 μM radiolabeled sphingomyelin, 0.1% (wt/vol) Triton X-100, and 150 mM sodium acetate at pH 5.1. On incubation, the reaction was stopped, and sphingomyelinase activity was determined as described in Experimental Procedures and expressed as nmol of sphingomyelin hydrolyzed/mg of protein/min. Inset: Replotting in the double-reciprocal Lineweaver–Burk presentation.

several similarities to the two sphingomyelinases described previously in tissues of neural origin. At pH 7.4, sphingomyelinase was stimulated by magnesium and strongly inhibited by EDTA, observations indicating that endogenous magnesium might be responsible for the remaining activity observed in the absence of this particular cation. Manganese was found to be a potent enhancer of the neutral sphingomyelinase, as was already shown in the adrenal medulla and the brain (Rao and Spence, 1976; Bartolf and Franson, 1986). The inhibitory effect of calcium (Table 1) suggests that it would bind to the divalent cation site of the enzyme without providing the stimulatory effect. Exogenous magnesium or manganese slightly stimulated the acidic sphingomyelinase activity, whereas EDTA had only a small effect. This suggests that the magnesium (manganese)-dependent sphingomyelinase retains some activity at acidic pH.

At neutral pH and under the conditions described herein, no linearity was obtained when increasing the protein concentration from 0.02 to 0.04 mg/ml and using either crude rat brain homogenates (data not shown) or purified rat brain microvessels as the source of enzyme (Fig. 1). Similar data have been reported by Gatt et al. (1978) using human brain neutral sphingomyelinase. In contrast, Rao and Spence (1976) determined that linearity with concentrations of crude human brain homogenate protein could be obtained up to 0.6 mg/ml with pH 7.4 sphingomyelinase. The same observation was made by Bartolf and Franson (1986) using adrenal medulla homogenate up to 0.16 mg of protein/ml. These conflicting observations, including ours, form the base of a poorly understood

issue. Using the work of Matheson et al. (1981), we calculated that endogenous sphingomyelin would represent no more than 15% of added radioactive sphingomyelin at 0.4 mg of protein/ml of microvessel homogenate, a result indicating that the measurement of sphingomyelinase activity in the brain microvessels would be little affected by isotopic dilution.

Kinetic parameter values of both acid and neutral sphingomyelinases in rat forebrain homogenates slightly differ from those determined in other studies using different sources of enzyme and different conditions of incubation. These conditions might account for the discrepancies in activities. Using bovine brain sphingomyelin, Rao and Spence (1976) calculated a K_m of 100 μM at pH 5.0 in human brain homogenate supernatant and 66 μM at pH 7.4 in human brain homogenate pellet; at both pH values, V_{max} was 3.3 nmol/mg of protein/min. In most studies, the initial velocity of sphingomyelinase activity at pH 5.0 and 7.4 ranged between 0.3 and 5.5 nmol/mg of protein/min, whereas the initial velocity of the neutral activity is higher than at acidic pH. This was the case with purified rat brain microvessels when incubation was performed with >0.05 mg of protein/ml. The V_{max} of the neutral enzyme in the microvessels is similar to that measured in the forebrain. Because the latter is an average of the high activity in the gray matter (Spence et al., 1978) and the low activity in the white matter, the microvessels exhibit an activity that is intermediate between those two structures and significant when compared with many other tissues. Indeed, the activity of the neutral sphingomyelinase in purified rat brain microvessels does not reflect exactly its activity in situ and in the absence of detergent. The presence of neutral sphingomyelinase in brain microvessels might be linked to transport processes existing at the level of this substructure of the brain. Bartolf and Franson (1986) suggested that it would occur through small changes in sphingomyelin content and phospholipid proportions altering membrane structure. The presence of the acidic activity was not surprising, because it has been already detected in almost every mammalian tissue. The neutral enzyme seems to have a more restricted distribution, and hence its presence in endothelial cells might eventually provide clues to its role in mammalian tissues.

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