Biochimica et Biophysica Acta, 777 (1984) 339–342
Elsevier

BBA Report

BBA 70185

EVIDENCE THAT NEUTRAL SPINGOMYELINASE OF CULTURED MURINE NEUROBLASTOMA CELLS IS ORIENTED EXTERNALLY ON THE PLASMA MEMBRANE

DEVAKI V. MOHAN DAS, HAROLD W. COOK and MATTHEW W. SPENCE *

Atlantic Research Centre for Mental Retardation, and Departments of Pediatrics and Biochemistry, Dalhousie University, Halifax, Nova Scotia B3H 4H7 (Canada)

(Received May 11th, 1984)

Key words: Sphingomyelinase; Enzyme orientation; Plasma membrane; (Neuroblastoma cell)

The activity of the neutral, Mg^{2+}-stimulated sphingomyelinase of cultured neuroblastoma cells (N1E-115) is enriched in the plasma membrane fraction and is reduced following treatment of intact or broken cells with trypsin, α-chymotrypsin, papain, and protease. Two protease-sensitive enzymes of the cell interior (lactate dehydrogenase and NADPH-cytochrome *c* reductase) are not affected by protease treatment of intact cells. These results indicate that the neutral, Mg^{2+}-stimulated sphingomyelinase is oriented externally on the plasma membrane of the cultured neuroblastoma cell.

Neutral, magnesium-stimulated sphingomyelinase (sphingomyelin phosphodiesterase, EC 3.1.4.12), a membrane-bound enzyme, is associated with neuron-rich areas of brain and is present in neuroblastoma cells and in reaggregating neural cells in culture [1–5]. Recently, we showed that this enzyme is localized predominantly in the plasma membrane fraction of cultured neuroblastoma cells [6]. Most of the enzymes concerned with lipid synthesis are membrane-bound, and several have been shown to be externally-oriented on the surface of microsomal vesicles [7]. 5′-Nucleotidase, $(Na^+ + K^+)$-ATPase, and several receptor proteins are localized on the external surface of the plasma membrane [8,9]. These studies of surface orientation or 'sidedness' have generally used selective inactivation of the enzyme or receptor by an impermeant probe such as a proteolytic enzyme to determine orientation. The topography of membrane-bound enzymes can provide valuable clues to their normal physiological function in the cell. The objective of these experiments was to determine the topography or orientation of neutral, Mg^{2+}-stimulated sphingomyelinase in the plasma membrane of cultured neuroblastoma cells. These cells were chosen in preference to brain because we had already established a predominantly plasma membrane localization of enzyme activity, and because they can be readily obtained intact from the culture vessels.

N1E-115 neuroblastoma cell lines were maintained as described earlier [10] and cells grown for 6 to 8 days from the last subculture were used in all experiments. Cells were harvested by scraping, washed and suspended in Tris-sucrose buffer (50 mM Tris-HCl (pH 7.4), containing 0.25 M sucrose, called buffer A). Aliquots containing approx. $2 \cdot 10^6$ cells and the required amount of proteolytic enzyme suspended in a 1 ml final volume of buffer A were incubated at 25 °C for 15 min in a shaking water bath. Following incubation, 10 ml of cold

* To whom correspondence should be addressed.

(4°C) buffer A was added, and the tubes were centrifuged at 1000 × *g* for 10 min to recover the treated cells. The cells were washed once and suspended in 1.0 ml of buffer A. Cell viability was checked by dye exclusion and was > 90%. The cells were disrupted in a sonic dismembrator (Fisher Scientific Co.) before enzyme determinations were made. In experiments where cell homogenates were treated with proteolytic enzymes, the reaction was stopped by cooling (0–4°C) and the enzyme activities were assayed as quickly as possible.

The following enzyme activities were measured: *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.30) [11], 5′-nucleotidase (EC 3.1.3.5) [12], alkaline phosphatase (EC 3.1.3.1) [13], NADPH-cytochrome *c* reductase (EC 1.6.2.4) [14], lactate dehydrogenase (EC 1.1.1.27) [15] and acid and neutral sphingomyelinase (EC 3.1.4.12) [1]. All enzyme activities were determined at protein concentrations at which the activities were linear and values represent the averages of activities obtained from two enzyme concentrations. Activity was linear with time over the period of assay for both control and treated cells. Protein was determined by the method of Lowry et al. [16] using bovine serum albumin as standard.

[*methyl*-^{3}H]Sphingomyelin was synthesized from bovine brain sphingomyelin [17]; [^{3}H(G)]adenosine 5′-monophosphate was from New England Nuclear (Canada) Limited and other substrates for enzyme analyses as well as trypsin, α-chymotrypsin (both from bovine pancreas), papain, and protease (from *Streptomyces griseus*) were from Sigma Chemical Co., St. Louis, MO. Dulbecco's minimal essential medium and fetal and newborn calf sera were from Grand Island Biological (Canada) Ltd., Toronto, ON.

Of the seven enzymes endogenous to the neuroblastoma cell that we examined, the neutral sphingomyelinase is the most sensitive to proteolytic inactivation (Table I). Further, it was sensitive to nonspecific proteases (protease), thiol proteases (papain) and serine proteases (trypsin and α-chymotrypsin). The loss of sphingomyelinase activity is due to proteolytic degradation and not to a non-specific effect of the enzyme protein or other components in the commercial preparation as heat-inactivated proteolytic enzyme or trypsin inactivated by soya bean trypsin inhibitor had little effect on sphingomyelinase activity. The other enzymes (5′-nucleotidase and alkaline phosphatase) that are also enriched in the plasma membrane were unaffected. At least for 5′-nucleoti-

TABLE I

THE EFFECT OF INCUBATION OF NEUROBLASTOMA CELL HOMOGENATES WITH EXOGENOUS PROTEOLYTIC ENZYMES ON THE ACTIVITY OF SOME ENDOGENOUS ENZYMES

Endogenous enzyme	Exogenous proteolytic enzyme			
	Trypsin [a]	α-Chymotrypsin	Papain	Protease
Alkaline phosphatase	95 ± 6 [b]	88 ± 5	89 ± 5	92 ± 9
5′-Nucleotidase	91 ± 8	91 ± 6	88 ± 5	96 ± 7
Neutral sphingomyelinase	6 ± 1	5 ± 1	32 ± 7	5 ± 2
Acid sphingomyelinase	68 ± 14	68 ± 19	97 ± 6	74 ± 17
N-Acetyl-β-D-hexosaminidase	84 ± 6	105 ± 4	97 ± 2	62 ± 20
NADPH-cytochrome *c* reductase	109 ± 12	48 ± 37	113 ± 20	16 ± 4
Lactate dehydrogenase	98 ± 3	107	105 ± 1	14 ± 10

[a] The concentration of exogenous proteolytic enzyme was 1 mg/ml except for protease (0.25 mg/ml).

[b] Values are expressed as percentages of control activities measured in homogenates that were not incubated with proteolytic enzymes. These control activities were: alkaline phosphatase, 135 ± 27 nmol/min per mg protein; 5′-nucleotidase, 4.5 ± 2 nmol/min per mg protein; neutral sphingomyelinase, 0.92 ± 0.05 nmol/min per mg; acid sphingomyelinase, 0.27 ± 0.11 nmol/min per mg; *N*-acetyl-β-D-hexosaminidase, 10 ± 5 nmol/min per mg; NADPH-cytochrome *c* reductase, 7.5 ± 7 units/min per mg; and lactate dehydrogenase, 2.24 ± 0.5 units/min per mg. ($n = 3$).

dase, this is probably due to the lack of sensitive linkages important for catalytic activity rather than simple limitation on accessibility in the membrane. Both 5′-nucleotidase and the neutral sphingomyelinase can be partially solubilized (50% and 35%, respectively) by treatment of the cells with 0.1% Triton X-100. Protease remains active on the Triton-solubilized proteins as judged by the loss of sphingomyelinase activity during protease digestion. However, the 5′-nucleotidase activity was unaffected.

The enzymes generally associated with the cell interior were variably affected by the proteolytic enzymes when the latter were incubated with the disrupted cells. Chymotrypsin affected acid sphingomyelinase and NADPH-cytochrome *c* reductase and trypsin affected hexosaminidase. The differences in sensitivity to proteolytic attack of the acid and neutral sphingomyelinases provides evidence additional to that in our previous studies [1] of the separate nature of these two activities. Protease was the most effective proteolytic enzyme among those studied, and as it markedly reduced the activity of neutral sphingomyelinase as well as the two internally situated enzymes (lactate dehydrogenase and NADPH-cytochrome *c* reductase), further experiments were done using this enzyme. These observations fit with the ability of protease to hydrolyse a variety of bonds in proteins [18].

Our studies (Fig. 1) of enzyme activities in cultured neuroblastoma cells following treatment of intact or disrupted cells with varying concentrations of protease (up to 0.5 mg/ml) indicate that most of the neutral, Mg^{2+}-stimulated sphingomyelinase is oriented externally on the surface of the plasma membrane. First, 85% of the sphingomyelinase activity in intact cells, and 95% in broken cells, was lost during proteolytic treatment. The difference between the two values is probably due to the activity associated with the microsomal fraction previously noted by us in these cells [6] which would not be affected in the intact cell. Second, there is no loss of activity from other protease-sensitive enzyme markers of intracellular components such as lactate dehydrogenase (cytosol) and NADPH-cytochrome *c* reductase (microsomes) in the intact cells. In broken cells, both of these endogenous enzyme activities are sensitive to proteolytic attack.

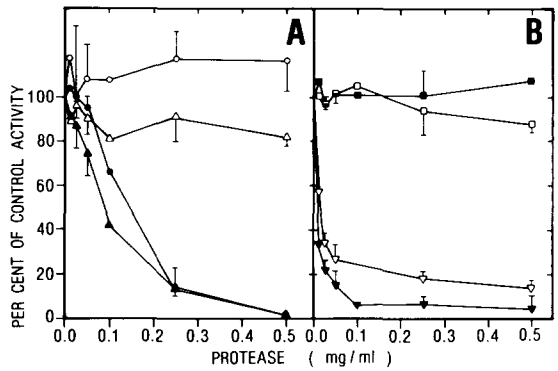

Fig. 1. The effect of varying protease concentrations on the activities of four endogenous enzymes in intact neuroblastoma cells (open symbols) and cell homogenates (closed symbols). Neuroblastoma cells or their homogenates containing about 1.2 mg protein were incubated with protease (0 to 500 μg) in a 1.0 ml final volume for 15 min at 25 °C. The results are expressed as percentages of the activities of untreated controls, and are mean ± S.D. of three or mean of two independent experiments. (A) Lactate dehydrogenase (○,●; control activity 2.36 ± 0.4 units/mg per min). NADPH-cytochrome *c* reductase (△, ▲; control activity 13.1 ± 2.85 units/mg per min). (B) Neutral sphingomyelinase (▽,▼; control activity 0.91 ± 0.25 nmol/min per mg). 5′-Nucleotidase (□,■; control activity 4.1 ± 1.04 nmol/min per mg).

We have previously shown that the neutral sphingomyelinase of human brain is firmly membrane-bound and that detergents are required to extract the activity [1,4]. The remarkable sensitivity of the neutral sphingomyelinase in neuroblastoma cells to proteolytic attack could be explained if this enzyme, in contrast to that in brain, were only loosely bound to membranes. However, treatment of cell lysates with low (50 mM) or high (1 M) salt buffers extracted less than 10% of activity, suggesting that the neuroblastoma enzyme is also tightly membrane-bound.

We have suggested that the neutral, Mg^{2+}-stimulated sphingomyelinase plays a specific role in the special physiological functions of brain based on its almost exclusive localization in brain, enrichment in grey matter and in certain grey matter areas in particular, the increase in activity in parallel with neuronal maturation, and the enrichment in the plasma membrane of the cultured neuroblastoma cell [4,6]. The present study indicates that the plasma membrane activity is oriented externally. It is of interest that

sphingomyelin is also oriented externally [19]. Whether the normal reaction catalysed by the neutral sphingomyelinase is within the same membrane or between cells is not known. However, the external orientation on a surface membrane that is the first interface in signalling between cells, in a tissue whose major role is intercellular signalling, seems unlikely to be fortuitous and further supports a role for this enzyme in the specialized physiological functions of the neuron.

The expert technical assistance of Robert Zwicker in culturing of cells is gratefully acknowledged. This work was supported by Grant PG-16 from the Medical Research Council of Canada. M.W.S. is supported by a Medical Research Council of Canada Career Investigator award.

References

1 Rao, B.G. and Spence, M.W. (1976) J. Lipid Res. 17, 506–515
2 Spence, M.W., Burgess, J.K. and Sperker, E.R. (1979) Brain Res. 168, 543–551
3 Spence, M.W. and Burgess J.K. (1978) J. Neurochem. 30, 917–919
4 Spence, M.W., Burgess, J.K., Sperker, E.R., Hamed, L. and Murphy, M.G. (1981) in Lysosomes and Lysosomal Storage Diseases, (Callahan, J.W. and Lowden, J.A., eds.), pp. 219–228, Raven Press, New York
5 Wenger, D.A., Wharton, C. and Seeds, N.W. (1979) Life Sci. 24, 679–684
6 Spence, M.W., Wakkary, J., Clarke, J.T.R. and Cook, H.W. (1982) Biochim. Biophys. Acta 719, 162–164
7 Bell, R.M., Ballas, L.M. and Coleman, R.A. (1981) J. Lipid Res. 22, 391–403
8 Salem, N., Jr., Lauter, C.J. and Trams, E.G. (1981) Biochim. Biophys. Acta 641, 366–376
9 Gorden, P., Carpentier, J.L., Fan, J.Y. and Orci, L. (1982) Metabolism 31, 664–669
10 Cook, H.W., Clarke, J.T.R. and Spence, M.W. (1983) J. Biol. Chem. 258, 7586–7591
11 Okada, S. and O'Brien, J.S. (1969) Science 165, 698–700
12 Avruch, J. and Wallach, D.F.H. (1971) Biochim. Biophys. Acta 233, 334–347
13 Ray, T.K. (1970) Biochim. Biophys. Acta 196, 1–9
14 Sottocasa, G.L., Kuylenstiernaa, B., Ernster, I. and Bergstand, A. (1967) J. Cell. Biol. 32, 415–438
15 Wacker, W.E.C., Ulmer, D.D. and Vallee, B.L. (1956) N. Engl. J. Med. 255, 449–456
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
17 Spence, M.W., Clarke, J.T.R. and Cook, H.W. (1983) J. Biol. Chem. 258, 8595–8600
18 Narahashi, Y. (1970) in Methods in Enzymology, Vol. 19 (Perlmann, G.E. and Lorand, L., eds.), pp. 651–664, Academic Press, New York
19 Den Kamp, J.A.F.O. (1979) Annu. Rev. Biochem. 48, 47–71