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Aliphatic aldehydes promote myelin basic protein-induced fusion of phospholipid vesicles

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Myelin basic protein induces slow and limited fusion of phospholipid vesicles composed of a mixture of phosphatidylcholine and phosphatidylethanolamine. Addition of palmitoyl aldehyde to these vesicles dramatically increases their ability to fuse in the presence of myelin basic protein. Compared to aliphatic aldehydes, fatty acids are much less potent promoters of myelin basic protein-induced membrane fusion. The ability of aliphatic aldehydes to promote myelin basic protein-induced membrane fusion may be of relevance to myelin structure and function and, particularly, to the pathology of demyelinating diseases such as multiple sclerosis.

Interaction of myelin basic protein with phospholipids is thought to play a crucial role in the formation and maintenance of the multilamellar structure of the myelin membrane [1–5]. It is well established that the basic protein associates strongly with acidic phospholipids, leading to changes in the molecular organization of the lipid bilayer (Ref. 1 and references therein). More recently studies indicate, however, that myelin basic protein is also able to interact with more abundant zwitterionic phospholipids, such as phosphatidylcholine [5–7] and sphingomyelin [8].

One of the ways in which the interaction of myelin basic protein with phosphatidylcholine manifests itself is in the protein-induced aggregation of vesicles [5–7]. In a previous study, we have shown that this aggregation is dramatically increased by the presence of long-chain aliphatic aldehydes in the bilayer [7]. The role of aliphatic aldehydes in promoting myelin basic protein-induced vesicle aggregation is particularly evident when, in addition to phosphatidylcholine, phosphatidylethanolamine is present as a second phospholipid constituent of the vesicles [7]. The ability of aliphatic aldehydes to enhance myelin basic protein-induced membrane-membrane interactions may be of considerable biological importance. Aliphatic aldehydes are formed from the breakdown of plasmalogens, a phospholipid which accounts for more than 30% of total myelin membrane phospholipid [9,10]. Moreover, the enzyme catalyzing the production of aliphatic aldehydes from plasmalogens is elevated in plaque tissue [11,12]. Thus, the expected increased concentration of aliphatic aldehydes in myelin plaques may be of particular importance to the pathology of demyelinating diseases such as multiple sclerosis. Our previous evidence of myelin basic protein-in-

Abbreviations; PC, egg yolk phosphatidylcholine; PE, egg yolk phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; N-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

duced aggregation of phospholipid vesicle containing aliphatic aldehydes was based on turbidimetric measurements [7]. This method, although useful for indicating increased membrane-membrane interactions, does not in general provide sufficient details about this interaction and, particularly, about the morphology of the final product. More important, it actually does not distinguish reversible aggregation from irreversible fusion involving lipid mixing and formation of larger liposomes. Therefore, further studies employing electron microscopy and fluorescence spectroscopy have been undertaken to elucidate the nature of myelin basic protein-induced membrane-membrane interactions in phospholipid vesicles containing aliphatic aldehydes.

Fig. 1 shows a typical freeze-fracture electron micrograph of sonicated vesicles composed of PC/DOPE/palmitoyl aldehyde (molar ratio, 1 : 1 : 0.1) after incubation with myelin basic protein. In addition to small vesicles, a number of larger liposomes with a diameter up to approx. 700 nm can be seen. No such larger structures were seen in electron micrographs of the same vesicles incubated without myelin basic protein. These latter micrographs revealed relatively homogeneous population of small unilamellar vesicles of the diameter in the range 20–50 nm. The dramatic increase in liposome size as a result of incubation with myelin basic protein indicates that the vesicles undergo fusion in the presence of myelin basic protein. Apparently several rounds of fusion of small vesicles must have occurred to produce such large liposomes as those shown in Fig. 1.

Myelin basic protein-induced vesicle fusion was studied in more detail using a fluorometric method based on resonance energy transfer from N-NBD-PE to N-Rh-PE (see legend to Fig. 2). This assay is based on the mixing of the lipid contents of different populations of vesicular membranes. Since we have demonstrated by freeze-fracture electron microscopy that the vesicles fuse, we can now use this assay to monitor this process in a quantitative manner. Assays based on the mixing of intravesic-

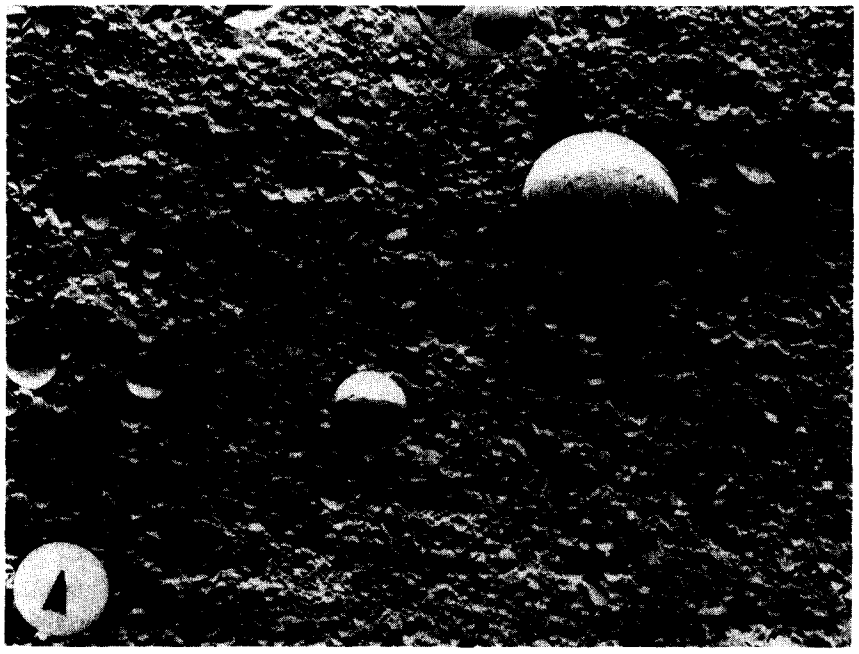

Fig. 1. Freeze-fracture electron micrographs of PC/DOPE/palmitoyl aldehyde (molar ratio 1 : 1 : 0.1) liposomes incubated with myelin basic protein. Sonicated vesicles (0.5 mg/ml) in buffer (0.1 M NaCl/0.01 M Hepes/0.002% NaN_3 (pH 7.4)) were incubated for 30 min in the presence of myelin basic protein (30 μg/ml). Vesicles were then concentrated by centrifugation in an Eppendorf centrifuge and samples for electron microscopy were prepared as described previously [13]. Magnification 30000× and the bar represents 1 μm. The arrow indicates the direction of platinum deposition.

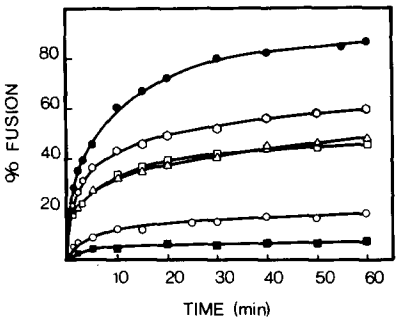

Fig. 2. Myelin basic protein-induced fusion of lipid vesicles of different composition as measured by the resonance energy transfer fusion assay. (○) PC/DOPE (1 : 1); (○) PC/DOPE/palmitoyl aldehyde (1 : 1 : 0.1); (●) PC/DOPE/palmitoyl aldehyde (1:1:0.2); (■) PC/DOPE (2:1); (□) PC/DOPE/palmitoyl aldehyde (2:1:0.3); (△) PC/PE/palmitoyl aldehyde (2:1:0.3). Vesicles were prepared by dissolving the appropriate mixture of lipids in chloroform, evaporating the solvent to dryness and resuspending the sample in buffer (0.1 M NaCl/0.01 M Hepes/0.002% NaN_3 (pH 7.4)). The suspension was subsequently sonicated under nitrogen to visual clarity in a bath-type sonicator for approx. 20 min. Fluorescence-labelled liposomes contained 1 mol% each of N-NBD-PE and N-Rh-PE and were prepared identically a the unlabelled liposomes. Membrane fusion was assayed by the loss of resonance energy transfer between two fluorophores incorporated into the same vesicle [14,15]. Upon excitation at 450 nm fluorescence spectra of vesicle preparations containing both fluorophores show emission maxima at 530 nm and at 585 nm. Essentially all of the fluorescence at 530 nm comes from N-NBD-PE, whereas the fluorescence at 585 nm arises almost exclusively from fluorescence energy transfer between the donor (N-NBD-PE) and acceptor (N-Rh-PE) pair. The ratio, R, of the fluorescence (corrected for light scattering) at 530 nm to that at 585 nm is a sensitive measure of the efficiency of the resonance energy transfer [15]. The fusion assay, based on dilution of membrane-incorporated fluorophores, was carried out as follows: 50 μl of labelled vesicles (phospholipid concentration of 1.5 mg/ml) and 50 μl of unlabelled vesicles (phospholipid concentration of 7.5 mg/ml) were mixed in a cuvette containing 2 ml of buffer and equipped with a stirring device. After measuring fluorescence at 530 and 585 nm 10 μl of 2 mg/ml solution of myelin basic protein was rapidly injected into the cuvette and the time dependence of R was measured. As the labelled liposomes fused with the unlabelled ones, dilution of fluorescent lipids occurred. This resulted in a decrease in the efficiency of energy transfer, and thus an increase in R. The final fluorophore concentration in fused vesicles was calculated using a calibration scale. For calibration purpose a series of vesicle samples were prepared containing fluorophores at different concentrations with respect to total lipid, and the dependence of R value on fluorophore concentration in the membrane was plotted. The extent of fusion was calculated as:

$$\% \text{ Fusion} = ((C_o/C)-1)100/(D-1)$$

where C_o is the initial fluorophore concentration in the membrane (before addition of myelin basic protein), C is the concentration of fluorophore in the membrane after a given time, D is the maximal possible dilution (six in our assay). All fluorescence measurements were performed at room temperature with a Perkin-Elmer MPF 44 spectrofluorimeter operating at a ratio mode. Phospholipids were purchased from Avanti Polar-Lipids, Inc. (DOPE, N-NBD-PE and N-Rh-PE) and from Sigma Chemical Co. (PC, PE and palmitic acid). Palmitoyl aldehyde was prepared by oxidation of hexadecanol (Sigma) according to the procedure of Corey and Suggs [26]. Myelin basic protein was prepared according to the method of Lowden et al. [27] and it was separated into various components as described by ion-exchange chromatography [22,28].

ular aqueous contents are generally more specific for fusion but they are applicable only to unleaky types of fusion. We have previously shown that aliphatic aldehyde-containing vesicles become leaky in the presence of myelin basic protein [7]. Thus the fusion phenomenon we demonstrate is not like exocytosis which is non-leaky. Nevertheless, it is a membrane fusion phenomenon which can drastically alter the properties of myelin.

Vesicles containing PC as the sole component did not exhibit any fusion in the presence of myelin basic protein. Addition of DOPE or PE to these vesicles results in some fusion in the presence of myelin basic protein. The extent of myelin basic protein-induced fusion of vesicles containing PE or DOPE admixed with PC increases with increasing phosphatidylethanolamine content, but even at a PC : DOPE ratio of 1 : 1 the incidence of fusion is very small (Fig. 2). The tendency of PC/DOPE and PC/PE vesicles to fuse in the presence of basic protein is consistent with the previous observation that pure phosphatidylethanolamine vesicles are readily fused by myelin basic protein [13]. The much smaller extent of fusion of mixed PC/phosphatidylethanolamine vesicles as compared to that of pure phosphatidylethanolamine vesicles most likely reflects the inhibitory effect of PC on membrane fusion [16].

Addition of palmitoyl aldehyde to PC/DOPE vesicles dramatically increases their ability to undergo fusion in the presence of myelin basic protein (Fig. 2). With PC/DOPE (1 : 1) vesicles the extent of fusion after 1 h incubation with myelin basic protein increases from 18% in the absence of aldehyde to about 60 and 87% in the presence of 5

and 10% of palmitoyl aldehyde, respectively. The fusion-promoting effect of palmitoyl aldehyde, also occurs at lower DOPE content. With vesicles containing PC/DOPE (2 : 1) and 10% palmitoyl aldehyde, the extent of fusion after 1 h incubation with myelin basic protein is 46% compared with only 7% without the aldehyde.

Some reports suggest that the role of phosphatidylethanolamine in certain types of membrane fusion may be related to the ability of this phospholipid to adopt a non-bilayer arrangement, although this has recently been questioned (Ref. 17 and references therein). We therefore examined the possible involvement of the hexagonal phase in the aliphatic aldehyde-promoted membrane fusion by using different types of phosphatidylethanolamines. Vesicles containing DOPE or PE respond almost identically to the basic protein (Fig. 2). At the temperature of present experiments, DOPE would be in the hexagonal phase whereas PE would be in the liquid-crystalline lamellar phase [18]. Thus, it is unlikely that the presence of phospholipids in the hexagonal phase is necessary for myelin basic protein-induced membrane fusion promoted by aliphatic aldehydes, since the DOPE-containing vesicles would be more likely to form such a phase. Recent studies indicate that Ca^{2+}-induced membrane fusion is also insensitive to the bilayer-hexagonal transition of phosphatidylethanolamine [19]. The role of this phospholipid in promoting membrane fusion is likely due to the poor hydration of its polar head group [17,19].

Myelin basic protein is known to undergo a number of post-translational chemical modifications which include phosphorylation, deamidation, methylation of arginine residues and C-terminal degradation [20,21]. Some of these modifications lead to alterations in the isoelectric point of the molecule, allowing for the separation of species of different charges. This microheterogeneity of myelin basic protein may be of functional significance. Particularly, it may affect the mode of protein interaction with phospholipids. Therefore, it was of interest to known whether different components of myelin basic protein differ in their ability to induce fusion of phospholipid vesicles in the presence of aliphatic aldehydes. There is essentially no difference between components I–IV in their ability to fuse vesicles composed of PC/DOPE/palmitoyl aldehyde (2 : 1 : 0.3) (Table I). Component VIII, on the other hand, appears to be a much less effective fusogen. This latter component is much less basic than the other components, and does not bind as readily to cation-exchange columns although it has an amino acid composition similar to that of the other components [22]. These results indicate that there is some degree of specificity in the observed fusion process. Replacement of aliphatic aldehyde with fatty acid in our system leads to a 50% reduction in the extent of fusion (Table I). This occurs despite the fact that the fatty acid-containing vesicles are negatively charged and might be expected to interact more strongly with the cationic myelin basic protein. This again emphasizes that a degree of specificity is required to obtain efficient fusion.

Previously myelin basic protein has been shown to fuse vesicles containing acidic phospholipids if the bilayer was additionally destabilized by the presence of lysophosphatidylcholine [23]. Our present study shows that membrane fusion induced

TABLE I

% FUSION AFTER 60 min INCUBATION IN THE PRESENCE OF MYELIN BASIC PROTEIN (MBP) (10 μg/ml)

Lipid composition	Fusion (%) in the presence of MBP component					
	Σ [b]	I	II	III	IV	VIII
PC/DOPE/palmitoyl aldehyde (2 : 1 : 0.3)	46 ± 5	48 ± 3	46 ± 3	45 ± 4	42 ± 3	24 ± 1
PC/DOPE/palmitic acid (2 : 1 : 0.3)	22 ± 3	[a]	[a]	[a]	[a]	[a]

[a] Not determined.

[b] Mixture of all components (unfractionated protein).

by myelin basic protein may be also strongly promoted by aliphatic aldehydes and, importantly, this fusion does not require the presence of acidic phospholipids in the bilayer. Aldehydes are known to react with proteins and it is possible that this reaction is involved in the mechanism by which aldehydes promote myelin basic protein-induced membrane fusion. It has been shown that small amounts of aldehyde-reacted proteins are present in human myelin [24]. If aldehyde-promoted fusion phenomenon were to occur in vivo, it would be expected to disturb the ordered multilamellar structure of myelin and lead to the formation of pores. This would explain the apparent relationship between plasmalogenase activity and demyelination [12,25].

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