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Does sphingomyelin inhibit the erythrocyte anion transport system?

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The anion transport protein of the human erythrocyte membrane, band 3, was incorporated into unilamellar sphingomyelin vesicles. The vesicles showed a rapid sulfate efflux which could be inhibited by specific inhibitors of the erythrocyte anion transport system. All band 3 molecules contributing to the inhibitor-sensitive flux component were arranged 'right-side-out'. The turnover number of the transport protein for sulfate transport was virtually identical to that in phosphatidylcholine bilayers and around 6 times larger than in human erythrocyte membranes. Thus, in contrast to other claims, sphingomyelin does not inhibit the erythrocyte anion transport system.

Sphingomyelin; Anion transport; Band 3 protein; Reconstitution; (Erythrocyte membrane)

1. INTRODUCTION

Phosphate exchange across erythrocyte membranes of different mammalian species was found to decrease with increasing sphingomyelin/phosphatidylcholine (SPH/PC) ratio of the membrane lipid [1]. The same was observed for the exchange of chloride against bicarbonate, which is the physiological function of the erythrocyte anion transport system [2]. Recent reconstitution experiments have yielded seemingly analogous results: in lipid vesicles of varying contents of SPH and PC (and, probably, cholesterol), band 3-mediated sulfate transport decreased down to zero with increasing SPH content [3]. Different interpretations of these observations have been considered. It was discussed whether the observed effects could be due simply to the differences in the fatty acid composition of the two phospholipids involved, the apparent inhibition of anion transport by SPH being caused by the high degree of saturation of its fatty acid chains [1,2]. This interpretation was, however, ruled out by studies on rat erythrocytes in which the fatty acid composition of the membrane phospholipid had been changed, by feeding the animals with diets deficient in unsaturated fatty acids [4]. It has also been suggested that SPH could influence anion transport by indirect effects involving other membrane components [5]. However, most authors have regarded a direct inactivation of the anion transport protein, band 3, by SPH as the most plausible explanation for the apparent negative correlation between anion transport and the membrane's SPH content [1–4]. Based on the known peculiarities of the structure of SPH as compared to that of the phosphoglycerides [6,7], possible molecular mechanisms have been discussed by which SPH could effect the presumed anion transport inhibition [3,4].

Our group has developed a new method for the reconstitution of the erythrocyte anion transport system in PC bilayers. The reconstituted system shows, for the first time, all major properties of the sulfate transport across the human erythrocyte membrane [8,9]. By applying this method (in a modified form), we have now succeeded in reconstituting band 3-mediated sulfate transport

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Abbreviations: SPH, sphingomyelin; PC, phosphatidylcholine; H_2DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate

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across lipid bilayer membranes with SPH as the only lipid constituent. We will show in this paper that there is no inhibitory influence of this phospholipid on the anion transport system.

2. MATERIALS AND METHODS

2.1. *Materials*

SPH from bovine spinal cord ('grade I', purity >99%) was purchased from Lipid Products (South Nutfield, England). Trypsin was obtained from Boehringer (Mannheim, FRG) and soybean trypsin inhibitor from Serva (Heidelberg, FRG). For the sources of all other materials used see [8].

2.2. *Reconstitution procedure*

The reconstitution protocol followed [8], with several modifications. 400 μg of ^{3}H-labelled band 3 protein, in a buffer containing 0.4% (w/w) Triton X-100 [8], were diluted to yield a protein concentration of 40 μg/ml in 10 mM Tris-HCl (pH 8.0), 25 mM NaCl, 0.1% Triton X-100. The solution was added to 20 mg SPH which had been dried from chloroform in a rotary evaporator, and gently shaken for 30–45 min in the presence of several glass beads. Then, 0.3 ml of 10% (w/w) octylglucopyranoside were added, followed by 1 g sucrose [9], and the sample was dialyzed for 64 h against two changes of 3 l of 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 0.5 mM EDTA, 15 mM β-mercaptoethanol, 10% (w/v) sucrose. For all subsequent steps see [8].

2.3. *Trypsin treatment and gel filtration*

In most experiments, the proteoliposomes isolated according to section 2.2 and [8] were incubated, after concentration by high speed centrifugation [8], with 1 mg trypsin per ml for 45 min at 30°C. After addition of 1 mg trypsin inhibitor per ml and incubation for another 15 min, the vesicles were again pelleted and resuspended [8]. A vesicle subpopulation of more narrow size distribution was then isolated by gel filtration on a 60 × 0.8 cm column of Sephacryl S-1000 (Pharmacia, Freiburg, FRG) which had been presaturated with SPH [9,10].

2.4. *Other methods*

Electron micrographs were obtained with a Philips 300 electron microscope as described in [8,11].

For the measurement and evaluation of sulfate efflux from the vesicles see [8]. Buffer during the flux measurements was 10 mM Hepes (pH 7.2), 10 mM Na_2SO_4, 0.5 mM EDTA.

3. RESULTS

3.1. *General structural and functional properties of the band 3/SPH recombinates*

The bilayer-micelle transition in egg PC/Triton X-100 mixtures occurs at a molar detergent/lipid ratio $R = 1.5$, whereas it occurs already at $R = 0.3$ when the lipid component is SPH [12]. As a consequence, our procedure for the reconstitution of the erythrocyte anion transport system from PC/Triton micelles [8] had to be modified in order to allow application to SPH.

The band 3/SPH recombinates obtained according to section 2.2 and [8] contained 30–50% of the protein and the lipid initially present. In electron micrographs of negatively stained samples, most of the recombined material had the form of unilamellar vesicles. Besides vesicles, some disk-shaped aggregates, as described by others [13], were also visible (fig.1).

Sulfate efflux under exchange conditions [8], for protein-containing and protein-free vesicles, is shown in fig.2. Efflux from the protein-free vesicles was slow on the time-scale of fig.2, whereas it was rapid from vesicles containing band 3 protein. The rapid protein-induced efflux could be strongly inhibited by two inhibitors of the erythrocyte anion transport system, H_2DIDS and flufenamate. This means that the transport system assembled in the liposomes represents a reconstitution of the native system. Importantly, inhibition by the combined action of H_2DIDS (which does not penetrate lipid bilayers) and flufenamate (which rapidly penetrates bilayer membranes) did not exceed that achieved by H_2DIDS alone (fig.2). This demonstrates that the orientation of virtually all functionally active band 3 molecules in the SPH vesicles is 'right-side-out', as in band 3/PC vesicles [8,9].

The temperature dependence of sulfate transport in the vesicles could be described by an activa-

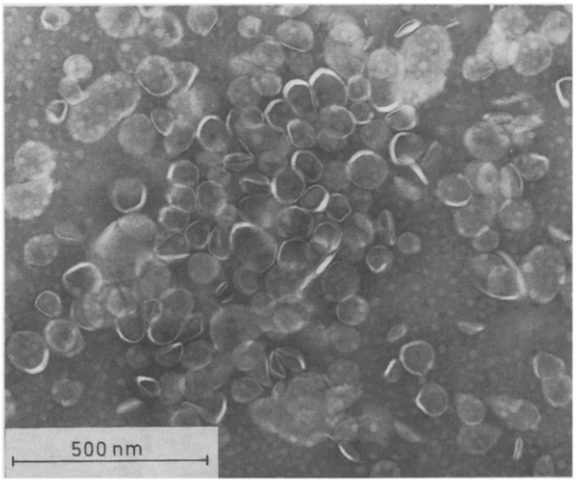

Fig.1. Electron micrograph of a negatively stained band 3/SPH recombinate.

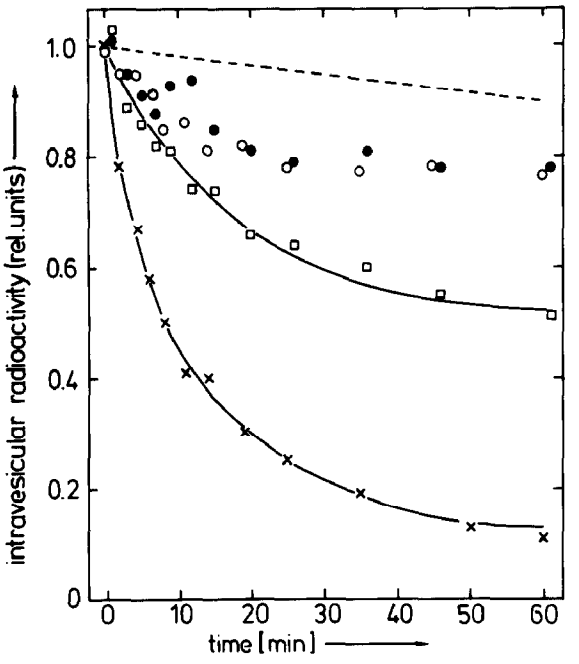

Fig.2. Sulfate efflux under exchange conditions from protein-free SPH vesicles (– – –), and from band 3/SPH vesicles either without added inhibitor (×), or after addition of 15 μM H_2DIDS (○), 5 μM flufenamate (□), and 15 μM H_2DIDS plus 5 μM flufenamate (●). T = 30°.

tion energy of 140 kJ/mol, as in erythrocytes [14] and band 3/PC vesicles [8]. The influence of other external parameters on the transport system has not been studied.

3.2. *Vesicle-associated non-functional band 3 protein*

The band 3/SPH vesicles contained, besides the functionally active band 3 protein arranged in right-side-out orientation, another band 3 population. Its presence could be demonstrated by treatment of the vesicles with trypsin: whereas addition of trypsin to intact erythrocytes or right-side-out vesicles derived from them does not affect the band 3 protein [14], it removed ~90% of the ^{3}H-label from the SPH vesicles. The occurrence of this band 3 population is probably due to the low Triton concentration at which, in the mixed SPH/Triton system, the bilayer-micelle transition occurs [12]: most of the protein seems to aggregate irreversibly at the reduced detergent concentration and afterwards to bind to the outer vesicle surface. The failure of other authors to reconstitute band 3 in SPH vesicles [3] may be due to similar effects.

There was no loss of ^{3}H-label when the band 3/PC vesicles described in [8,9,11] were treated with trypsin.

3.3. *The turnover number for sulfate transport in band 3/SPH vesicles*

The average 'turnover number' τ for band 3 protein, in those SPH vesicles which contribute to the protein-mediated sulfate transport, was determined from the rate constant k_1 of the H_2DIDS-sensitive component of the sulfate efflux, the sulfate concentration c, the specific internal volume v_i of the vesicles (in l per mol of phospholipid), and the molar protein/lipid ratio α in their membranes applying equation (1):

$$\tau = \frac{k_1 \cdot c \cdot v_i}{\alpha} \qquad (1)$$

[3,8,11]. In order to ensure the applicability of equation (1), the following procedures and precautions were used:

(a) The band 3 molecules adsorbed to the outer vesicle surface were degraded by trypsin, and the residual ^{3}H-label in the vesicles was assumed to indicate the content of functionally active band 3 molecules. (This procedure may overestimate α, since some of the ^{3}H-labelled peptides may not be released from the membranes.)

(b) All measurements were performed on a vesicle subpopulation of a narrow size distribution, as isolated from trypsin-treated vesicles by gel filtration [9,11]. Neither the trypsin treatment nor the gel filtration led to significant changes in the efflux curves or in their sensitivity towards inhibitors, as compared to fig.2 (not shown).

(c) v_i was calculated from the average vesicle diameter D, as determined from electron micrographs of freeze-dried and Pt-C shadowed samples (fig.3). D was found to be between 100 and 130 nm, depending on the preparation. Using the average value, D = 115 nm, and the data in [15], and considering that the area per lipid molecule in bilayer membranes is by ~25% smaller for SPH than for PC [7], we obtained v_i = 2.65 l per mol of phospholipid.

(d) In our preparations of both band 3-containing and protein-free SPH vesicles, ~85% of the vesicles were so leaky for sulfate that they

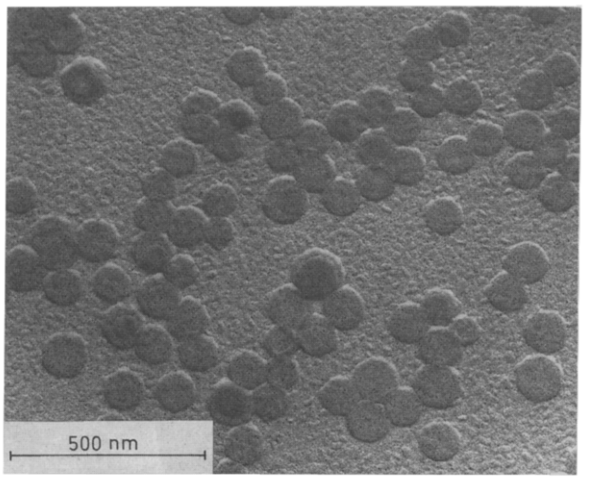

Fig.3. Electron micrograph of a sample from the pooled peak fractions from a Sephacryl S-1000 column [9] after freeze-drying and Pt-C shadowing [11].

did not contribute to the observed fluxes (subpopulation 4 of [8]). This was apparent from the measured trapped volume for the anion [8] of only 0.2–0.6 l per mol of phospholipid. We have separated sealed and leaky band 3-containing vesicles according to [11] and have found that both had virtually the same (average) molar protein/lipid ratio α. Thus, the value of α for the sealed vesicles, which needs to be inserted into equation (1), is virtually identical to that obtained with the unfractionated samples. Possible variations of α within the population of the sealed vesicles (probably represented by a Poisson distribution [16]) were neglected in our evaluations.

The values of k_1, α and τ at 37°C obtained in this way, for 4 different preparations, are shown in table 1. The individual turnover numbers τ ranged from 460 to 670 min^{-1}, with a mean of 520 min^{-1}. We estimate that the uncertainty of the latter value does not exceed ± 50%, at least with respect to its lower limit. Under the same experimental conditions, the turnover number of band 3 for sulfate transport in the human erythrocyte membrane is 85 min^{-1} (as calculated from the data in [17,18]). The corresponding figure for band 3 protein reconstituted in PC vesicles is 430–1000 $\cdot min^{-1}$, with a mean of 590 $\cdot min^{-1}$ (n = 5) [11].

Table 1

Values of k_1, α and τ obtained with 4 different vesicle preparations

Prep. no.	k_1 (min^{-1})	α	τ (min^{-1})
1	0.490	2.83×10^{-5}	459
2	0.550	3.15×10^{-5}	464
3	0.677	2.69×10^{-5}	668
4	0.581	3.11×10^{-5}	495

T = 37°C

4. DISCUSSION

As shown above, the turnover number of band 3 protein for sulfate transport in an SPH environment is virtually identical to that in an environment of egg PC and is ~6 times larger than the corresponding figure for band 3 protein in the human erythrocyte membrane. Thus, SPH does not inhibit the erythrocyte anion transport system, in contrast to other claims.

The parameters which lead to the apparent correlation between anion transport and the SPH content of the erythrocyte membrane have still to be identified, as well as those which lead to the increased turnover numbers in the reconstituted system as compared to the native system [11].

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