USE OF A FLUORESCENT STEROL TO PROBE THE TRANSBILAYER DISTRIBUTION OF STEROLS IN BIOLOGICAL MEMBRANES

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1. Introduction

Although considerable insight into the asymmetric distribution of proteins, carbohydrates and phospholipids in surface membranes has been obtained, little is known of the transbilayer distribution of sterol [1–3]. X-Ray analysis of myelin [4] and electron microscopic investigations of red blood cell membranes [5] provided the first results indicating a preferential distribution of cholesterol in the outer monolayer. The only report localizing cholesterol in the inner monolayer of any membrane system was in model membranes of egg lecithin, containing >30 mol% cholesterol [6]. This investigation represents the first data using a fluorescent sterol analogue to probe the asymmetric distribution of sterol in a biological membrane. With mouse and rat red blood cells, this technique confirmed the presence of more sterol in the outer monolayer. However, the data obtained with the tumorigenic LM fibroblasts were unique in that they demonstrate a preferential distribution of sterol in the inner monolayer of this plasma membrane.

2. Materials and methods

Dehydroergosterol was a generous gift of Dr Loran L. Bieber (Department of Biochemistry, Michigan State University) and Dr Edward H. Goh (Section of Pharmacology, Indiana University).

LM cells, a strain of transformed murine fibroblasts, were obtained from the American Type Culture Collection (CCL 1.2) and were grown in suspension (1×10^6 cells/ml) as in [7]. Dehydroergosterol was incorporated into LM cells as follows: Dehydroergosterol was dissolved in ethanol and added to the culture medium (0–20 μg dehydroergosterol/ml medium) such that the concentration of ethanol was $\leq$0.5% in the medium. After 72 h the cells were washed and divided into 3 aliquots. One aliquot was designated as control; one aliquot was treated under non-penetrating conditions (4°C) with 4 mM trinitrobenzenesulfonic acid (Sigma Chemical Co., St Louis MO) [8]; and the last aliquot was treated under penetrating conditions (37°C) as in [8]. Plasma membranes were then isolated as in [8]. Lipids were extracted, separated by silicic acid chromatography, and sterols were identified and quantitated by gas chromatography [7].

C_3H/MTV mice (23 g body wt) and Sprague Dawlet rats (120 g body wt) were obtained from Dr A. B. Kier and Dr Joseph Wagner (Research Animal Diagnostic Laboratory, University of Missouri School of Veterinary Medicine, Columbia MO). The animals were bled and blood was collected in citrated test tubes. The plasma was removed and the red blood cells were washed as in [9]. The dehydroergosterol was incorporated into plasma lipoproteins as in [10]. The plasma containing dehydroergosterol was incubated at 37°C for 20 h with the red blood cells as in [10]. The red blood cells were then treated with trinitrobenzenesulfonic acid as above (except that 23°C and 37°C were used), lysed, washed 3 times, and resealed as right-side out vesicles in phosphate-buffered saline [9]. Protein was determined by the method in [11]. Absorbance, absorption corrected fluorescence (corrected for instrumental variables and for inner filter effect), relative fluorescence efficiency, and corrected fluorescence emission were determined as in [12]. Excitation was 325 nm and emission was 380 nm. Light scattering was corrected by appropriate cutoff filters.

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3. Results and discussion

Dehydroergosterol is a 5,7,9 (11)-trienol with an unsaturated alkyl side chain and structurally resembles desmosterol, the sterol synthesized by LM cells [13]. The dehydroergosterol did not adversely affect the growth rate of LM fibroblasts over a wide concentration range (table 1). Dehydroergosterol was not fluorescent in aqueous solution but fluoresced when associated with membranes of LM cells (fig.1). The maximum excitation of dehydroergosterol in the plasma membranes occurred at 325 nm while the maximum emission occurred at 380 nm. The dehydroergosterol was indeed a plasma membrane component. Lipid extraction of the plasma membranes and gas chromatographic analysis indicated that the dehydroergosterol was not metabolized significantly by the LM cell over 72 h (not shown). The dehydroergosterol was rapidly taken up by the cells in a biphasic manner as a function of time (fig.2). The uptake of dehydroergosterol into both cells and plasma membranes was linear with increasing concentration of dehydroergosterol in the medium (fig.3). The dehydroergosterol rapidly replaced the endogenous sterol, desmosterol, as the primary sterol in the LM plasma membrane (fig.2). Greater than 85% of the native sterol was replaced in 72 h. This replacement had no significant effect on the sterol/phospholipid ratio, the phospholipid composition, or the activity of Na^+,K^+-ATPase and 5′-nucleotidase in the LM plasma membrane (not shown). The fluorescence emission of

Table 1
Effect of dehydroergosterol on LM cell number doubling time[a]

Dehydroergosterol (μg/ml medium)	Doubling time (days)
0	1.20 ± 0.10
1	1.33 ± 0.08
3	1.25 ± 0.12
4	1.10 ± 0.06
5	1.12 ± 0.15
10	1.11 ± 0.09
15	1.12 ± 0.07
20	1.06 ± 0.12

[a] LM cells growing in exponential phase were resuspended in fresh medium at 1 × 10^6 cells/ml. Dehydroergosterol was added as an ethanolic solution (≤0.5% of the medium). Cell number was determined for 3 days and doubling times were calculated

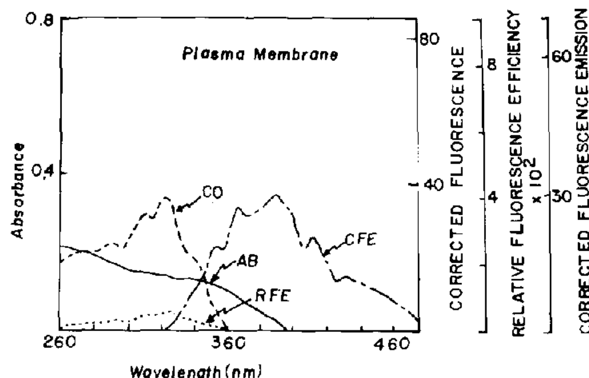

Fig.1. Spectral properties of dehydroergosterol in LM fibroblast membranes. LM cells (1 × 10^6 cells/ml) were cultured for 3 days with dehydroergosterol (20 μg/ml). Plasma membranes were isolated and absorbance (AB, — —), absorption corrected fluorescence (CO, ---), relative fluorescence efficiency (RFE, . . .) and corrected fluorescence emission (CFE, — - —) were determined as in section 2.

dehydroergosterol overlaps with the absorbance of trinitrophenyl groups [12,14]. This overlap results in efficient energy transfer and reduction in the quantum yield of fluorescence of a variety of probe molecules [12,14]. We have shown elsewhere that the trinitrophenyl group is an excellent quencher of the

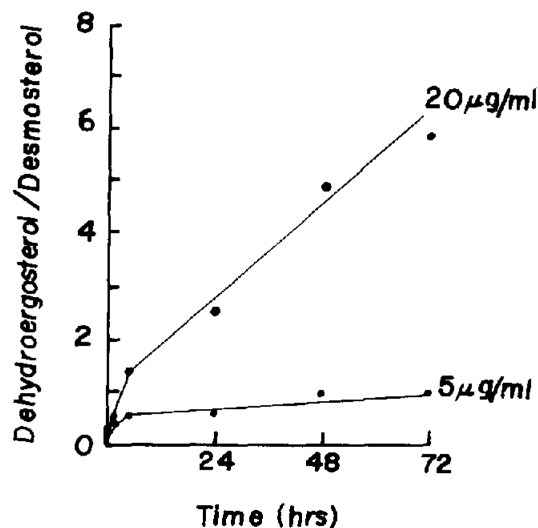

Fig.2. Replacement of LM cell desmosterol by dehydroergosterol. LM cells were cultured for varying times with dehydroergosterol (5 or 20 μg/ml medium), lipids were extracted, and sterol composition was determined as in section 2.

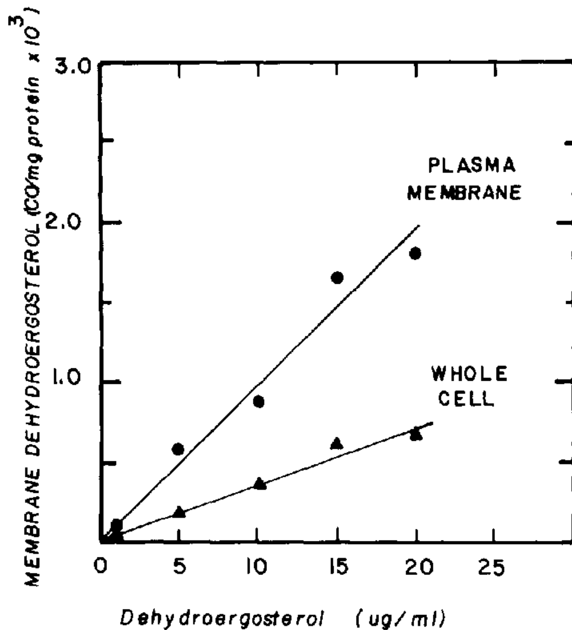

Fig.3. Effect of medium dehydroergosterol concentration on incorporation of dehydroergosterol into LM cells and LM plasma membranes. All procedures were as in fig.1 except that LM cells were incubated for 72 h with dehydroergosterol and plasma membranes were isolated as in section 2.

fluorescence of the closely related cholesta-5,7,9 (11)-trienol and can completely quench the fluorescence of this sterol in a monolayer [14]. The surface membrane of LM fibroblasts can easily be trinitrophenylated using the chemical labeling reagent trinitrobenzenesulfonic acid. Under non-penetrating conditions (4 mM trinitrobenzenesulfonic acid, 4°C, pH 8.5, 80 min) trinitrobenzenesulfonic acid reacts with free amino groups of proteins, phosphatidylethanolamine and phosphatidylserine in the outer monolayer of the LM plasma membrane [8] and completely quenches the fluorescence of a variety of probe molecules in this monolayer [12,14]. As shown in table 2, under non-penetrating conditions the trinitrophenyl groups quenched ~20% of the dehydroergosterol fluorescence in the isolated plasma membrane. In contrast, when both sides of the plasma membrane were trinitrophenylated (4 mM trinitrobenzenesulfonic acid, 37°C, pH 8.5, 80 min) the fluorescence of dehydroergosterol was quenched >95%. The fluorescence quenching by trinitrophenyl groups under either of these treatment conditions with trinitrobenzenesulfonic acid was not dependent on the mol% dehydroergosterol present over 1–85 mol%. This indicates that excess trinitrophenyl quenching groups were present. The trinitrophenyl groups were covalently linked either to only the outer membrane monolayer (treatment of LM cells with trinitroben-

Table 2
Quenching of dehydroergosterol fluorescence in surface membranes[a] (% decrease in absorption-corrected fluorescence)

Cell	Treatment temperature with trinitrobenzene-sulfonic acid (°C)	Exposure time to dehydroergosterol			
		1 h	4 h	20 h	72 h
Mouse LM fibroblast	14°C	18 ± 4	19 ± 3	–	20 ± 3
Mouse LM fibroblast	37°C	94 ± 6	96 ± 3	–	95 ± 3
Mouse red blood cell	23°C	–	–	75 ± 3	–
Mouse red blood cell	37°C	–	–	96 ± 2	–
Rat red blood cell	23°C	–	–	69 ± 4	–
Rat red blood cell	37°C	–	–	98 ± 2	–

[a] LM fibroblasts (1 × 10^6 cells/ml) were cultured for 1, 4 and 72 h with dehydroergosterol (20 μg/ml). This resulted in plasma membrane dehydroergosterol mol% of 1, 10 and 85, respectively. At each time point part of the culture was removed and divided in 3 aliquots. One part was left untreated while the other 2 aliquots were exposed to trinitrobenzenesulfonic acid (pH 8.5, 4 mM) at either 4°C or 37°C for 80 min as in section 2. Plasma membranes were then isolated from all 3 aliquots and absorption corrected fluorescence of dehydroergosterol was determined also as in section 2. The mol% dehydroergosterol in the total sterol was determined by gas chromatography. Red blood cells were obtained and processed as in section 2. Values represent the mean ± SEM (n = 3)

zenesulfonic acid at 4°C) or to both monolayers (treatment of LM cells with trinitrobenzenesulfonic acid at 37°C) of the plasma membrane [8,11]. A single fluorescence lifetime for the dehydroergosterol in untreated plasma membranes was determined with a SLM sub-nanosecond fluorimeter (SLM Instr., Champaign-Urbana IL) to be 2.25 ± 0.20 ns while in the trinitrobenzenesulfonate (4°C) treated plasma membranes, the lifetime was 2.40 ± 0.25 ns, not significantly different. If only partially quenching had occurred in the outer monolayer, the fluorescence lifetime would have decreased. Thus, the results presented here indicate that ~20% of the mouse LM plasma membrane sterol is in the outer monolayer while ~80% is in the inner monolayer. The opposite result was obtained with red blood cell membranes, as expected [1–5]. The outer monolayer of the mouse and rat red blood cell membrane was enriched with sterol. These findings would predict that the inner monolayer of the LM plasma membrane may be more rigid than the outer monolayer while the opposite should be true for the red blood cell membrane. Results consistent with both possibilities have appeared from our laboratory [12–15] and others [16]. These data indicate that the fluorescent sterol, dehydroergosterol, in combination with quenching techniques can be used to determine the distribution of sterol across a mammalian plasma membrane. The technique has significant advantages in that once the sterol is incorporated into the plasma membranes, its distribution across the surface membrane in response to experimental manipulation can be monitored in seconds. The finding that the inner monolayer of the LM cell plasma membrane is enriched in sterol may represent an observation unique to this cell line since it synthesizes desmosterol instead of cholesterol.

Acknowledgements

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