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Expression of liver fatty acid binding protein alters plasma membrane lipid composition and structure in transfected L-cell fibroblasts

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Liver fatty acid binding protein, L-FABP, is an abundant protein that binds fatty acids in vitro. The effects of L-FABP on plasma membrane lipid composition, distribution, and physical structure were determined in intact L-cell fibroblasts transfected with cDNA encoding L-FABP. L-FABP expression altered plasma membrane phospholipids by decreasing both phosphosphatidylethanolamine and esterified oleic acid content, and increasing sphingomyelin. L-FABP also binds sterols and stimulates sterol uptake and esterification. The fluorescent sterol dehydroergosterol was used to examine sterol distribution in the transfected cell plasma membrane. Dehydroergosterol codistributed equally with the cholesterol in both the bulk membrane and the individual bilayer leaflets. The sterol/phospholipid ratio was decreased in the inner leaflet due to sterol depletion. Concomitantly, intermembrane sterol transfer from the rapidly exchangeable lateral sterol domains as measured by exchange of dehydroergosterol, was reduced. The fluidity of the plasma membrane was measured with the fluorescent molecule diphenylhexatriene by multifrequency (1–250 MHz) phase and modulation fluorometry. Both the bulk plasma membrane and the plasma membrane outer leaflet lipids were fluidized in transfected cells. These alterations of plasma membrane structure and composition are consistent with a role for L-FABP in regulating intracellular sterol and fatty acid distribution and thereby membrane lipid domain structure.

Introduction

Fatty acid binding proteins (FABPs) belong to a ubiquitous family of lipid binding proteins discovered approximately thirty years ago (reviewed in Ref. 1). These proteins are found in many tissues including liver, heart, and intestine where the typical content is 2–8% depending on cell type. Although a great deal of information has been obtained in vitro regarding the function of FABPs, it is not known if these functions also occur in vivo. At least three important lipid classes serve as FABP ligands and FABPs have been shown to be involved in their metabolism in vitro. First, FABPs bind both long chain (> 16 carbons) fatty acids and acyl-CoAs with binding constants in the micromolar range [2,3]. Previous work has shown FABPs stimulate in vitro fatty acid transfer [4] and in vitro fatty acid metabolism via activation of enzymes producing triglyceride and phospholipids such as diacylglycerol acyltransferase [5] and *sn*-glycerol-3-phosphate transacylase [6]. L-FABP also binds cholesterol [7,8] and fluorescent sterols [9,10] in a 1:1 complex with $K_d = 0.3$–$1.5\ \mu M$. Sterol binding gives L-FABP the additional in vitro capabilities of sterol transfer and metabolic acti-

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Abbreviations: FABP, fatty acid binding protein; L-FABP, liver fatty acid binding protein; BSA, bovine serum albumin; TNBS, trinitrobenzylsulfonate; diphenylhexatriene, 1,6-diphenyl-1,3,5-hexatriene; dehydroergosterol, $\Delta^{5,7,9(11),22}$-ergostatetraen-3β-ol; dimethyl POPOP, 1,4-bis(2-(4-methyl-5-phenyloxazol-2-yl))benzene; PIPES, piperazine-*N*,*N*-bis[2-ethanesulfonic acid]; ACAT (EC 2.3.1.26), acyl-CoA : cholesterol acyltransferase; PE, phosphatidylethanolamine; SM, sphingomyelin.

vation [11,12]. However, it has been shown that binding of sterol in vitro is insufficient to definitively assign in vivo effects on sterol composition or metabolism. For example, ligandin [13] and elastase [14], both isolated because of lipid binding capability in vitro, are not involved in lipid metabolism in vivo.

Because of its in vitro sterol binding capabilities, L-FABP may also be involved in intracellular sterol trafficking. Intracellular sterol distribution is asymmetric between the subcellular membranes [15], the leaflets within the membranes [16–18], and even within the lateral plane of each leaflet [11]. Study of the maintenance of the unequal intracellular distribution of sterols has generally focused on two areas; vesicular transport [19], and cytosolic sterol binding proteins [1,11]. The revelation of L-FABP binding sterol adds L-FABP to the latter category [8,10,20]. Presented herein are results indicating L-FABP functions to modify L-cell plasma membrane sterol domains in intact cells.

Early attempts at identifying L-FABP function in vivo were performed by changing the nutritional conditions of the cell (reviewed in Ref. 21). However, conclusions reached are complicated by other parameters, independent of L-FABP, which may also be affected by changing nutrition. Recently, Jefferson et al. [22] transfected an L-cell line with cDNA encoding rat liver L-FABP. In these whole cell studies, the authors found increased sterol uptake and internalization, as well as increased phospholipid content. Since the transfected cells but not the non-transfected cells expressed L-FABP, the above differences were due to an increased level of this protein. The studies reported here extend the previous whole cell work by examining the lipid domain composition and individual leaflet structure of plasma membranes isolated from these transfected cells.

Materials and Methods

Materials

BSA (fatty acid free, lot 75F-0007), TNBS (lot 94F-0265), ergosterol (lot 81F-0116), cholesterol (lot 82C-0990), L-α-phosphatidylcholine (lot 112F-8425), L-α-phosphatidyl-L-serine (lot 123F-8370), L-α-phosphatidylinositol (lot 105F-8350), and *N*-stearoyl-α-sphingomyelin (lot 116F-4015) were obtained from Sigma, St. Louis, MO. Both desmosterol (lot 2976) and fatty acid methyl ester standards (mix K102) were purchased from Applied Science, Deerfield, IL. Fetal bovine serum (lot 12103221) was from Hazleton (Denver, PA) while BF_3 was from Supelco (Bellefonte, PA). The diphenylhexatriene from Eastman (Rochester, NY) was stored in tetrahydrofuran at −20°C. Dehydroergosterol was synthesized and purified as reported by Fischer et al. [20,23]. For sterol exchange assays dehydroergosterol and cholesterol were recrystallized from ethanol to remove potential oxidized sterols. The latter can dramatically accelerate sterol exchange between membranes [24]. All other chemicals were of reagent grade or better.

Cells

Mouse L-cells (L aprt$^-$tK$^-$) were a generous gift of Dr. David Chaplin, Washington University, St. Louis, MO. The cells were transfected with plasmid pLFABP-MT8 [22]. Briefly, a plasmid of the full length L-FABP gene was inserted downstream of human MT-II_A promoter segment [25] and upstream of splice and polyadenylation sequences derived from SV40. Cells were grown in Higuchi medium containing 10% fetal bovine serum [26]. Earlier work from this laboratory showed that the transfection itself did not effect lipid composition or structure [22]. These parameters were not significantly different between control nontransfected cells and transfected cells expressing low levels of L-FABP. Cells that contain 0 to 0.008% cytosolic protein as L-FABP were designated as low expression. Cells with 0.3 to 0.5% cytosolic protein as L-FABP were designated as high expression.

Incorporation of fluorescent sterols into L-cells

Using the methods of Hale and Schroeder [27], L-cells grown to exponential phase were harvested and subcultured at 1:3 dilution ($10 \cdot 10^6$ cells/ml in 75 cm^2 Corning tissue culture dishes, Corning, NY). Dehydroergosterol was prepared in stock solution of 4 mg/ml nondenatured ethanol. At the time of subculture, the cells were cultured in the presence and absence of dehydroergosterol (final concentration, 20 μg/ml). The final ethanol concentration, maintained constant at 0.05%, did not adversely affect the cells. Cells were grown for three days and plasma membranes were isolated as described below.

Plasma membrane preparation

Cell surface membranes were isolated per the procedures of Schroeder et al. [26]. Briefly, cells in exponential growth were sedimented, suspended in sucrose homogenization medium, and disrupted in a tight fitting Dounce homogenizer. The membranes were separated using differential and sucrose gradient centrifugation. A marker enzyme was used to identify and establish purity of the plasma membrane fraction. The specific activity of ouabain sensitive Na^+,K^+-ATPase was increased 6.5-fold in purified plasma membranes as compared to crude homogenate. The fold purification of plasma membranes was essentially the same for high and low expression cells. Relative subcellular contamination and fold purification utilizing this method have not differed despite significant membrane compositional differences [28,29]

Plasma membrane lipid composition

All organic solvents were glass distilled and glassware was H_2SO_4/chromate (Chromerge, Fisher, Pittsburgh, PA) washed. Sterols and phospholipids were extracted by the methods of Bligh and Dyer [28] as modified by Schroeder et al. [26]. The lipids were extracted with $CHCl_3$/MeOH, then separated by elution from a 100–200 mesh unisil column (Clarkson, Williamsport, PA) using $CHCl_3$ for neutral lipids and MeOH for phospholipids. Phospholipid content was determined using the procedures of Ames [31]. Sterol content was measured by HPLC as described by Fischer et al. [20].

Phospholipid headgroup composition was determined using 2-D thin-layer chromatography with 10 × 20 cm HPTLC plates (Silica gel 60, CAMAG Scientific, Wrightsville Beach, NC) [32]. The solvent in the first dimension consisted of $CHCl_3$/MeOH/NH_4OH (67.5:30:5, v/v) and in the second dimension was $CHCl_3$/MeOH/acetone/CH_3COOH/ammonium acetate (70:30:27.5:1.75:5, v/v). The phospholipid R_f values were compared to those of known standards. The phospholipid fatty acid composition was determined per the procedures of Schroeder and Gardiner [28]. The fatty acids were converted to methyl esters with BF_3/MeOH before application to a 100/120 chromosorb WAW column (Supelco, Bellefonte, PA) on a Sigma 2 gas chromatograph (Perkin Elmer, Norwalk, CT). The retention times and relative response factors were determined by comparison with standards.

Fluorescence intensity

An SLM 4800 subnanosecond spectrofluorometer (SLM Instruments, Urbana, IL) equipped with a 450 mW xenon-arc lamp was used in all experiments. The temperature was maintained at 37°C by a Neslab Instruments cooling system (Plymouth, NH). Excitation of diphenylhexatriene was at 355 nm, while that of dehydroergosterol was at 325 nm. The inner filter effect was avoided by maintaining absorbance at the excitation wavelength less than 0.15. Light scatter was reduced with narrow excitation bandwidths and GG-375 (Janos Technology, Townshend, VT) sharp cut-off filters in the emission lightpath. Polarization and intensity measurements were obtained with the instrument in the T format.

Fluorescence lifetime

Fluorescence lifetimes were obtained by multifrequency phase and modulation fluorometry [33]. A SLM 4800 subnanosecond spectrofluorometer (SLM Instruments, Urbana, IL) was upgraded to multifrequency 1–250 MHz capability by ISS Instruments (Urbana, IL). For lifetime measurements, the light source was a model 4240NB He/Cd laser (Liconix, Santa Clara, CA). Fluorescence lifetimes of diphenylhexatriene were obtained using dimethyl POPOP in HPLC grade ethanol as the reference solution [32]. Data were obtained at 14 frequencies (30–140 MHz) and analyzed by non-linear least-squares and continuous distributional analyses using ISS Lifetime Software (ISS Instruments). The continuous distribution was calculated using Equation 1:

$$f(\tau) = A/\{1+[(\tau - C)/(W/2)]^2\} \quad (1)$$

where A is a constant obtained from normalization, τ is the lifetime, C is the center of the distribution, W is the width of the distribution at one-half height, and $f(\tau)$ is the function that minimizes the reduced χ^2 [34]. The centers obtained from continuous analysis were basically the same as those obtained using the non-linear least-squares method, thus the centers from the continuous analysis are reported here.

Differential polarized phase fluorometry

Because anisotropy is comprised of both static and dynamic components, anisotropy was resolved into two components, limiting anisotropy and rotational relaxation time. Limiting anisotropy is indicative of the degree of resistance to probe motion while the rotational relaxation time measures the rate of probe motion. Although limiting anisotropy is more sensitive to fluidity, both are expected to decrease as fluidity increases. Differential polarized phase fluorometry was used to resolve the anisotropy into two components, limiting anisotropy and rotational relaxation time as first described by Weber [35] and Lakowicz [36]. Limiting anisotropy was calculated using Equation 2 below:

$$r_\infty = r + (r - r_0)/6R\tau \quad (2)$$

where r_∞ is the limiting anisotropy, r is the steady-state anisotropy, r_0 is the anisotropy in the absence of rotational motion, R is the rotational rate in rad/s, and τ is the lifetime.

Rotational relaxation time was calculated using the following equations.

$$(m \tan \Delta)(2R\tau)^2 + (C \tan \Delta - A)(2R\tau) + (D \tan \Delta - B) = 0 \quad (3)$$

$$A = 3B = \omega\tau(r_0 - r) \quad (3a)$$

$$C = (1/3)(2r - 4r^2 + 2) \quad (3b)$$

$$D = (1/9)(m + m_0\omega^2\tau^2) \quad (3c)$$

$$m = (1+2r)(1-r) \quad (3d)$$

where Δ is the phase shift angle, ω is the circular modulation frequency, and the rest are as defined for Eqn. 2. Individual plasma membrane leaflet fluidity was determined as described earlier [37].

Plasma membrane transbilayer sterol domains

To resolve the transbilayer leaflet sterol distribution, cells grown in the presence and absence of the fluorescent sterol dehydroergosterol were treated with trinitrobenzyl sulfonate at 4°C to covalently label amino groups located in the plasma membrane outer leaflet [27,37]. The presence of trinitrophenyl groups selectively quenches fluorescence of dehydroergosterol located in the outer leaflet. Detailed justification of the trinitrobenzyl sulfonate method is shown in Sweet et al. [37].

Lateral sterol domains

Cells were grown in the presence and absence of dehydroergosterol to obtain donors containing up to 35% mole fraction of total lipid as dehydroergosterol and acceptors containing 35% cholesterol (no dehydroergosterol). Sterol exchange assays were performed as described for model membranes [38] adapted to L-cell plasma membranes with slight modification. Briefly, polarization of donor membranes containing dehydroergosterol in 10 mM Pipes buffer (pH 7.3) was measured using a Xe-arc lamp to establish baseline polarization. Then, a 10-fold excess of acceptor membranes lacking dehydroergosterol was added and the polarization measured over a period of 4 h. Dehydroergosterol exchange from donor to acceptor plasma membrane vesicles decreases dehydroergosterol concentration in the donors. This transfer decreases dehydroergosterol self-quenching in the donor plasma membranes and is detected as an increase in dehydroergosterol fluorescence polarization. The data was corrected for light scatter and exchange curves were fit to a multi-exponential equation using a VAX computer system [38].

Miscellaneous methods

Protein concentrations were determined by the method of Lowry et al. [39]. Na^+,K^+-ATPase assays were performed as in Schroeder et al. [26]. Comparison of data sets were evaluated using the standard Student's *t*-test as found in general statistics texts.

Results

Phospholipid composition

To determine whether L-FABP affects plasma membrane phospholipid composition through stimulation of phospholipid synthesis [6], the phospholipid composition of isolated plasma membranes from L-cells and L-cells transfected with the cDNA encoding for L-FABP was determined. Five phospholipid species comprised the majority of phospholipid in the plasma membrane. These were approx. 40% phosphatidylcholine, 30% phosphatidylethanolamine, 14% sphingomyelin, 10% phosphatidylserine, and 7% phosphatidylinositol. Two statistically significant differences ($P < 0.05$) between control and transfected cells were observed, Table I. Phosphatidylethanolamine (PE) decreased from 30.29 ± 1.86 to $25.77 \pm 0.70\%$. Sphingomyelin (SM) content increased from 13.98 ± 0.78 to $16.71 \pm 0.82\%$. Significant changes were not observed in the other phospholipid species. Despite the changes in PE and SM, the overall ratio of neutral/acidic phospholipids remained unchanged within experimental error.

TABLE I

Phospholipid composition of plasma membrane from L-cells expressing L-FABP

Phospholipid composition of plasma membranes was determined using 2-D thin-layer chromatography, as described in the Materials and Methods. Values represent the means ± S.E. ($n = 8$–13). An asterisk represents $P < 0.05$ as compared to control.

Phospholipid	% composition	
	control	transfected
Phosphatidylcholine	39.74 ± 1.41	40.85 ± 1.61
Phosphatidylethanolamine	30.29 ± 1.86	25.77 ± 0.70 *
Sphingomyelin [a]	13.98 ± 0.78	16.71 ± 0.82 *
Phosphatidylserine	9.83 ± 1.34	11.36 ± 1.02
Phosphatidylinositol	7.48 ± 1.47	5.92 ± 0.79
Neutral zwitterionic/acidic [b]	1.16	1.10

[a] Reported previously by Jefferson et al. [45].

[b] Neutral zwitterionic = phosphatidylcholine + sphingomyelin, acidic = phosphatidylethanolamine + phosphatidylserine + phosphatidylinositol.

Phospholipid fatty acid composition

As L-FABP selectively binds unsaturated fatty acids, the phospholipid fatty acid side chain composition was measured to determine if L-FABP altered the overall fatty acid content of the plasma membrane. Only one fatty acid showed a significant change; oleic acid (18:1) decreased from 34.49 ± 2.0 to $27.72 \pm 2.37\%$ ($P < 0.05$), Table II. The unsaturation ratios were calculated to be 0.72 ± 0.04 and 0.62 ± 0.06 for control and transfected cells, respectively, while the double-bond indices were 0.79 ± 0.04 and 0.68 ± 0.06, neither of which represents a statistically significant decrease. In conclusion, in intact cells L-FABP expression does not alter the equilibrium unsaturated fatty acid content of plasma membrane phospholipids at the L-FABP levels expressed.

Sterol / phospholipid in plasma membrane

As L-FABP has also been shown to bind sterol in vitro, the possibility of L-FABP directed alterations of plasma membrane sterol/phospholipid was examined. The ratio was decreased in the bulk membrane from 0.60 ± 0.07 to 0.36 ± 0.06 ($P < 0.01$), Table III. The phospholipid content remained unchanged in the

TABLE II

Phospholipid fatty acid composition of plasma membranes from L-cells expressing L-FABP

Fatty acid composition of plasma membrane phospholipids was determined by gas chromatography after conversion to methyl esters as described in the Materials and Methods. Values represent the means ± S.E. ($n = 13$–21). An asterisk represents $P < 0.05$ as compared to control.

Fatty acid	% composition	
	control	transfected
14:0	5.18 ± 1.06	8.58 ± 2.6
15:0	0.17 ± 0.09	0.04 ± 0.01
16:0	24.49 ± 1.42	23.98 ± 1.70
16:1	2.06 ± 0.41	2.41 ± 0.33
18:0	26.54 ± 0.47	26.98 ± 1.50
18:1	34.49 ± 2.0	27.72 ± 2.37 *
18:2	1.16 ± 0.29	1.31 ± 0.40
18:3	0.99 ± 0.22	1.26 ± 0.31
22:1/22:0	3.25 ± 0.93	4.57 ± 0.66
24:1	0.45 ± 0.19	0.93 ± 0.39
22:5	0.48 ± 0.13	0.65 ± 0.37
Unsaturated/saturated	0.72 ± 0.04	0.62 ± 0.06
Double bond index	0.79 ± 0.04	0.68 ± 0.06

plasma membrane as measured by protein concentration. Thus, this decrease is due to L-FABP effects on sterol distribution.

Plasma membrane fluidity

As shown above, L-FABP expression alters SM, PE, and oleic acid contents, as well as the sterol/phospholipid ratio of the plasma membrane. Although the sterol/phospholipid ratio is a major determinant of membrane fluidity, the alterations in SM and oleic acid content would be expected to have an opposing effect on the membrane fluidity [39]. Thus it is not possible to accurately predict the extent of alteration in membrane fluidity arising from these compositional changes. The fluorescent probe diphenylhexatriene, which reports on overall membrane fluidity, was monitored in L-cell plasma membranes.

TABLE III

Sterol / phospholipid ratio in plasma membrane from transfected L-cells

Sterol/phospholipid was determined in plasma membranes isolated from L-cells using HPLC as described in Materials and Methods. Values represent the means ± S.E. ($n = 15$). An asterisk refers to $P < 0.01$, as compared to control. A dagger refers to $P < 0.01$ as compared to the outer leaflet of the same cell line.

Membrane leaflet	Cell line	
	control	transfected
Bulk	0.60 ± 0.07	0.36 ± 0.06 *
Outer	0.19 ± 0.04	0.20 ± 0.04
Inner	1.00 ± 0.07 *,†	0.58 ± 0.07 †

TABLE IV

Static and dynamic properties of diphenylhexatriene in plasma membrane from transfected L-cells

Fluorescence polarization, lifetime, lifetime distributional width, limiting anisotropy, and rotational relaxation time were determined in plasma membrane vesicles as described in Materials and Methods. Only the major lifetime component representing greater than 96% of total is given. Values represent the means ± S.E. ($n = 6$–22). An asterisk, double asterisk, and triple asterisk represent $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively, as compared to control. A dagger and double dagger refer to $P < 0.05$ and $P < 0.01$, respectively, as compared to the outer leaflet of the same cell line.

Parameter	Membrane leaflet	Cell line	
		control	transfected
Polarization	bulk	0.264 ± 0.003	0.250 ± 0.003 **
Limiting anisotropy	bulk	0.186 ± 0.003	0.173 ± 0.003 **
	outer	0.211 ± 0.006	0.165 ± 0.005 **
	inner	0.194 ± 0.006	0.189 ± 0.005 ††
Rotational relaxation time (ns)	bulk	1.19 ± 0.06	1.31 ± 0.13
	outer	1.81 ± 0.06	1.33 ± 0.14 *
	inner	0.83 ± 0.08 ††	0.80 ± 0.13 ††
Lifetime (ns)	bulk	9.72 ± 0.30	9.85 ± 0.61
Lifetime distributional width (ns)	bulk	0.230 ± 0.039	0.128 ± 0.034 ***
	outer	0.350 ± 0.090	0.052 ± 0.011 *
	inner	0.130 ± 0.044 †	0.180 ± 0.072 †

First, the polarization decreased from 0.264 ± 0.003 to 0.250 ± 0.003 ($P < 0.01$), Table IV. Fluorescence polarization is due to the restriction of rotation during the timescale of emission measurement. The greater the freedom of order or rotation (e.g., the larger the fluidity) the smaller the polarization will be.

Second, the anisotropy was resolved into the two components, limiting anisotropy and rotational relaxation time. The limiting anisotropy decreased from 0.186 ± 0.003 to 0.173 ± 0.003 ($P < 0.01$), while the rotational relaxation time showed no statistically significant changes, see Table IV. Thus, the change in polarization was due to a change in the ordering of the diphenylhexatriene rather than rotational dynamics.

Third, the lifetime of a fluorescent molecule, the average time spent in the excited state before fluorescence emission, is also indicative of membrane structure. Although the discrete lifetime of diphenylhexatriene was found to be equal in both sets of cells (9.8 ns), the lifetime distributional width, which is obtained from a continuous distribution analysis of the data, was seen to decrease from 0.230 ± 0.039 to 0.128 ± 0.034 ns ($P < 0.01$). The distribution of lifetimes reveals the range of time spent by individual molecules in the excited state. A larger distributional width indicates the population of molecules is heterogenous, which is consistent with a more highly ordered membrane system.

Plasma membrane transbilayer fluidity

Transbilayer cholesterol, SM, and PE distribution in L-cells is asymmetric [41–43]. Since these lipids have diverging effects on fluidity, it is important to determine experimentally the individual leaflet fluidity in the plasma membrane. The structure of these leaflets from control and transfected cell plasma membranes was resolved as described in the Materials and Methods. The resolved components of the polarization are listed in Table IV. As compared to controls, the limiting anisotropy was significantly decreased in the outer leaflet of the transfected cell from 0.211 ± 0.006 to 0.165 ± 0.005 ($P < 0.01$). That of the inner leaflet remained unchanged at 0.194 ± 0.006 and 0.189 ± 0.005 in control and transfected membranes, respectively. Thus, the rigidity of the plasma membrane outer leaflet is decreased in the transfected cells.

Although the rotational relaxation time of diphenylhexatriene in bulk plasma membrane did not change significantly, the rotational relaxation time of diphenylhexatriene in the outer leaflet actually decreased from 1.81 ± 0.06 to 1.33 ± 0.14 ns ($P < 0.05$) in transfected cell membranes (Table IV). The relaxation time in the inner leaflet remained unchanged at 0.83 ± 0.08 and 0.80 ± 0.13 ns in plasma membranes from control and transfected cells, respectively. Therefore, although these effects were not observable when examining bulk membrane, the outer leaflet membrane structure was altered.

The diphenylhexatriene lifetime distributional width in individual leaflets of the plasma membrane was also determined. A decrease in lifetime width was observed in the outer leaflet. The width decreased from 0.350 ± 0.090 to 0.052 ± 0.011 ns ($P < 0.05$). In contrast, the width in the inner leaflet remained unchanged at 0.13 ± 0.04 and 0.18 ± 0.07 ns in control and transfected membranes, respectively. The ratio of inner/outer distributional width was effectively inverted upon transfection, i.e., inner/outer < 1 in control, > 1 in transfected plasma membrane.

Plasma membrane transbilayer distribution of sterols

Since the major alteration in transfected L-cells is a large change in sterol/phospholipid, it is important to know if this change in sterol content was restricted to one leaflet or another. The transbilayer distribution of sterol in the plasma membrane of L-cells was determined using dehydroergosterol, a fluorescent sterol analog, as described in the Materials and Methods [27]. Measurements of transbilayer sterol distribution with dehydroergosterol assume that dehydroergosterol and cholesterol codistribute. The codistribution of dehydroergosterol with cholesterol is similar in both cell systems. The mole fraction of dehydroergosterol is 0.60 ± 0.04 in control cell bulk plasma membrane and 0.63 ± 0.05 in transfected. The codistribution within each leaflet is also similar between the two cell lines, control vs. transfected, with 0.51 ± 0.05 vs. 0.54 ± 0.04 and 0.62 ± 0.06 vs. 0.66 ± 0.04 mole fraction in the outer and inner leaflets, respectively. Since 50–60% of the sterol in each leaflet was dehydroergosterol, the two species codistribute, i.e., there is no incorporation of dehydroergosterol at the expense of cholesterol in either leaflet.

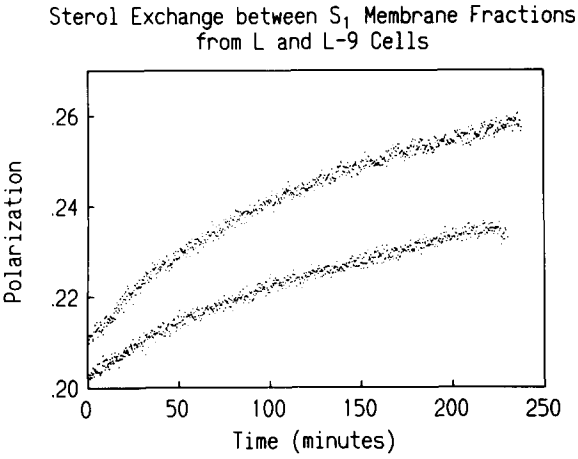

Fig. 1. Sterol exchange assay of dehydroergosterol. Exchange assays were performed using plasma membranes isolated from L-cells as described in Materials and Methods. The top curve is control and the bottom curve is transfected L-cells expressing high L-FABP.

Sterol transfer from lateral sterol domains

This and other laboratories have shown that in addition to transbilayer domains, there exist lateral sterol domains (reviewed in Ref. 11). One way to examine lateral sterol domains is by kinetics of sterol exchange utilizing the fluorescent dehydroergosterol [24,38]. The dehydroergosterol exchange assays measure sterol transfer by release from self-quenching when the sterol is transferred from donor to acceptor membranes. This is observed by a continuous increase of dehydroergosterol fluorescence polarization upon addition of acceptors lacking dehydroergosterol. As indicated in Fig. 1, the rate of fluorescence polarization change was less rapid for plasma membranes from transfected cells, (bottom curve). The initial rate decreased from $(4.12 \pm 0.37) \cdot 10^{-4}$ to $(3.18 \pm 0.22) \cdot 10^{-4}$ min^{-1} ($P < 0.10$).

When the polarization data were fit to a multi-exponential equation as described in Materials and Methods, the data best fit two exchangeable exponents and one additional pool that was not exchangeable. These three kinetic pools of sterol were described as rapidly, slowly, and very slowly (non-exchangeable) exchangeable domains; with exchange halftimes near 30 min, 150 min, and $> > 24$ h, respectively. The halftime of exchange from the rapidly exchangeable pool de-

TABLE V

Effect of L-FABP expression on plasma membrane sterol domains

Exchange assays were performed by measurement of dehydroergosterol polarization increase upon transfer from donor to acceptor membranes as described in the Materials and Methods. Values represent means ± S.E. ($n = 4$–16). An asterisk represents $P < 0.025$, as compared to control, respectively.

Parameter	Cell line	
	control	transfected
Halftime ($t_{1/2}$)		
rapid (min)	24.3 ± 3.1	3.0 ± 1.7 *
slow (min)	150 ±10	167 ±29
Pool size (f)		
rapid (f_1)	4.2 ± 0.5	0.5 ± 0.2 *
slow (f_2)	30.1 ± 1.6	25.1 ± 1.1 *
nonexchangeable (f_3)	66.1 ± 1.6	77.7 ± 1.7 *

creased from 24.3 ± 3.1 to 3.0 ± 1.7, see Table V. The relative size of the nonexchangeable pool increased from 66.1 ± 1.6 to 77.7 ± 1.7%. This was at the expense of both the rapidly and slowly exchangeable pools which decreased from 4.2 ± 0.5 to 0.5 ± 0.2% and from 30.1 ± 1.6 to 25.1 ± 1.1%, respectively. Thus, the sterols in the plasma membrane appear to have reorganized laterally. If diphenylhexatriene is not uniformly distributed into all of the sterol domains, alterations in the lateral sterol domains of the outer leaflet may account for the increased fluidity observed above.

Structural properties of sterols in plasma membrane domains

Both sterol-rich and sterol-poor domains co-exist in L-cell plasma membranes [11]. Diphenylhexatriene is expected to partition equally into all phospholipid domains, however, it may not report on sterol-rich domains. Dehydroergosterol was used to probe the structure of sterol-rich domains in the outer vs. inner leaflets of the L-cell plasma membranes, Table VI. The dehydroergosterol polarization appears to decrease in the plasma membrane of transfected cells vs. those of controls. This was observed in both the bulk membrane, 0.231 ± 0.006 to 0.222 ± 0.008, and in the individual leaflets; 0.266 ± 0.010 to 0.250 ± 0.012 (outer leaflet), and 0.224 ± 0.010 to 0.203 ± 0.011 (inner leaflet). However, the differences were all within experimental error preventing any conclusions from being drawn.

TABLE VI

Polarization of dehydroergosterol in plasma membranes from transfected L-cells

Fluorescence polarization of dehydroergosterol was determined in plasma membranes isolated from L-cells as described in Materials and Methods. Values represent the means ± S.E. ($n = 6$). A dagger refers to $P < 0.05$ as compared to the outer leaflet of the same cell line.

Membrane leaflet	Cell line	
	control	transfected
Bulk	0.231 ± 0.006	0.222 ± 0.008
Outer	0.266 ± 0.010	0.250 ± 0.012
Inner	0.224 ± 0.010 †	0.203 ± 0.011 †

Discussion

Many capabilities of lipid binding proteins have been well characterized in vitro. L-FABP, by virtue of fatty acid and fatty acyl-CoA binding is capable of stimulation of fatty acid metabolic enzymes such as diacylglycerol acyltransferase [5] and *sn*-glycerol-3-phosphate transacylase [6]. While all FABPs bind fatty acids, L-FABP is also capable of binding sterols. As a consequence of its sterol binding ability, L-FABP is also capable of activating sterol metabolic enzymes, such as ACAT in vitro and in intact cells [12,44]. Another potential function of the fatty acid binding proteins studied in vitro is the ability to transfer lipids between membranes. Increased fatty acid exchange between membranes in the presence of L-FABP was documented by Peeters et al. [4]. L-FABP also stimulates sterol exchange between biological membranes, though not between model vesicle membranes [11].

Although much has been determined in vitro, very little is known about the physiological function of these proteins. It seems probable that they may be important in fatty acid and sterol transport, substrate delivery, protection from free fatty acids, regulation of sterol uptake, or maintenance of intracellular sterol asymmetry. However, one must be careful in assigning in vivo function based on in vitro properties. Two earlier proteins, ligandin and elastase were both isolated due to lipid binding properties yet were later found to have no role in lipid metabolism [13,14]. Thus in vitro capability does not confer physiological significance.

The experiments reported herein were performed specifically to examine the potential physiological function(s) of one of these lipid binding proteins, L-FABP, in intact cells. Mouse L-cells containing extremely low levels of endogenous FABP proteins were previously transfected with the cDNA coding for L-FABP [22,45]. Changes between control and transfected cells were due to the presence of L-FABP in the cell. This allowed the first examination of in vivo functions and effects of L-FABP.

In this study we have extended the earlier whole cell studies by focusing on physical characteristics and composition of plasma membrane individual leaflets. Diphenylhexatriene, which partitions into both fluid

and solid regions and reports overall membrane rigidity, indicated the fluidity of the bulk plasma membrane increased. Examination of the individual leaflets revealed that nearly the entire fluidity change occurred in the outer leaflet of the transfected cell plasma membrane.

There are two possible mechanisms to explain the increased fluidity of the plasma membrane outer leaflet. The first is alteration of the lipid composition. Although the bulk plasma membrane sterol/phospholipid ratio exhibited a decrease in transfected cells, this change was localized to the inner leaflet. The ratio in the outer leaflet was maintained constant and thus cannot account for the fluidity of the outer leaflet. Changes in phospholipid headgroup and fatty acid composition also occurred in the bulk plasma membrane. However, the changes observed would suggest a decreased fluidity [40].

The second mechanism is a redistribution of lipid between the plasma membrane transbilayer leaflets and/or within the leaflets. Indeed, the sterol content and sterol/phospholipid ratio were depleted in the inner leaflet, but constant in the outer leaflet. Thus ruling out fluidizing effects by reduced sterol/phospholipid ratio in the outer leaflet of the bilayer. While phospholipids do not readily redistribute from one leaflet to the other in L-cells [41,42] the following alternate possibilities must be considered.

First is the possibility of fatty acid redistribution or compositional changes specific to an individual leaflet. Because the phospholipids exhibit a preference for specific membrane leaflets, fatty acid content, and fatty acid unsaturation [43], the alteration in PE and SM content may have profound effects on the fatty acid composition in the individual leaflets. Such transbilayer distribution of fatty acids is beyond the scope of the present study and will be addressed in future studies.

Second, differences between control and transfected cell plasma membranes may be due to the apparent association of SM and cholesterol [45–48]. Changes in SM content may alter sterol lateral domains within the leaflet. Indeed, the greater polarization of dehydroergosterol in the outer leaflet may be interpreted as resulting from an interaction which hinders sterol motion. Increased association with SM due to higher SM content could account for this hindrance. As 82–100% SM is reported to reside in the outer leaflet, it is likely that the increased SM content also resides preferentially in this leaflet [50]. If the SM and sterol are associated, the SM content in the rest of the leaflet would be depleted. As phosphatidylcholine, the other major phospholipid component of the outer leaflet, is more fluid than SM [51,52], the fluidity of diphenylhexatriene would be expected to increase as was observed.

DHE polarization is dependent upon both fluidity and the local DHE concentration. Increased association with SM would be expected to reduce the fluidity resulting in increased polarization. However, at the same time, the relative size of the nonexchangeable sterol domain (or sterol-rich region) is increased. As a larger population of DHE experiences self-quenching the polarization is expected to decrease. Thus, the two effects have cancelled out and an expected DHE polarization increase upon association with SM was not observed.

The above alterations in lateral sterol domains due to association with SM was also manifested in sterol exchange between transfected plasma membranes. The exchange assay monitors three sterol domains described as rapidly exchangeable, slowly exchangeable, and non-exchangeable. The relative amount of nonexchangeable sterol increased, however, the exchange halftime from the first pool was dramatically decreased. In contrast, Clejan and Bittman [53] previously demonstrated a similar increase of exchange halftime in model membranes with increasing SM content. The exchangeable sterol may also have been affected due to the depletion of sterol in the inner leaflet. This depletion would render replenishment of outer leaflet sterol through transbilayer migration from the inner leaflet more difficult.

The above changes in lateral sterol domains were due to alterations in the membranes themselves and should not be confused with previous studies where L-FABP was acutely present in the in vitro assay mixture [11]. In the present study, the changes were due to alterations in the membrane from chronic endogenous L-FABP exposure, whereas the previous study monitored a stimulation of exchange due to L-FABP transport activity in the mixture [11].

In conclusion, the effects of L-FABP expression on plasma membrane composition and characteristics were examined. The overall fluidity was increased due to an increase in the outer leaflet. One potential mechanism resulting in increased fluidity which appears to be operative in the transfected cell line, is the increased association of SM and cholesterol due to increased SM levels in the outer leaflet. This serves to lower the rigidifying effects of both cholesterol and SM in the bulk plasma membrane. A further effect of this association and inner leaflet sterol depletion is hindered intermembrane sterol exchange between transfected plasma membranes.

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