Biochimica et Biophysica Acta 812 (1985) 473–484
Elsevier

BBA 72411

Calcium alters the acyl chain composition and lipid fluidity of rat hepatocyte plasma membranes in vitro

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(Received July 17th, 1984)
(Revised manuscript received October 2nd, 1984)

Key words. Ca^{2+}, Membrane fluidity, Lipid composition, (Rat hepatocyte)

Calcium ion decreases the lipid fluidity of isolated rat hepatocyte plasma membranes by modulating the activity of membrane enzymes which alter the lipid composition. To explore the mechanism of the effect of the cation, eight fluorophores were used to assess lipid fluidity via estimations of either steady-state fluorescence polarization or excimer fluorescence intensity. The results demonstrate that the reduction in fluidity occurs in the hydrophobic interior of the bilayer and that both the dynamic and static (lipid order) components of fluidity are affected by treatment with calcium. Analysis of the membrane lipids demonstrates that calcium treatment decreases the arachidonic acid content of the polar lipid fraction and, thereby, reduces the double-bond index of the fatty acids. This change in composition, which is expected to reduce the lipid fluidity, may result from activation by calcium of the endogenous hepatocyte plasma membrane phospholipase A_2.

Introduction

The addition of calcium ion to suspensions of isolated rat hepatocyte plasma membranes decreases the membrane 'lipid fluidity', defined below, by two distinct mechanisms [1]. To a minor extent the fluidity change results from direct binding of the cation to phospholipid headgroups, an interaction which is well established for bilayer phospholipids [2–4] and readily reversed by chelation of the cation with EDTA. Most of the decrease in membrane fluidity, however, is not reversed by EDTA and results from metabolic alteration of the lipid composition owing to calcium-dependent, endogenous membrane enzymes. Given the general importance of calcium in the regulation of membrane enzymes and functions [5,6] and its known effects on hepatocyte plasma membrane activities such as the $(Na^+ + K^+)$-dependent adenosine triphosphatase [7] and adenylate cyclase [8], the present studies were undertaken to explore the mechanisms responsible for the calcium-modulated alterations of hepatocyte plasma membrane fluidity. The experiments below demonstrate that the cation decreases the arachidonic acid content and the overall double bond index of the membrane lipids, changes which can account at least partially for the reduction in fluidity.

The term 'lipid fluidity' as applied to anisotropic bilayer membranes is used in this report to denote the relative motional freedom of the lipid molecules or substituents thereof. As evaluated by steady-state fluorescence polarization of lipid fluorophores, the 'fluidity' is assessed via the parameters of the modified Perrin equation described below (Methods). An increase in 'fluidity' corresponds to a decrease in either the correlation time,

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τ_c, or the limiting hindered anisotropy, r_∞, of the fluorophore. Hence the term combines the concepts of the dynamic and static (lipid order) components of fluidity.

Methods

Membrane preparations. Albino male rats of the Sherman-Wistar strain weighing 225–300 g were maintained on a standard chow diet (NIH Rodent Diet, Agway, Inc., Syracuse, NY) and water ad libitum. Groups of four animals were killed by exsanguination, the livers perfused in situ via the inferior vena cava to remove blood and excised. Suspensions of hepatocyte plasma membranes were prepared by a modification of the method of Nigam et al. [9], as described previously [1,10]. Purity of the membrane suspensions and the extent of contamination with intracellular organelles were assessed by marker enzyme assays. The plasma membrane marker, 5′-nucleotidase, was assayed as described by Morre [11], using the method of Gomori [12] to quantify the inorganic phosphate released. Mitochondrial and microsomal membranes, respectively, were monitored by estimation of succinate dehydrogenase [13] and glucose-6-phosphatase [11]. The specific activity ratios (purified plasma membranes)/(homogenate) of 5′-nucleotidase for 17 preparations were 14.7 ± 1.5 (mean $\pm$ S.E.). The corresponding values for the mitochondrial and microsomal marker enzymes were 0.4 ± 0.1 (12 preparations) and 0.3 ± 0.1 (10 preparations), respectively. Liver homogenates were also fractionated by differential centrifugation into nuclear, mitochondrial-lysosomal and microsomal fractions according to the method of De Duve et al. [14] as modified by Amar-Costesec et al. [15].

Liposome preparations. Small unilamellar vesicles of defined lipid composition were prepared from pure phospholipids (Avanti Polar lipids, Inc., Birmingham, AL) and free fatty acids (Nu-Chek Prep, Elysian, MN). Purity of these compounds was confirmed by gas-liquid chromatography, as described below, and by argentation thin-layer chromatography [16]. The unilamellar vesicles were prepared as described by Huang and Thompson [17] using various mixtures of phosphatidylcholines, lysophosphatidylcholine, palmitic acid and arachidonic acid. Although either free fatty acids or lysophosphatidylcholines alone form micelles above their critical micellar concentrations in aqueous media, mixtures of the two lipid classes do form bilayers [18,19]. Where free fatty acids and lysophosphatidylcholine were components, therefore, the vesicle suspensions were fractionated by gel filtration through a 22×0.9-cm column of Sepharose 4B (Pharmacia, Piscataway, NJ) to ensure removal of micellar particles. The elution volume/void volume ratio (V_e/V_0) for the vesicles in the eluate was approx. 1.45 and the corresponding values for lysophosphatidylcholine micelles was 2.50. Peak fractions of the vesicles were pooled for fluorescence polarization studies, as described below, and for lipid analysis to ensure that the vesicle composition corresponded to that of the original mixture used.

Lipid analyses. Total lipids were extracted from plasma membrane preparations by the method of Folch et al. [20]. Extracts prepared from the pooled membranes of four livers were used to quantify the fatty acid composition of the total lipids. Extracts prepared from the pooled membranes of 12 livers were separated into neutral and polar lipid fractions by silicic acid column chromatography using chloroform and methanol to elute the neutral and polar fractions, respectively. Each separation was complete as monitored by thin-layer chromatography on Silica Gel G Chromatoplates (Applied Sciences) using a modification of the method of Ando et al. [21] described in detail in the following paragraph. The fatty acid composition of each fraction was determined by gas-liquid chromatography. Fatty acid methyl esters were prepared using boron trifluoride (14%, w/v, in methanol) as catalyst [22], and the derivatives analyzed in a JEOL JGX-20K gas chromatography equipped with a flame ionization detector and interfaced with a Hewlett-Packard 3390A integrator. The esters were resolved on a 6-foot glass column (outer diameter 4 mm, inner diameter 2 mm) packed with 10% Silar 10C on 100–120 mesh Gas Chrome Q (Applied Science Laboratories, College Park, PA). Column temperature was 165°C for 16 min followed by an increased of 2 deg. C per min to 195°C. Injection temperature was 195°C and carrier gas pressure 1.8 kg/cm^2. Authentic standards were purchased from Supelco, Inc. (Belefonte, PA)

and sample peaks identified by retention times. Unidentified peaks comprised less than 2% of the total areas and were disregarded.

Column fractions containing small unilamellar vesicles were extracted by the method of Folch et al. [20] and the extracts separated by a modification of the method of Ando et al. [21]. Samples and standards were applied to 20 × 20 cm Silica Gel G Chromatoplates (Applied Sciences) which had been washed in $CHCl_3/CH_3OH$ (2:1, v/v) and activated at 110°C for 60 min. Two-step development was used to separate polar and nonpolar lipid components. The polar solvent system (chloroform/methanol/acetic acid/formic acid/water, 35:15:6:2:1, v/v) was developed to half the plate height; the plate was air-dried and the nonpolar solvent system (hexane/ethyl ether/acetic acid, 70:30:1, v/v) was developed to the entire height of the plate. Lipids were visualized by spraying with 50% H_2SO_4 and charring on a hot plate. Densitometric scans (Transidyne, Inc.) were quantified by cutting and weighing the peaks.

Fluorescence polarization studies. Except as indicated, plasma membrane suspensions were incubated in a Tris-buffered saline consisting of 5 mM Tris of pH 7.4 containing 146 mM NaCl and 4 mM KCl. To study the effects of calcium ion the membranes were suspended at a density of 0.1–0.2 mg of membrane protein per ml of Tris-buffered saline containing 0.1 mM sodium EGTA (pH 7.4), and either no $CaCl_2$ or 4 mM $CaCl_2$. After incubation with shaking at 37°C for 2 h, sodium EGTA (pH 7.4), was added to a final concentration of 8 mM in order to eliminate effects owing to direct binding of Ca^{2+} and to limit the changes observed to the indirect effects on lipid composition [1]. For fluorescence polarization studies the membranes were incubated with calcium and a fluorophore simultaneously. The following lipid-soluble fluorophores were obtained from Molecular Probes (Junction City, OR) and solutions in ethanol (0.5–1.0 mM) prepared just before use: 1,6-diphenyl-1,3,5-hexatriene; 2-(9-anthroyloxy)stearate; 7-(9-anthroyloxy)stearate; 9-(9-anthroyloxy)stearate; 12-(9-anthroyloxy)stearate; and 16-(9-anthroyloxy)palmitate. Sufficient probe solution was added with vigorous mixing to the membrane suspensions in the Tris-buffered saline described above to yield final fluorophore concentrations of 2–10 μM. After incubation at 37°C (30–120 min) the membranes were pelleted by centrifugation (40 000 × *g*, 20 min, 5°C) and resuspended in fresh Tris-buffered saline to a final concentration of 50–100 μg/ml of membrane protein. Corrections for light scattering (membrane suspensions minus probe) and for fluorescence in the ambient medium (quantified by pelleting the membranes) were made routinely, and the combined corrections were < 5% of the total fluorescence intensity observed for diphenylhexatriene-loaded membranes and < 10% of that observed for (anthroyloxy)stearate-loaded suspensions. Membranes were treated similarly with the cationic fluorophore 1-(4′-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (Molecular Probes), except that a stock solution, 0.1 mM in Tris-buffered saline, was stored frozen at −20°C between use.

Estimations of the steady-state fluorescence anisotropy, *r*, were made at 24°C, using an SLM-Aminco (Urbana, IL) model 4000 or 4800 polarization spectrophotometer, as described previously [10]. The results were interpreted according to the modified Perrin relationship [23,24]:

$$r = r_\infty + (r_0 - r_\infty)[\tau_c/(\tau_c + \tau_F)]$$

where r_0 is the maximal limiting anisotropy, taken as 0.365 for diphenylhexatriene [25] and 0.285 for the anthroyloxy probes [26], respectively, r_∞ is the limiting hindered anisotropy; τ_c is the correlation time and τ_F the mean lifetime of the excited state. The lifetime, τ_F, was estimated by phase fluorometry at 30 mHz [27,28] in the SLM 4800 polarization spectrophotometer. Values of r_∞ for diphenylhexatriene were calculated from the fluorescence anisotropy *r*, and from estimates of tan Δ obtained by differential polarized phase fluorometry and the modulation lifetimes, as described by Lakowicz et al. [28]. Using diphenylhexatriene, the static component of membrane fluidity was assessed by an order parameter, *S*, where $S = (r_\infty/r_0)^{1/2}$, as described previously [23,24].

Intramolecular excimer fluorescence. The foregoing fluorescence polarization studies monitor rotational diffusion of lipid fluorophores as an index of lipid fluidity. The dynamic component of membrane fluidity was also assessed by estimates of the

excimer fluorescence of an intramolecular excimer-forming fluorophore, 1,3-di(1-pyrenyl)propane, introduced by Zachariasse [29] and applied to monitor short-range lateral diffusion of the pyrene substituents in erythrocyte membranes [30]. A solution of 0.15 mM dipyrenylpropane (Molecular Probes) in ethanol was prepared fresh and 4 μl were added to 0.4 ml of buffer containing membranes equivalent to 400 μg of membrane protein and prewarmed to 37°C. The mixture was stirred vigorously for 20 s and then incubated with shaking at 37°C for 1 h. 3 ml of deoxygenated Tris-buffered saline (buffer evacuated and flushed with Airco highly-purified N_2 for 30 min) were added to each sample and 2 ml of 20% (w/v) Dextran 500 (Pharmacia) were layered under the membrane suspension to separate the suspension from any insoluble aggregates of the probe, which can adhere to the walls of the tube in the course of the incubation. After standing for 10 min the upper phase containing the membranes was removed and the membranes were pelleted and washed twice with 5 ml of deoxygenated Tris-buffered saline by centrifugation (40000 × *g*, 20 min, 20°C). The washed pellet was suspended in 1.5 ml of the deoxygenated buffer, allowed to stand at room temperature for at least 1 h and fluorescence emission spectra were recorded at 25°C in a Perkin-Elmer 650-40 fluorescence spectrophotometer using an excitation wavelength of 345 nm. Excimer and monomer fluorescence peak intensities were estimated at 485 nm and 396 nm, respectively, and corrections were made for membranes carried through the procedure without probe and for the probe added to Tris-buffered saline alone. The observed excimer/monomer intensity ratios were also expressed in Poise values by reference to a standard curve of dipyrenylpropane dissolved in sorbitol/ethylene glycol solutions of known viscosities (Schachter, D. and Abbot, R.E., unpublished observations).

Effects of pH. To examine the effects of calcium on hepatocyte plasma membranes incubated at various pH values, the membranes were suspended and incubated as described above in either 5 mM 4-morpholineethanesulfonic acid (Calbiochem; pK_a 6.15) in 0.15 M NaCl for pH values of 5.5 and 6.5; or in 5 mM Tris in 0.15 M NaCl for pH values of 7.4, 8.1 and 9.0. After incubation the membranes were pelleted by centrifugation, washed once with 20 volumes of Tris-buffered saline (pH 7.4), and suspended in the same buffer for fluorescence studies

Results

Fluorescence polarization studies

To characterize further the effects of calcium treatment on the lipid fluidity of isolated hepatocyte plasma membranes, suspensions in Tris-buffered saline containing 0.1 mM EGTA were incubated for 2 h at 37°C in the presence or absence of 4 mM $CaCl_2$. The membranes were also loaded with one of eight fluorophores, and at the end of the incubation period excess EGTA was added to chelate the calcium (Methods) and the fluorescence anisotropy, *r*, was quantified. Values of *r* for each probe are listed in Table I. With the exception of 2- and 7-anthroyloxystearate (see below), each fluorophore reported a calcium-dependent decrease in lipid fluidity, as indicated by an increment in *r*. In agreement with previous results [1], the diphenylhexatriene fluorescence anisotropy was increased by approx. 9.0% ($P < 0.001$). The corresponding increment for trimethylammonium diphenylhexatriene, a cationic derivative which is probably localized closer to the aqueous interfaces of the membrane [31], was only 2.6% ($P < 0.05$), suggesting that calcium affects mainly the hydrophobic interior of the membrane bilayer.

The fluorescence lifetime, τ_F, hindered anisotropy, r_∞, and order parameter, *S*, of diphenylhexatriene were also examined as functions of the calcium treatment, and the results are summarized in Table II. Calcium did not affect the fluorescence lifetime values and did increase significantly both r_∞ ($P < 0.025$) and *S* ($P < 0.025$). Thus the cation increases lipid order and the static component of membrane fluidity [32].

In contrast to the rod-shaped diphenylhexatriene whose fluorescence depolarization in bilayer membranes is determined largely by r_∞ [23,24,32], i.e., by the constraint of rotation, the depolarization of the disc-shaped anthroyloxy fluorophore is influenced more by the correlation time, τ_c, i.e., by the rate of rotation, and the r_∞ values are relatively less significant [33,34]. Thus if τ_F is relatively constant, the Ca^{2+}-dependent in-

TABLE I

EFFECTS OF CALCIUM TREATMENT ON THE FLUORESCENCE ANISOTROPY OF VARIOUS FLUOROPHORES IN RAT HEPATOCYTE PLASMA MEMBRANES

Membranes were incubated in 0 1 mM EGTA with or without 4 mM $CaCL_2$ for 2 h at 37°C (Methods) and then treated with 8 mM EGTA prior to estimation of the fluorescence anisotropy at 24°C Values are means

Probe	N [a]	Fluorescence anisotropy, r		S E of difference [b]	P [b]
		0 1 mM EGTA	4 mM Ca^{2+}		
Diphenylhexatriene	40 (18)	0.244	0.266	0 002	< 0.001
TMA-diphenylhexatriene [c]	3 (3)	0 304	0 312	0.003	< 0 05
2-Anthroyloxystearate	6 (4)	0 075	0.077	0.002	n.s.
7-Anthroyloxystearate	3 (3)	0 078	0.080	0.012	n s
9-Anthroyloxystearate	3 (3)	0.095	0 101	0 002	< 0.05
12-Anthroyloxystearate	6 (4)	0 059	0 066	0 001	< 0.005
16-Anthroyloxypalmitate	5 (4)	0 060	0.069	0.002	< 0.01

[a] Number of determinations (number of preparations in parenthesis).
[b] S.E of differences and P values calculated by Student's t-test of paired comparisons, n s , not significant
[c] Trimethylammonium diphenylhexatriene

crements in the r values of 9- and 12-anthroyloxystearate and of 16-anthroyloxypalmitate (Table I) point to increases in τ_c, i.e., decreases in the dynamic component of membrane fluidity. This interpretation was supported by estimating τ_F for 12-anthroyloxystearate in control and Ca^{2+}-treated plasma membranes. Values (mean $\pm$ S.E.; four determinations of two membrane preparations) of the modulation lifetimes for control and Ca^{2+}-treated membranes, respectively, were 7.5 ± 0.2 and 7.3 ± 0.1 ns; corresponding values of the phase lifetimes were 5.7 ± 0.0 and 5.5 ± 0.1 ns, respectively.

The series of anthroyloxy derivatives yield information on lipid environments at various depths from the aqueous interfaces of the membrane [35]. When the Ca^{2+}-dependent increments in r of Table I are plotted against the acyl attachment site of the fluorophores (Fig. 1), it is apparent that the effects of Ca^{2+} are greatest in the hydrophobic interior of the bilayer, as suggested by the comparison of diphenylhexatriene and its trimethylammonium derivative above. Calcium increased the r value of 16-anthroyloxypalmitate, 12- and 9-anthroyloxystearate by approx. 15% ($P < 0.01$), 12% ($P < 0.005$) and 6% ($P < 0.05$), respectively, whereas the cation did not affect the values of 2- or 7-anthroyloxystearate significantly.

Intramolecular excimer studies

The effects of calcium treatment on the dynamic component of membrane fluidity were also assessed by estimates of the excimer/monomer fluorescence intensity ratio of dipyrenylpropane.

TABLE II

EFFECTS OF CALCIUM TREATMENT ON DIPHENYLHEXATRIENE FLUORESCENCE PARAMETERS IN RAT HEPATOCYTE PLASMA MEMBRANES

Membranes were incubated in 0.1 mM EGTA with or without 4 mM $CaCl_2$ for 2 h at 37°C and then treated with 8 mM EGTA prior to assay of fluorescence at 24°C Values are means $\pm$ S.E for six determinations (three membrane preparations)

Treatment	Fluorescence lifetime, τ_F (ns)		Hindered anisotropy, r_∞	Order parameter, S
	Modulation	Phase		
0 1 mM EGTA	$8.6 \pm 0 1$	$7 1 \pm 0.3$	$0 150 \pm 0.004$	0.644 ± 0.008
4 mM Ca^{2+}	8.6 ± 0.1	$6.9 \pm 0 4$	0.162 ± 0.002 [a]	$0.669 \pm 0 004$ [a]

[a] $P < 0 025$ for difference owing to calcium.

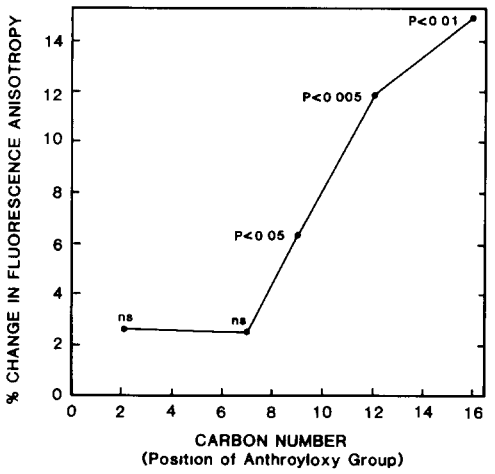

Fig 1 Effects of calcium on the fluorescence anisotropy of various anthroyloxy-fatty acid derivatives in rat hepatocyte plasma membranes Values listed in Table I were used to calculate the % change in r owing to Ca^{2+} for 2-, 7-, 9-, and 12-anthroyloxystearate and for 16-anthroyloxypalmitate P values indicate significant effects of Ca^{2+} on the anisotropy parameter, n s , not significant.

As shown in Table III, the treatment decreased the excimer/monomer ratio at 37°C by approx. 15% ($P < 0.005$). Based on a standard reference curve of this fluorophore in sorbitol/ethylene glycol (Methods), the apparent microviscosity values of the membranes incubated with 0.1 M EGTA or 4 mM Ca^{2+} were 1.25 and 1.66 Poise ($P < 0.025$), respectively.

TABLE III

EFFECTS OF CALCIUM TREATMENT ON FLUORESCENCE EXCIMER INTENSITY OF DIPYRENYLPROPANE IN RAT HEPATOCYTE PLASMA MEMBRANES

Values are means ± S.E. for four determinations (four membrane preparations) at 37°C The excimer/monomer ratio was estimated as the ratio of the peak emission intensity at 485 nm/396 nm. Poise values were estimated from a reference curve of dipyrenylpropane in sorbitol/ethylene glycol solvents of known viscosities.

Treatment	Fluorescence intensity ratio excimer/monomer	Viscosity (Poise)
0 1 mM EGTA	0 40 ± 0 03	1.25 ± 0.17
4 mM Ca^{2+}	0 34 ± 0 02 [a]	1 66 ± 0.26 [a]

[a] $P < 0$ 025 for difference owing to calcium, as calculated by Student's t-test of paired comparisons (S.E. for excimer/monomer ratio and Poise values, respectively, is 0.008 and 0.11)

Effects of pH

The changes in fluidity observed above were demonstrated in the presence of excess EGTA and are not owing to direct binding of calcium. Accordingly, they support the hypothesis that Ca^{2+} decreases the fluidity by regulating endogenous membrane enzymes which alter the lipid composition [1]. To determine whether these enzyme activities are dependent on pH, isolated plasma membranes were incubated in the presence or absence of 4 mM Ca^{2+}, as described above, except that the pH of the incubation medium was varied (Methods) and the effects on the fluorescence anisotropy of diphenylhexatriene monitored. The Ca^{2+}-dependent increments in r at pH 5.5, 6.5, 7.4, 8.1 and 9.0, respectively, were 0.012, 0.015, 0.020, 0.013 and 0.006 (mean values of two experiments). Thus an optimal pH in the vicinity of the physiological value was observed.

Fatty acid composition

Prior studies [1] indicate that calcium treatment of rat hepatocyte plasma membranes in vitro does not change the cholesterol content, cholesterol/phospholipid molar ratio or sphingomyelin content of the lipid. Accordingly, studies were undertaken to determine whether the decrease in fluidity is related to changes in fatty acid composition. Isolated membranes suspended in Tris-buffered saline containing 0.1 M EGTA of pH 7.4 were incubated for 2 h at 37°C in the absence and presence of 4 mM $CaCl_2$. Thereafter lipid extracts were prepared, separated into neutral and polar lipid fractions and the fatty acid composition determined (Methods). The results in Table IV indicate that calcium treatment did not change significantly the acyl chain composition or double bond index of the neutral lipid fraction. In the polar lipid fraction, by contrast, calcium decreased the arachidonic acid content by approx. 33% ($P < 0.025$) and the double-bond index by 18% ($P < 0.05$). Nine additional membrane preparations were also examined to determine the effects of the calcium treatment on the fatty acid composition of the total membrane lipid. As indicated in Table V, calcium decreased the arachidonic acid content by approximately 19% ($P < 0.005$) and the double-bond index by 11% ($P < 0.005$); the ratio of saturated/unsaturated fatty acids was concom-

TABLE IV

EFFECTS OF CALCIUM TREATMENT ON THE FATTY ACID COMPOSITION OF THE POLAR AND NEUTRAL LIPID FRACTIONS OF RAT HEPATOCYTE PLASMA MEMBRANES

Mean values ± S.E. for three membrane preparations are listed. Each preparation consisted of plasma membranes isolated from 12 livers. Membranes were incubated 2 h at 37°C in the presence or absence of 4 mM $CaCl_2$ and lipid extracts were prepared and fractioned as described in Methods

Component	Neutral lipids		Polar lipids	
	No Ca^{2+}	Ca^{2+}	No Ca^{2+}	Ca^{2+}
Fatty acids (% by wt.)				
16·0	26 9 ±2 5	29 2 ±0.9	24.8 ±2.2	24.6 ±3 6
16 1	2 4 ±0 2	1.5 ±0.8	1.3 ±0.0	1.4 ±0.5
18:0	13.2 ±3.2	12.1 ±2.3	25.0 ±1.4	25.4 ±2 0
18·1	27.2 ±4.1	26.9 ±4.4	14.5 ±1.0	14.2 ±1.0
18 2	18.3 ±2.4	17.7 ±1 5	15.4 ±0.2	13.6 ±0 8
20·4	1 7 ±0 6	2 6 ±1 2	14 0 ±1 2	9 4 ±1.0 [a]
Double-bond index	0.72 ± 0.07	0 73 ± 0 08	1.05 ± 0.07	0.86 ± 0 05 [b]

[a] $P < 0.025$ by Student's t-test of paired comparisons

[b] $P < 0.05$ by t-test of paired comparisons.

itantly increased by 15% ($P < 0.001$). Lastly, four additional membrane preparations were treated with calcium as described above and compared with both unincubated (zero time) and incubated (2 h, 37°C) controls. The arachidonic acid content (% by weight of total fatty acids) was similar, 15.2% versus 14.7%, in the zero time and incubated preparations, but significantly lower, 11.5% ($P < 0.01$), in the calcium-treated samples.

TABLE V

EFFECTS OF CALCIUM TREATMENT ON THE FATTY ACID COMPOSITION OF RAT HEPATOCYTE PLASMA MEMBRANES

Values are means ± S.E. for 11 determinations (9 membrane preparations). Membranes were treated as described in Table I prior to extraction of total lipids

Component	Treatment	
	0.1 mM EGTA	4 mM Ca^{2+}
Fatty acids (% by wt.)		
16:0	29.5 ±1.1	31 2 ±1.3
16.1	7.6 ±0 8	5.9 ±0.6
18:0	21.9 ±0.7	24.5 ±1 1
18:1	17.7 ±1.2	17.1 ±1.5
18.2	12.3 ±0.8	12.6 ±0.8
20:4	11.1 ±0.8	9.0 ±1.2 [a]
Ratio saturated/ unsaturated fatty acids	1.08 ± 0.07	1.24 ± 0.07 [b]
Double-bond index	0.95 ± 0.03	0.85 ± 0.05 [a]

[a] $P < 0.005$ by t-test of paired comparisons

[b] $P < 0.001$ by t-test of paired comparisons.

Arachidonic acid and membrane fluidity

The foregoing decreases in membrane arachidonic acid content and double-bond index could underlie the reductions in membrane lipid fluidity owing to calcium. Changes in fluidity have been observed to accompany significant alterations, i.e., 10% or more, in the double-bond index of a number of membrane types [36–39]. A reasonable working hypothesis for the action of calcium is that stimulation of phospholipase A_2, an enzyme known to be present in rat hepatocyte plasma membranes and activated by Ca^{2+} [40], leads to cleavage and loss of arachidonic acid residues from the membrane phospholipids. To predict the resulting effects on lipid fluidity, however, one must take into account the lysophosphatide products of the hydrolysis, which are reported to fluidize bilayers [41,42]. Accordingly, experiments with liposomes of defined composition were undertaken to evaluate the net effects on fluidity of cleavage followed by loss of phospholipid arachidonoyl residues. The influence of arachidonoyl residues on bilayer fluidity was examined by preparing small unilamellar vesicles from mixtures of 1,2-dipalmitoylphosphatidylcholine (DPPC) and 1-palmitoyl-2-arachidonoylphosphatidylcholine

(PAPC). (PAPC was chosen because phospholipids of biological membranes typically contain the arachidonoyl residue in the *sn*-2 position and a saturated acyl chain in the *sn*-1 position,) Bilayer fluidity was monitored by estimates of diphenylhexatriene *r* and the results are illustrated in Fig. 2. Increasing the mole fraction of PAPC decreases *r*. The fluidizing effects of the arachidonoyl residues were also demonstrated throughout the temperature range of 0 to 40°C, i.e., both above and below the phase transition temperatures of DPPC and mixed DPPC/PAPC vesicles. Arrhenius plots of the dependence of diphenylhexatriene fluorescence anisotropy on 1/K yielded midpoint transition temperatures (transition temperature range) of 38°C (34–42°C), 32°C (25–40°C), 27°C (18–36°C) and 20°C (9–32°C), respectively, for unilamellar vesicles composed of DPPC alone; DPPC/PAPC, 68 : 32; DPPC/PAPC, 36 : 64; and PAPC alone. Thus, increasing the number of arachidonoyl residues lowered the midpoint transition temperature and broadened the transition range.

A second series of experiments were designed to determine the net effects on bilayer fluidity of cleavage at the *sn*-2 position of either DPPC or

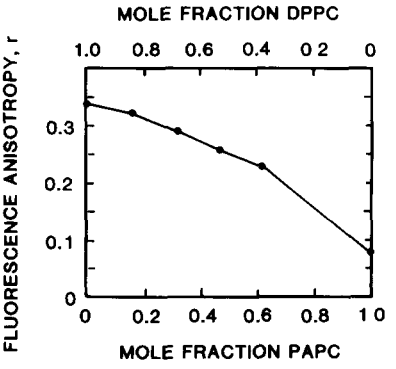

Fig 2. Fluorescence anisotropy (24°C) of diphenylhexatriene in small unilamellar vesicles composed of dipalmitoylphosphatidylcholine (DPPC), 1-palmitoyl-2-arachidonoylphosphatidylcholine (PAPC), and various mixtures of the two

PAPC followed by loss of the acyl chain. Small unilamellar vesicles of DPPC/1-(16 : 0)lysophosphatidylcholine (lysoPC)/PAPC/arachidonic acid in various proportions were prepared. To compare the bilayer composed of intact phospholipid molecules with that resulting from (a) cleavage of 50% of the *sn*-2 acyl residues and (b) cleavage of 50% of the acyl residues followed by loss of 40%, the composition (molar ratios) of the vesicle prepara-

TABLE VI

FLUORESCENCE ANISOTROPY OF DIPHENYLHEXATRIENE IN SMALL UNILAMELLAR VESICLES OF DEFINED COMPOSITION

Mean values ± S.E. for four different preparations tested at 25°C are shown Preparations A and D represent unhydrolyzed phospholipid bilayers, B and E represent 50% cleavage at the *sn*-2 position with no loss of the products; C and F represent 50% cleavage followed by loss of 40% of either palmitic or arachidonic acids.

Prepn	Composition (molar ratios)	Fluorescence anisotropy, *r*
A	Dipalmitoylphosphatidylcholine alone	0 272 ± 0.030
B	Dipalmitoylphosphatidylcholine/1-palmitoyl-lysophosphatidylcholine/palmitic acid, (1 : 1 : 1)	0.322 ± 0.003
C	Same as B, (1 : 1 : 0.6)	0.318 ± 0.003
D	Dipalmitoylphosphatidylcholine/1-palmitoyl--2-arachidonoylphosphatidylcholine (1 : 1)	0.139 ± 0 011
E	Dipalmitoylphosphatidylcholine/1-palmitoyl-lysophosphatidylcholine/arachidonic acid (1 : 1 · 1)	0.148 ± 0.024
F	Same as E, (1 . 1 0.6)	0 186 ± 0.028 [a]

[a] $P < 0\ 02$ for difference from preparation E (paired *t*-test, S E. of difference, 0 007)

TABLE VII

EFFECTS OF CALCIUM TREATMENT ON THE FLUORESCENCE ANISOTROPY OF DIPHENYLHEXATRIENE IN LIVER HOMOGENATE FRACTIONS

Mean values for estimations at 24°C are shown Each preparation was treated as described in Table I prior to quantification of the fluorescence anisotropy

Fraction	N [a]	Fluorescence anisotropy, r		S E of difference [b]	P [b]
		0.1 mM EGTA	4 mM Ca^{2+}		
Nuclear	3 (3)	0 180	0 185	0 002	n s
Mitochondrial-lysosomal	3 (3)	0.184	0.193	0 002	< 0 05
Microsomal	8 (6)	0 173	0 178	0 004	n s

[a] Number of determinations (number of preparations in parenthesis).
[b] Calculated by Student's t-test of paired comparisons; n s , not significant

tions were: DPPC alone; DPPC/lysoPC/palmitic acid = (1 : 1 : 1); and DPPC/lysoPC/palmitic acid (1 : 1 : 0.6). Corresponding compositions of the mixed DPPC/PAPC vesicles were: DPPC/PAPC (1 : 1); DPPC/lysoPC/arachidonic acid (1 : 1 : 1); and DPPC/lysoPC/arachidonic acid (1 : 1 : 0.6). Table VI summarizes the results of estimating the fluorescence anisotropy, r, of diphenylhexatriene in these six types of unilamellar vesicles. Cleavage alone of the acyl residues, either palmitoyl or arachidonoyl, had no significant effect on the r values. Cleavage and loss of palmitic acid from the bilayers also had no effect, whereas loss of arachidonic acid increased r significantly ($P < 0.02$), i.e., decreased the lipid fluidity.

Effects on intracellular membranes

To characterize further the specificity of the effects of Ca^{2+} on hepatocyte plasma membrane fluidity, liver homogenates were fractionated into microsomal, mitochondrial-lysosomal and nuclear suspensions and these were treated with 4 mM Ca^{2+} as described above. Diphenylhexatriene r values were estimated to monitor fluidity changes and the results are listed in Table VII. The microsomal and nuclear fractions exhibit no effects of the Ca^{2+} treatment, while the mitochondrial-lysosomal fractions show a relatively small increase in r of 4.9% ($P < 0.05$). These results indicate that the effects of calcium treatment are relatively specific for the plasma membranes and not due to admixture of the preparations with intracellular membranes.

Discussion

The foregoing studies focus on the mechanisms by which calcium alters hepatocyte plasma membrane lipid composition and, thereby, lipid fluidity. Possible effects on fluidity of direct binding of the cation were prevented by treating the membranes with excess EGTA prior to fluidity assays [1]. The fluorescence methods employed provide three concordant lines of evidence to demonstrate the Ca^{2+}-dependent decrease in hepatocyte plasma membrane fluidity. The degree of order of the lipids, or static component of membrane fluidity [23,24,32], was increased, as indicated by the increments in diphenylhexatriene r_∞ and S (Table II). The dynamic component of fluidity was assessed by estimation of the fluorescence anisotropy of anthroyloxy-fatty acid derivatives. The results (Table I and Fig 1) demonstrate Ca^{2+}-dependent reductions in fluidity as monitored by 9- and 12-anthroyloxystearate and by 16-anthroyloxypalmitate, probes which report from the hydrophobic interior of the bilayer. On the other hand, values obtained with 2- and 7-anthroyloxystearate, whose fluorophores are localized in more superficial domains, showed no significant effect of the cation. This pattern of change in fluidity is consistent with the alterations in arachidonic acid content and double bond index of the membrane lipids (Table IV). Thulborn et al. [43] compared the fluorescence anisotropy of a similar series of anthroyloxy-fatty acids in vesicles of distearoylphosphatidylcholine and dioleoylphosphatidylcholine and

reported that the double bond in the latter increased the fluidity in the hydrophobic interior of the bilayer. A similar effect of *cis*-monounsaturation on the pattern of mobility of substituents of phospholipid acyl chains was described by Seelig and Seelig [44] using deuterium nuclear magnetic resonance. Lastly, the decrease with calcium treatment of the excimer/monomer fluorescence intensity ratio of dipyrenylpropane confirms the reduction in the dynamic component of membrane fluidity, in this case by monitoring short-range lateral diffusion of the fluorophores.

The lipid composition studies indicate that calcium treatment decreases the arachidonic acid content and the double-bond index of the polar fraction of the membrane lipids. It is reasonable to propose, therefore, that these compositional changes underlie the reduction in membrane fluidity. A relationship between phospholipid acyl chain unsaturation and fluidity is supported by studies of model and natural membranes. *cis*-Unsaturation of phospholipid acyl chains increases the molecular packing areas in monolayers [45,46] and bilayer liposomes [45] and enhances the fluidity of model membranes [44,47]. Changes in the fluidity of biological membranes owing to alterations in the content of unsaturated acyl chains have been observed by a number of investigators [36–39] but not by others [48–50]. The apparent inconsistency can be resolved by taking into account the extent of the overall change in acyl unsaturation as monitored, for example, by the double-bond index. In the first group of studies, changes in lipid fluidity were observed in association with alterations of the double-bond index amounting to 10% or more. King et al. [37] and King and Spector [38], who studied plasma membranes isolated from Ehrlich's ascites cells grown in mice, reported fluidity differences with changes in double-bond index of approx. 18% and 33%. Storch and Schachter [39] observed significant increases in the fluidity of rat hepatocyte plasma membranes when the animals were maintained on a starve-refeed regimen which increased the double-bond index by 20%. Cossins and Prosser [51] noted positive correlations between fluorescence polarization values of diphenylhexatriene and the ratios of saturated/unsaturated fatty acids in the choline and ethanolamine phosphoglycerides of synaptosomal membranes of various animal species. From their published data we calculate that a detectable (approx. 3%) change in diphenylhexatriene fluorescence polarization would require a change in the ratio of saturated/unsaturated fatty acids of approx. 10–12%. A change in double-bond index of 10% or more was not demonstrated in the studies which report no difference in fluidity secondary to modulation of acyl chain unsaturation. Stubbs et al. [48] detected no differences in diphenylhexatriene fluorescence polarization on comparison of plasma membrane fractions of calf thymus lymphocytes grown in media enriched with either linoleic or palmitic acid. As compared to control cultures, the phospholipid double-bond index was changed by only 9% and 5%, respectively, in the media enriched with linoleic and palmitic acid. The results of McVey et al. [49] and Poon et al. [50] are more difficult to evaluate, since the double-bond index cannot be calculated nor the purity of the plasma membrane fractions assessed quantitatively from the data reported. On the basis of the available evidence, therefore, it is reasonable to conclude that the calcium-dependent reduction of 18% in the double-bond index of the hepatocyte plasma membrane polar lipids (Table IV) accounts at least partially for the decrease in lipid fluidity.

The results of the model bilayer studies (Fig. 2, Table VI) support the suggestion that cleavage and loss of phospholipid arachidonoyl residues can decrease the membrane fluidity. The fluidizing effects of arachidonoyl residues are illustrated in Fig. 2. Cleavage and loss of these residues, though not cleavage alone, decreased the fluidity (Table VI), despite the presence of lysophosphatide, which is reported to fluidize and destabilize membranes [41,42,52]. It is noteworthy that membrane destabilization has been observed on treatment of model bilayers or erythrocytes with exogenous lysophosphatides. The hemolytic action of exogenous lysophosphatide is considerably diminished below the critical micellar concentration [42]. Klopfenstein et al. [53] reported that inclusion of lysophosphatidylcholine in dipalmitoylphosphatidylcholine vesicles at a molar ratio of 1 : 1 hardly altered the phase transition temperature, and similar findings were noted by Blume et al. [54]. Further, recent studies demonstrate that aqueous mixtures of equimolar 1-acyllysophosphatidylcholine

and free fatty acids associate to form bilayers rather than micelles [18,19], apparently because a 'close-packing dimer' [19] is formed which is similar in shape and size to the diacylphosphatidylcholine. In summary, cleavage alone of phospholipid acyl residues need not alter bilayer fluidity, and no change was observed in our model studies (Table VI); subsequent loss of arachidonoyl residues, however, can decrease the fluidity. A similar mechanism can also account for the results of Simpkins et al. [55], who reported that treatment of human erythrocyte and lobster axonal membranes with exogenous phospholipase A_2 decreases membrane lipid mobility as monitored by electron spin resonance.

Acknowledgment

The support of National Institutes of Health research grants AM21238, AM01483 and HL16851 and of training grant AM07330 is gratefully acknowledged.

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