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Fluidity characteristics of bovine thyroid plasma membranes

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Highly purified plasma membranes of bovine thyroid were obtained by differential pelleting followed by discontinuous gradient centrifugation in a swing-out rotor. Subfractions of plasma membranes were prepared by affinity chromatography on Con A-Sepharose. The final membrane fractions were enriched 25–30-fold over homogenate in 5′-nucleotidase and alkaline phosphatase and displayed a protein to phospholipid ratio of 1.67 and a cholesterol to phospholipid molar ratio of 0.55. The phospholipid composition did not deviate appreciably from that of whole tissue except for the higher sphingomyelin level (22.5 vs. 14.0%). The predominant fatty acids were palmitic (16:0), oleic (18:1), stearic (18:0) and linoleic (18:2) acid. The physical state of the membrane was studied by (i) calculation of the lipid structural order parameter S_{DPH} from steady-state fluorescence anisotropy determinations of the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH); (ii) estimation of the lateral diffusion coefficient of pyrene following excimer formation. These parameters were determined in native thyroid plasma membranes and in reconstituted vesicles, obtained by detergent dialysis from octylglucoside solubilized membrane components. The presence of membrane protein or neutral lipids induced more restraint on the movements of the fluorophores. The lipid order parameter, S_{DPH} was mainly determined by the neutral lipids. Subfractions of plasma membrane enriched in luminal membranes have a slightly lower fluidity (higher S_{DPH} and lower D_{diff} values) than subfractions enriched in basolateral membranes. This difference appears to be due to both differences in lipid as well as protein composition. Under physiological conditions, no significant alterations in probe dynamics could be observed upon addition of thyrotropin or cholera toxin, even at micromolar concentrations.

Introduction

Several studies have provided evidence indicating that thyroidal plasma membrane lipids might play a modulating role in the state of the thyrotropin receptor and its ability to bind thyrotropin [1–4]. Furthermore, an important role for phospholipids has been implicated in the thyrotropin-responsive adenylate cyclase system [5–10]. From these studies it has been suggested that certain phospholipids (phosphatidylcholine and phosphatidylserine) are involved in the coupling mechanism between the occupied receptor and the catalytic component. A recent study [11] has shown that thyrotropin is able to perturb the

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Abbreviations: N, nuclear fraction; M, mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; P_2, highly purified plasma membranes; PM_1 and PM_2, subfractions of highly purified plasma membranes; I_D/I_M, ratio of fluorescence intensities of pyrene excimer to pyrene monomer; D_{diff}, lateral diffusion coefficient; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene when incorporated into a strain of functioning rat thyroid cells in culture ($FRTL_5$). It was suggested that this receptor-mediated modification of the physical state of the plasma membrane might be a means of initiating and/or regulating non-cAMP-mediated cellular responses. In these studies, however, the nature and physical state of the plasma membrane lipid matrix have not received detailed attention. More specifically, the relation between plasma membrane composition and its 'structural' and 'fluidity' characteristics has not been considered. As part of a systematic study of lipid-protein interactions involved in thyrotropin action, we describe in detail the lipid composition and some physical properties of highly purified plasma membrane fractions and subfractions of bovine thyroid. In order to study the effect of plasma membrane composition on the physical state, reconstitution with the nonionic detergent octylglucoside was used as an approach. The physical characteristics were evaluated by following the motional characteristics of the apolar fluorescent probes diphenylhexatriene and pyrene. The potential perturbing effects of ligands such as thyrotropin and cholera toxin were also investigated.

Materials and Methods

Chemicals. Diphenylhexatriene was obtained from Aldrich. Pyrene was purchased from Janssen Chimica. Thyrotropin came from Armour and cholera toxin from Schwarz/Mann. Boehringer was the source of octyl-β-D-glucopyranoside. Con A-Sepharose was obtained from Pharmacia and α-methyl-D-mannoside from Sigma.

Preparation of highly purified plasma membranes. Subcellular fractionation of bovine thyroid as described by Dierick and Hilderson [12] resulted in a quantitative isolation of five subcellular fractions (N, M, L, P and S). Hypotonic washing (10 mM Tris-HCl (pH 7.4)) of an (L + P) fraction followed by discontinuous sucrose gradient centrifugation in a swing-out rotor (96 300 × *g* for 15 h) resulted in a highly purified plasma membrane fraction P_2 [13].

Subfractionation of plasma membranes. Plasma membranes were subfractionated by affinity chromatography on Con A-Sepharose [14] yielding a nonbinding fraction PM_1 and a binding fraction PM_2. A suspension of 1 mg thyroid plasma membranes in 10 ml buffer 1 (0.02 M Hepes (pH 7.0)/0.14 M KCl) was mixed with 25 ml Con A-Sepharose gel by stirring for 2 min. The mixture was poured into a column and allowed to stand for 20 min at 4°C. A nonbinding fraction (PM_1) was eluted from the column using buffer 1. The column was subsequently exhaustively washed with the same buffer (at least 3-times the bed volume). A second fraction (PM_2) could be eluted with buffer 1, supplemented with 0.1 M α-methylmannoside (buffer 2). To this end, the gel was transferred to a beaker, stirred for 2 min with 10 ml buffer 2, transferred again to the column and eluted. A second binding plasma membrane fraction could eventually be obtained after stirring the gel for an additional period of 15 min.

Chemical analyses. Protein was measured according to the method of Lowry et al. [15] with bovine serum albumin as a standard. Phospholipid phosphorus was assayed according to Rouser et al. [16]. Cholesterol was determined by the method of Hanel and Dam [17]. Sialic acid was assayed according to Warren [18]. Sphingosine was measured following the procedure of Kisic and Rapport [19]. The phospholipid composition of plasma membranes was assayed by performing two-dimensional thin-layer chromatography on precoated silica gel 60 plates (Merck, 10 × 10 cm) on a Bligh and Dyer [20] lipid extract of highly purified plasma membranes using the following solvent systems: (A) chloroform/methanol/methylamine 40% (13 : 6 : 1.5, v/v) and (B) chloroform/acetone/methanol/acetic acid/H_2O (100 : 40 : 20 : 30 : 10, v/v). The fatty acid composition of total phospholipids of plasma membranes was assayed by gas liquid chromatography using a polar capillar column (Carbowax 20). Total phospholipids were transmethylated by heating at 80°C overnight in a 5% sulphuric acid solution in methanol [21]. Identification of unsaturated fatty acids was done by hydrogenation [22].

Determination of enzyme activities. Glucose-6-phosphatase and 5'-nucleotidase were determined according to Morré et al. [23], ($Na^+ + K^+$)-ATPase according to Dornand et al. [24], alkaline phosphatase according to Kind and King [25] and

leucine aminopeptidase according to Berger and Broida [26].

Extraction and isolation of thyroid plasma membrane constituents. The total lipid extract, obtained according to Bligh and Dyer [20], of a P_2 fraction was dissolved in a minimal volume 'pure' chloroform, applied to a silicic acid column and eluted to obtain neutral lipids, neutral glycolipids and phospholipids [2]. Acidic glycolipids were obtained by lipid extraction according to Rouser et al. [27], followed by Folch partition [28], dialysis (48 h) and were finally lyophilized. Glycoproteins were isolated by continuous sucrose gradient centrifugation with octylglucoside as described by Petri and Wagner [29]. Fractions from the protein peak of the gradient were used for the reconstitution studies.

Preparation of large lipid vesicles. Large vesicles were prepared by detergent dialysis with octylglucoside as described by Gonzales-Ros et al. [30]. The final lipid phosphorus concentration was always adjusted to 50 μM. This was important in securing constant 'dilute' anisotropy values.

Diphenylhexatriene and pyrene labelling procedures. Diphenylhexatriene was made up in tetrahydrofuran at a concentration of $4 \cdot 10^{-4}$ M. For labelling, this solution was diluted 1000-fold by adding to a vigourously stirred vesicle buffer solution (1 mM EDTA/10 mM Tris (pH 7.4)/5 mM KCl/262 mM NaCl/2 mM NaN_3). Diphenylhexatriene was added to the lipid vesicles or native membranes at a ratio of 1 molecule diphenylhexatriene for every 250 phospholipid molecules. A stock solution of 1 μM pyrene was prepared by diluting 1000-fold a 1 mM pyrene solution in ethanol with vesicle buffer. The molar ratio of pyrene-to-lipid was always 0.01 (unless otherwise indicated). A plot of I_D/I_M vs. pyrene concentration showed linearity for concentrations up to 10 μM. Fluorescence measurements were performed after 2 h incubation at 37°C of vesicles and probes. Thyrotropin or cholera toxin was added to labelled native membranes or to reconstituted vesicles directly into the cuvette. After 2 min at 37°C under continuous stirring, the fluorescence measurements were performed.

Fluorescence measurements. Static and dynamic fluorescence parameters were recorded on a SLM 4800 spectrofluorometer equipped with a Hewlett-Packard 85 calculator and a 7225A plotter. Temperature was controlled by a Lauda thermostated water-bath and measured inside the fluorescent cuvettes with an AD 590 probe (Analog Devices). Fluorescence lifetimes were determined by the cross-correlation phase method described by Spencer and Weber [31]. The average of the phase and modulation values relative to a glycogen scattering solution was used for excited-state lifetime calculations. For diphenylhexatriene anisotropy and lifetime measurements, the following instrumental conditions were utilized: excitation wavelength was 360 nm and Schott KV399 filters were used in both emission beams. For pyrene, the excitation wavelength was 338 nm and the monomer and excimer fluorescence intensities were measured at 393 and 474 nm, respectively. The excimer lifetime was measured with a Schott KV450 filter in the emission beam. The physical state of thyroid membrane preparations and liposomes with different degrees of reconstitution was evaluated by measuring the steady-state fluorescence anisotropy (r_s) of diphenylhexatriene. This can be resolved into a fast-decaying component r_f (dynamic part) and an infinitely slow-decaying component r_∞ (static part) [32–34]:

$$r_s = r_f + r_\infty$$

where $r_f = (r_0 - r_\infty)/(1 + \tau/\phi)$ and hence $\phi = \tau(r_s - r_\infty)/(r_0 - r_s)$. The term r_0 is the initial anisotropy of fluorescence before any molecular motion takes place (0.395 for diphenylhexatriene), τ is the lifetime and ϕ the rotational correlation time for reorientation along the long molecular axis [35]. The static part r_∞ can be obtained from the empirical relation between r_s and r_∞ [36,37]:

$$r_\infty = \tfrac{4}{3} r_s - 0.10$$

for the range $0.13 < r_s < 0.28$. The lipid order parameter S_{DPH} can be determined from r_∞, since it has been shown for a rodlike probe such as diphenylhexatriene, which is supposed to be aligned with the acyl chains that the limiting fluorescence anisotropy r_∞, is proportional to the square of the lipid order parameter S_{DPH} [32,33]:

$$S^2_{DPH} = \frac{r_\infty}{r_0}$$

Because of the lack of a correlation between fluorescence polarization parameters and lateral diffusion rates of lipids or proteins in cell membranes [38], the lateral diffusion coefficient of pyrene was also determined by following pyrene excimer formation. Coefficients of lateral diffusion (D_{diff}) were calculated according to Galla and Sackmann [39]:

$$D_{diff} = \frac{I'}{\kappa \cdot I} \times \frac{1}{40 \cdot \tau_{excim} \cdot C}$$

where κ is the spectral proportionality coefficient ($\kappa = 0.8$), I the fluorescence intensity of the monomer, I' the fluorescence intensity of the excimer, τ_{excim} the modulation lifetime of the excimer (excitation light modulated at 6 MHz) and C the pyrene concentration defined as the number of pyrene molecules per Å^2. The label concentrations in membranes containing cholesterol is given by $C = R/(F + \alpha F_c)$ where R is the molar ratio of pyrene-to-lipid, α the molar ratio of cholesterol to lipid, F the area per lipid molecule (approx. 58 Å^2) and F_c the area per cholesterol molecule (approx. 33 Å^2).

Results

Subcellular fractionation of a bovine thyroid homogenate by differential pelleting followed by discontinuous sucrose gradient centrifugation yielded a plasma membrane fraction P_2 enriched 25–30-fold in terms of 5′-nucleotidase and alkaline phosphatase (Table I). The purity of the membrane preparations was assessed by measuring the activities of marker enzymes. The relevant data are summarized in Table I. The enrichment factor for succinate dehydrogenase, a mitochondrial marker, was 0.7. There was however still a significant contamination by β-hexosaminidase (lysosomal marker) and glucose-6-phosphatase (endoplasmic reticulum marker) with enrichement factors of 1.4 and 3.9, respectively. Further subfractionation of P_2 by means of affinity chromatography on Con A-Sepharose 4B allowed the separation of these membranes into a nonbinding fraction PM_1 and a binding fraction PM_2. In Table I, the chemical composition of P_2, PM_1 and PM_2 is compared. The cholesterol to phospholipid molar ratio and the protein to phospholipid ratio (w/w) of PM_1 are similar to those found in P_2. This is not surprising, as in terms of proteins, PM_1 represents about 75% of P_2. These ratios however are higher in PM_2.

The chemical composition of whole bovine thyroid tissue and of highly purified plasma membrane (P_2 fraction) is summarized in Table II. The plasma membranes displayed a protein to phospholipid ratio (w/w) of 1.67 and a cholesterol to phospholipid molar ratio of 0.55. The phospholipid composition of plasma membranes did not deviate appreciably from that of whole tissue ex-

TABLE I

CHARACTERIZATION OF THYROID PLASMA MEMBRANES AND PLASMA MEMBRANE SUBFRACTIONS

Specific activities are expressed as nmol/min per mg protein. Cholesterol/phospholipids is a molar ratio. The number of experiments was five, statistics refer to the average deviation.

Fraction	Homogenate	P_2	PM_1	PM_2
% Protein	–	100	72 ± 1	14 ± 1
5′-Nucleotidase	7.7 ±0.5	202.5 ±14.9	235.7 ±15.3	158.2 ±6.4
Alkaline phosphatase	1.2 ±0.4	35.8 ± 6.5	29.9 ± 6.1	53.7 ±7.1
$(Na^+ + K^+)$-ATPase	2.4 ±0.5	51.2 ± 7.8	59.4 ± 7.6	37.8 ±5.4
Leucine aminopeptidase	0.6 ±0.1	11.1 ± 0.9	10.8 ± 0.8	19.7 ±2.1
Glucose-6-phosphatase	2.7 ±0.4	10.5 ± 0.5	11.4 ± 0.6	9.7 ±0.5
β-Hexosaminidase	29.0 ±2.2	40.6 ± 2.9	45.2 ± 5.4	37.7 ±3.8
Succinate dehydrogenase	0.38 ± 0.05	0.27 ± 0.08	–	–
Cholesterol/phospholipids	–	0.55 ± 0.07	0.56 ± 0.06	0.66 ± 0.07
Protein/phospholipids	–	1.67 ± 0.11	1.80 ± 0.13	2.02 ± 0.15

TABLE II

CHEMICAL COMPOSITION OF BOVINE THYROID

All values are expressed as μg/mg protein. All data are average values from three separate experiments. CHE, cholesteryl ester.

Compound	Whole tissue	P_2 fraction
Total phospholipids	61 ±8	592 ±81
Cholesterol (+CHE)	12 ±2	153 ±22
Lipid-bound sialic acid	0.13±0.04	5.2± 1.1
Total asialoglycolipids	0.16±0.02	5.9± 0.5

cept for the higher sphingomyelin level (22.5 vs. 14.0%; Table III). The fatty acid composition of total phospholipids of plasma membranes differed from that of whole tissue in C16:1 fatty acids being present in much higher amounts and in C20:0, C20:4 and C22:6 fatty acids being present in much smaller amounts (Table IV). The ratio of unsaturated over saturated fatty acids was 1.50 for whole tissue and 1.37 for plasma membranes.

The subcellular fractions of bovine thyroid obtained after differential pelleting (M, L and P) displayed considerable differences in fluorescence polarization of diphenylhexatriene (data not shown). In this respect, the L fraction, which is most enriched in plasma membranes, appeared to be the least 'fluid' fraction, whereas the P fraction showed the highest fluidity. Highly purified plasma membranes (P_2 fraction) were still less fluid than the L fraction.

TABLE III

PHOSPHOLIPID COMPOSITION OF BOVINE THYROID

All values are expressed as percent of total phospholipid phosphorus. All data are average values for three separate experiments.

Compound	Whole tissue	P_2 fraction
Phosphatidylinositol	6.5±0.7	5.5±0.6
Phosphatidylserine	5.6±0.8	6.1±0.9
Sphingomyelin	14.0±1.8	22.5±2.5
Lysophosphatidylcholine	43.0±2.0	1.2±0.4
Phosphatidylcholine		38.7±3.4
Alkenylphosphatidylethanolamine	18.3±1.4	15.4±1.2
Phosphatidylethanolamine	9.9±0.7	8.5±0.9
Cardiolipin	2.8±0.5	–
Other phospholipids	<1	2.0±0.3

TABLE IV

FATTY ACID COMPOSITION OF BOVINE THYROID

All values are expressed as percent of total fatty acids. All data are average values from three separate experiments. A and B, aldehydes, hexadecanal and octadecanal; tr., trace.

Fatty acid designation	Whole tissue	P_2 fraction
14:0		3.3±0.4
A	2.3±0.4	2.8±0.2
16:0	22.0±0.5	23.3±1.2
16:1	tr.	7.9±0.8
B	1.5±0.3	1.4±0.2
18:0	13.1±1.1	10.9±0.8
18:1	28.2±0.9	28.9±1.3
18:2	10.6±0.4	11.2±0.6
20:0	1.7±0.3	1.0±0.3
20:4	11.1±0.7	3.4±0.9
22:0	1.7±0.3	1.9±0.4
22:6	7.8±0.9	4.1±1.1

The influence of each membrane constituent on the 'fluidity' was studied by preparing large unilamellar vesicles, obtained by detergent dialysis from octylglucoside-solubilized membrane constituents. All native membrane constituents were incorporated in these large vesicles according to their ratios in a P_2 fraction. The temperature dependence of the fluorescence excited-state lifetime (τ) of diphenylhexatriene, embedded in these large vesicles with a different degree of reconstitution is shown in Fig. 1. The excited-state lifetime seemed to vary considerably not only with temperature but also with membrane composition. Large lipid vesicles prepared from phospholipids alone displayed the lowest τ value. Incorporation of neutral lipids, acidic glycolipids or membrane proteins led to an increase of the τ value, whereas the addition of neutral glycolipids to a decrease. The higher the degree of reconstitution the more the value of the lifetime found in the P_2 fraction was approached. The τ value for the total lipid extract (according to Bligh and Dyer [20]) of a P_2 fraction was intermediate between the values for vesicles prepared from phospholipids alone and from the P_2 fraction. The temperature dependence of the structural order parameter (S_{DPH}) of diphenylhexatriene embedded in these vesicles with a different degree of reconstitution is shown in Fig. 2a,

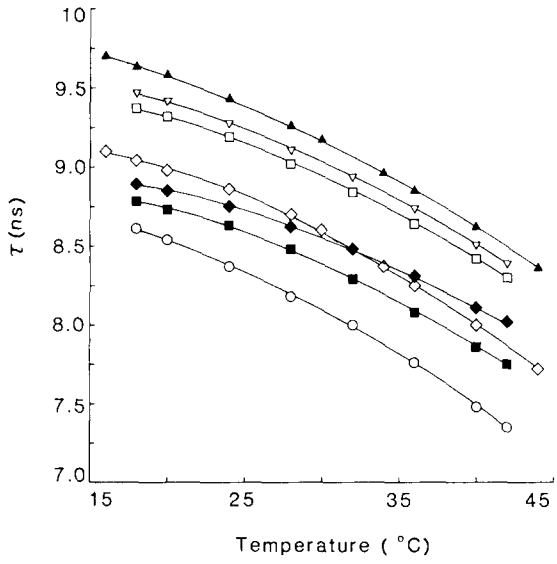

Fig. 1. Temperature dependence of the fluorescence excited-state lifetime τ of diphenylhexatriene embedded in large vesicles prepared by detergent dialysis from a P_2 fraction (▲———▲); a total lipid fraction of P_2 (◇———◇); a phospholipid extract of P_2 (○———○); a mixture of phospholipids/neutral lipids (□———□); a mixture of phospholipids/neutral lipids/neutral glycolipids (■———■); a mixture of phospholipids/neutral lipids/neutral glycolipids/acidic glycolipids (◆———◆); and a mixture of phospholipids/neutral lipids/neutral glycolipids/acidic glycolipids/glycoproteins (▽———▽). All these native membrane constituents were mixed according to their ratio in the P_2 fraction. The diphenylhexatriene to lipid ratio was always kept at 1/250.

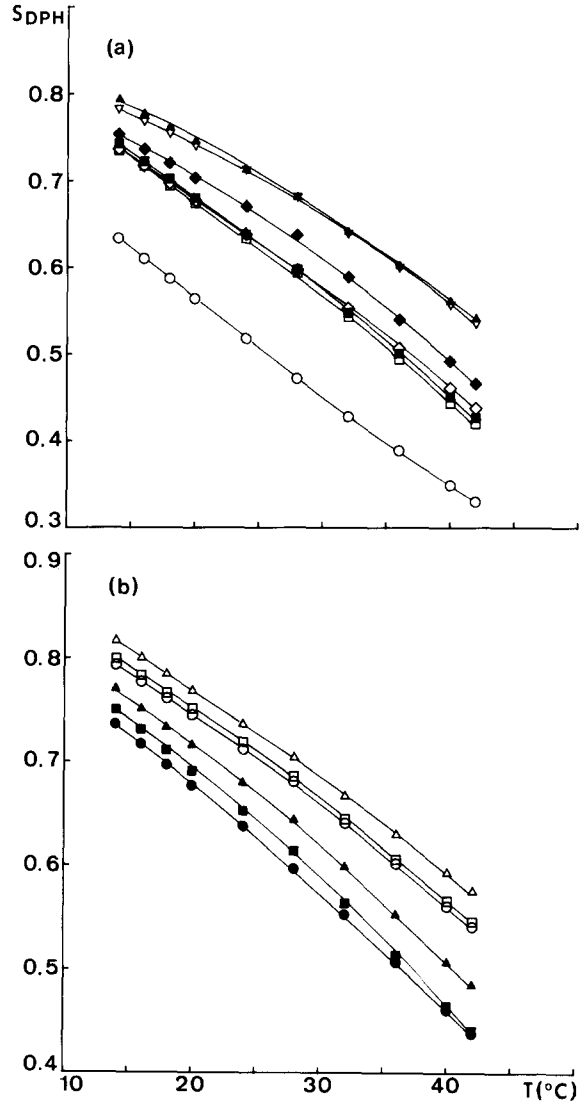

Fig. 2. Temperature dependence of the structural order parameter (S_{DPH}) of diphenylhexatriene embedded in large vesicles prepared by detergent dialysis from: (a) the same reconstituted systems as in Fig. 1. For explanation symbols see legend Fig. 1. (b) a P_2 fraction (○———○); a PM_1 fraction (□———□); a PM_2 fraction (△———△); total lipids P_2 fraction (●———●); total lipids PM_1 fraction (■———■) and total lipids PM_2 fraction (▲———▲). All the native membrane constituents were mixed according to their ratio in a P_2 fraction. The diphenylhexatriene to lipid molar ratio was always 1/250.

revealing no significant breakpoint. Large lipid vesicles prepared from phospholipids alone displayed the lowest S_{DPH} value. Incorporation of neutral lipids, acidic glycolipids or membrane proteins in the phospholipid matrix according to their native ratios provoked an increase in the S_{DPH} value, whereas neutral glycolipids had no distinct effect on this value. The same phenomenon occurred when acidic glycolipids, neutral glycolipids or membrane proteins were incorporated into vesicles containing both phospholipids and neutral lipids (data not shown). After complete reconstitution of all the plasma membrane components, a S_{DPH} value was obtained corresponding nicely to this in native plasma membranes (P_2 fraction). This was also the case when S_{DPH} of vesicles prepared from total lipids of plasma membranes was compared to S_{DPH} found in a reconstituted system containing phospholipids + neutral lipids + neutral glycolipids.

The lateral diffusion coefficient (D_{diff}) of pyrene

was followed as a function of temperature in the same reconstituted systems. Similar fluidity trends were observed as with S_{DPH} and τ, apart from the fact that the incorporation of acidic glycolipids produced an increase in D_{diff} and that the effects of neutral glycolipids and membrane proteins were more pronounced than the effect of neutral lipids. Complete reconstitution again produced a D_{diff} value very similar to that found in native membranes. This was also the case for large lipid vesicles prepared from the total lipid extract of the P_2 fraction and a reconstituted system containing phospholipids + neutral lipids + neutral glycolipids. The excited-state lifetime of the excimer of pyrene had a more pronounced temperature dependency than that of diphenylhexatriene (data not shown) and its increase also paralleled the decrease in fluidity.

A summary of all the fluorescence parameters investigated is given in Table V. From this it is clear that the fluorescence anisotropy r_s is mainly determined by the neutral lipids. The increase in steady-state anisotropy with a higher degree of reconsitution is due to an increase in the static part r_∞ and hence to S_{DPH} (lipid order). The presence of neutral lipids or membrane proteins actually leads to a decrease in the rotational correlation time ϕ (dynamic effect). The lipid order parameter (S_{DPH}) is mainly determined by the neutral lipids, whereas the D_{diff} is more sensitive to membrane proteins. Incorporation of membrane proteins markedly decreases D_{diff} by approx. 50% but provokes a 6% increase in S_{DPH}. Neutral glycolipids do not display any significant effect on S_{DPH}. However, they increase D_{diff}. Acidic glycolipids enhance S_{DPH} as well as D_{diff}. Thus, an increase in S_{DPH} is not always paralleled by a decrease in D_{diff}.

The fluidity characteristics of plasma membrane subfractions (PM_1 and PM_2) and unilamellar vesicles prepared from their total lipid extracts were also studied. The temperature dependence of S_{DPH} for the PM_1 and PM_2 fractions again did not reveal any significant breakpoint (Fig. 2b). From Table V it is evident that PM_1 membranes are slightly more fluid than those in PM_2. As shown in Fig. 3b, nearly identical D_{diff} values are found in large unilamellar vesicles prepared from the lipid extracts of both PM_1 and PM_2, suggesting that membrane proteins and/or acidic glycolipids could be responsible for the difference in fluidity. However, the difference in S_{DPH} values obtained in PM_1 and PM_2 was also found in vesicles prepared from their total lipid extracts.

TABLE V

FLUORESCENCE PARAMETERS FOR DIPHENYLHEXATRIENE AND PYRENE IN BOVINE THYROID PLASMA MEMBRANES AND IN RECONSTITUTED LARGE UNILAMELLAR VESICLES AT 24°C

For experimental details and definitions of r_s, r_f, r_∞, S_{DPH}, etc. see Materials and Methods. Results are means of three separate experiments. PL, phospholipids; NL, neutral lipids; AGL, acidic glycolipids; NGL, neutral glycolipids; MP, membrane proteins; tlip, total lipids.

	r_s	r_∞	r_f	S_{DPH}	D_{diff} (cm^2/s)($\times 10^8$)	τ (ns)	ϕ (ns) $r_\infty \neq 0$
P_2	0.225 ± 0.003	0.200 ± 0.004	0.025 ± 0.005	0.712 ± 0.007	0.98 ± 0.13	9.43 ± 0.21	1.39 ± 0.28
PM_1	0.229 ± 0.003	0.205 ± 0.004	0.024 ± 0.005	0.720 ± 0.007	1.01 ± 0.12	9.22 ± 0.25	1.33 ± 0.28
PM_2	0.234 ± 0.004	0.212 ± 0.005	0.022 ± 0.006	0.733 ± 0.009	0.82 ± 0.11	9.34 ± 0.23	1.28 ± 0.34
tlip P_2	0.196 ± 0.007	0.161 ± 0.009	0.035 ± 0.011	0.638 ± 0.018	1.78 ± 0.26	8.84 ± 0.36	1.55 ± 0.42
tlip PM_1	0.202 ± 0.006	0.169 ± 0.008	0.033 ± 0.010	0.654 ± 0.015	1.76 ± 0.22	8.29 ± 0.35	1.41 ± 0.42
tlip PM_2	0.214 ± 0.007	0.185 ± 0.009	0.029 ± 0.011	0.684 ± 0.017	1.72 ± 0.24	8.03 ± 0.37	1.29 ± 0.48
PL	0.155 ± 0.005	0.107 ± 0.007	0.048 ± 0.009	0.520 ± 0.017	1.51 ± 0.18	8.37 ± 0.11	1.67 ± 0.32
PL + NL	0.194 ± 0.003	0.159 ± 0.004	0.035 ± 0.005	0.634 ± 0.008	1.12 ± 0.16	9.20 ± 0.41	1.60 ± 0.23
PL + NL + NGL	0.196 ± 0.004	0.161 ± 0.005	0.035 ± 0.006	0.638 ± 0.010	1.82 ± 0.22	8.65 ± 0.21	1.52 ± 0.26
PL + NL + NGL + AGL	0.208 ± 0.003	0.177 ± 0.004	0.031 ± 0.005	0.669 ± 0.008	2.15 ± 0.32	8.75 ± 0.22	1.45 ± 0.23
PL + NL + NGL + AGL + MP	0.226 ± 0.007	0.201 ± 0.009	0.025 ± 0.011	0.713 ± 0.016	1.04 ± 0.20	9.27 ± 0.38	1.37 ± 0.59

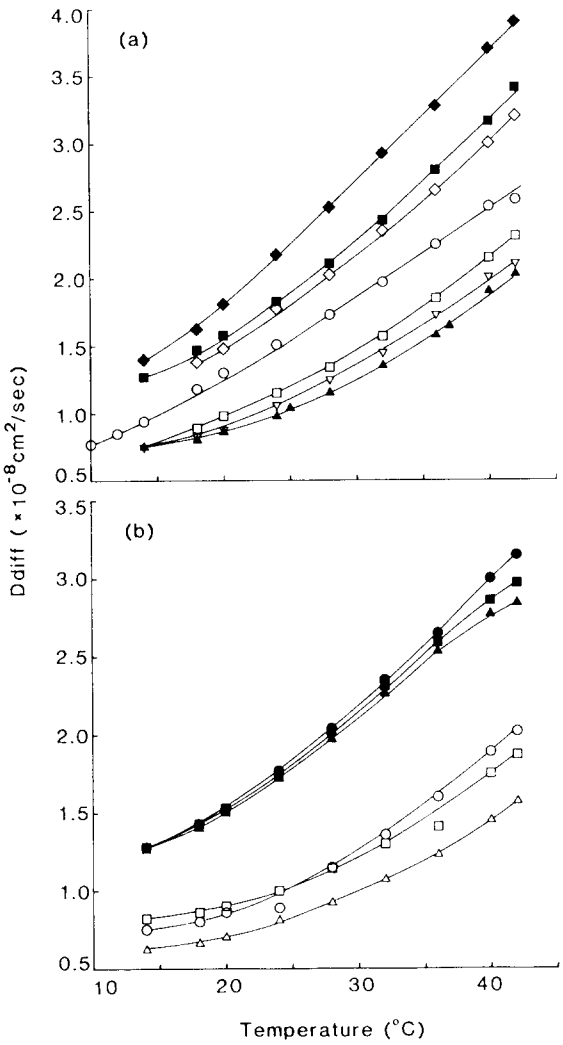

Fig. 3. Temperature dependence of the lateral diffusion coefficient (D_{diff}) of pyrene embedded in large unilamellar vesicles prepared by detergent dialysis from: (a) the same reconstituted systems as in Fig. 1 (for explanation of symbols, see legend Fig. 1); (b) a P_2 fraction (○———○); a PM_1 fraction (□———□); a PM_2 fraction (△———△); total lipids P_2 fraction (●———●); total lipids PM_1 fraction (■———■) and total lipids PM_2 fraction (▲———▲). All the native membrane constituents were mixed according to their ratio in a P_2 fraction. The pyrene to lipid molar ratio was always 0.01.

Therefore, it is suggested that both lipid as well as protein are at the basis of the observed differences in fluidity. This view is supported by the different protein to phospholipid (w/w) and cholesterol to phospholipid molar ratios in PM_1 and PM_2.

Finally, under physiological conditions, addition of thyrotropin and of cholera toxin at different concentrations up to micromolar levels did not show any detectable effect on the probe dynamics for the different plasma membrane (sub)fractions and reconstituted systems studied.

Discussion

Although several methods for the isolation of plasma membranes from thyroids have been reported [40], their chemical composition and physical state have not been analyzed in detail. In the present report, bovine thyroid plasma membranes were isolated by differential pelleting followed by discontinuous sucrose gradient centrifugation. Based on 5′-nucleotidase and alkaline phosphatase specific activities these membranes were purified 25–30-fold over the homogenate. The phospholipid composition did not deviate appreciably from that of whole tissue except for the higher sphingomyelin level. This composition as well as the molar ratios of cholesterol to phospholipids, sphingomyelin to phospholipids and the protein to phospholipid ratio all closely resemble those reported for rat liver plasma membranes [41,42]. Subfractionation of plasma membranes by affinity chromatography on Con A-Sepharose 4B yielded fractions enriched in luminal membranes and basolateral membranes, respectively, as indicated by high and low leucine aminopeptidase activities. Indeed, subfractions of plasma membranes which can be separated may reflect the existence of membrane heterogeneity. For thyroid, this view is substantiated by the different distribution patterns observed for several plasma membrane associated enzymes (Table I). The specific activities of 5′-nucleotidase and of $(Na^+ + K^+)$-ATPase are enhanced in PM_1, whereas those of leucine aminopeptidase and alkaline phosphatase are enriched in PM_2. In several epithelial systems, leucine aminopeptidase has been shown to be mainly located in the apical cell surface [43]. Recent electron microscopic studies using horseradish peroxidase-labelled antibodies directed against leucine aminopeptidase (Herzog, V., personal communication) strongly suggest that also the thyroidal leucine aminopeptidase is predominantly located at the apical cell surface. $Na^+ + K^+$-dependent ouabain-sensitive ATPase at the other hand ap-

pears to be mainly located in basolateral membranes [44]. Therefore, from the distribution of these enzymes over PM_1 and PM_2 it is suggested that the nonbinding fraction PM_1 is enriched in basolateral membranes and the binding fraction PM_2 in apical membranes.

The physical nature of bovine thyroid plasma membranes and their subfractions was investigated by measuring the anisotropy of the fluorescence from the apolar probe molecule diphenylhexatriene. This probe partitions equally between gel and liquid crystalline phases of model bilayer membranes [45]. Time-resolved fluorescence anisotropy decay measurements [34] and differential polarized phase fluorometry studies [46] using diphenylhexatriene have shown that the degree of hindrance of its rotation more than the rate of rotation is altered by the physical state of a membrane with a relatively high degree of order. Therefore, in this study parameters giving a good indication of the order of the membrane lipids (S_{DPH}, r_∞), parameters more related to the rates of motion of the fatty acid chains in the bilayer (r_f, ϕ) and parameters depending on both range and rate of motion of the fatty acid chains in the bilayer (r_s) were calculated. Since the mobility characteristics of diphenylhexatriene do not necessarily reflect lateral translational diffusion rates of lipids or proteins in cell membranes [38] the lateral diffusion coefficient of the hydrophobic fluorescent probe pyrene was also measured by following its excimer formation.

Our data clearly indicated that the excited-state lifetime of diphenylhexatriene varied considerably with both temperature and membrane composition. Heterogeneity analysis of phase and modulation-average lifetimes for diphenylhexatriene in these different reconstituted bilayer systems and native membranes were consistent with a monoexponential decay model. This is in contrast with several pulse fluorimetric studies which revealed biexponential decay kinetics for diphenylhexatriene in artificial vesicle-preparations as well as native membranes [47]. It should, however, be noted that, using analogous systems, with the phase and modulation technique in most cases no similar biexponential decays were observed [47]. High standard errors in lifetime determinations were thought to be partially responsible for this discrepancy [47].

When diphenylhexatriene was incorporated into aliquots of different subcellular fractions of bovine thyroid tissue at the same probe to lipid ratio it was observed that the steady-state anisotropy r_s was considerably higher in fractions enriched in plasma membranes (L fraction and P_2 fraction). This indicated that the structural order of thyroid plasma membranes is greater than that of the intracellular membranes which is in agreement with their higher cholesterol to phospholipid and sphingomyelin to phospholipid molar ratios. Subfractions of thyroid plasma membranes enriched in luminal membranes (PM_2) displayed a somewhat lower fluidity than their basolateral counterpart (enriched in PM_1) as evidenced by all fluorescence parameters studied. It is rather unlikely that this difference in fluidity is the result of a preferential retention of contaminating membrane fragments in PM_1 or PM_2 because of the lack of any significant differential enrichment of contaminating membrane markers in each subfraction. The difference in fluidity could however be a consequence of differences in protein composition of PM_1 and PM_2 which are probably maintained in vivo by their links to elements of the cytoskeleton as well as of differences in lipid composition. These results point to the presence of a lateral fluidity gradient in the plasma membranes of thyroid follicular cells. The existence of a similar fluidity gradient has already been proposed for rat enterocytes [48] and dog kidney epithelial cells [49]. The physiological relevance of such a fluidity gradient remains to be established. At the other hand, the lower fluidity in PM_2 could also reflect a general property of microvillus membranes. Indeed, the canalicular fraction of rat hepatocyte plasma membranes, microvillus, also displays less fluidity than their other plasma membrane counterparts [42,50].

Plots of S_{DPH} and D_{diff} for plasma membranes and their subfractions as a function of temperature did not reveal any significant breakpoints, indicating that in the temperature range studied no lipid phase transitions would occur. Similar results were already reported for other types of biological membranes [36]. In order to study the relation between thyroid plasma membrane composition and their physical characteristics, large unilamellar vesicles were prepared by detergent dialysis using

octylglucoside. It has been shown that in these artificial vesicles glycoproteins and glycolipids have the same topological distribution as their native counterparts [29]. Moreover, upon complete reconstitution of thyroid plasma membranes a S_{DPH} value was obtained almost identical to that found for native plasma membrane vesicles. As a consequence, large vesicles prepared by detergent dialysis of octylglucoside are suitable systems for studying thyroid plasma membrane fluidity and the contribution of plasma membrane constituents to this fluidity. When using such vesicles for diphenylhexatriene anisotropy measurements it was noted that the standard deviations for r_s were on the average 10-times lower than when using native plasma membrane vesicles. This probably resulted from the strong reduction in curvature in these large vesicles. Plots of S_{DPH} and D_{diff} for artificial vesicles with different degree of reconstitution again did not reveal any significant breakpoint in function of temperature.

Native plasma membrane vesicles showed the highest lipid order in comparison to intracellular membranes and to reconstitution systems. This phenomenon was paralleled by a higher molar ratio of cholesterol to phospholipids. The fluidity of plasma membranes of bovine thyroid is less than average and similar to that observed for rat liver plasma membranes and mouse thymocytes plasma membranes [36] (S_{DPH} bovine thyroid plasma membranes is 0.71; S_{DPH} rat liver plasma membranes is 0.74 and S_{DPH} mouse thymocyte plasma membranes is 0.71). As for other membrane systems [36], the lipid order parameter S_{DPH} of bovine thyroid plasma membranes was mainly determined by the neutral lipids. The latter however did not seem to increase the viscous drag imposed on the rotation of diphenylhexatriene as evidenced by the decrease in rotational correlation time ϕ upon addition of neutral lipids. A further decrease of ϕ was observed upon incorporation of plasma membrane proteins in artificial vesicles with different degree of reconstitution, in contrast with the data reported by Heyn [33].

The lowest D_{diff} of pyrene was found in native plasma membranes. Upon complete reconstitution, again an almost identical value was obtained. The difference in D_{diff} values obtained for native plasma membranes and artificial vesicles prepared from a total lipid extract of plasma membranes was however much more pronounced that the difference in their corresponding S_{DPH} values. This indicates taht D_{diff} in contrast to S_{DPH} is strongly dependent on plasma membrane proteins. Furthermore, in contrast to what one would expect from their effects on S_{DPH}, neutral glycolipids and to a smaller extent acidic glycolipids markedly increase D_{diff}. This discrepancy was observed upon incorporation of neutral glycolipids and acidic glycolipids into artificial phospholipid vesicles as well as in artificial (phospholipids + neutral lipids) vesicles. Although it has been suggested that the structural order parameter S_{DPH} is a better indicator of membrane fluidity than does microviscosity [36], there is not always a good correlation between changes in S_{DPH} and changes in D_{diff}.

Both neutral lipids and proteins have a major effect on thyroid plasma membrane fluidity. However, estimation of their relative contribution to membrane fluidity is dependent on the type of fluorescence probe technique applied, S_{DPH} being mainly affected by the presence of neutral lipids and D_{diff} being more sensitive to plasma membrane proteins.

In contrast to the rat liver system where it has been shown that insulin produces a rapid and marked increase in membrane fluidity [51], addition of thyroid effectors such as thyrotropin and cholere toxin did not result in a significant effect on bovine thyroid plasma membrane fluidity. These data are in contrast to those reported by Beguinot et al. [11] where it was shown that TSH caused a significant increase in the fluorescence polarization of diphenylhexatriene when incorporated into a strain of functioning rat thyroid cells. However, one has to consider that Beguinot et al. were using viable cells where during the 30 min preincubation at 37°C a progressive incorporation of this fluorescent probe in intracellular membranes (endocytosis, membrane fusion, recycling etc.) might have occurred. Thyrotropin, in turn, by affecting these membrane phenomena, might change the fluidity values which are in fact a weight average of all labelled lipid domains [52].

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