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LIPID FLUIDITY OF HEPATOCYTE PLASMA MEMBRANE SUBFRACTIONS AND THEIR DIFFERENTIAL REGULATION BY CALCIUM

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Rat hepatocyte plasma membranes were subfractionated by several methods into canalicular, sinusoidal and mixed contiguous plus sinusoidal membranes. Assessment of lipid fluidity by steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene and 12-(9-anthroyloxy)stearate indicates that the canalicular fraction is less fluid than the other membranes. Incubation with calcium decreases the fluidity of the sinusoidal and contiguous membranes by altering the lipid composition, an action which is not reversed by subsequent chelation of the cation. This effect of calcium is not observed in canalicular membranes.

Prior studies demonstrate that protein-mediated functions of rat hepatocyte plasma membranes can be regulated by the motional freedom, or 'fluidity' **, of the membrane lipids [1–4]. Moreover, calcium ions, which regulate many membrane processes [5,6], decrease the lipid fluidity of hepatocyte plasma membranes in vitro by influencing membrane-bound enzymes to alter the lipid composition [7,8]. Inasmuch as the hepatocyte plasma membrane consists of morphologically distinct regions [9,10], i.e. canalicular, sinusoidal and contiguous membranes, it was of interest to separate the membrane fractions and to compare the lipid fluidity and the response to calcium described above. The experiments below demonstrate that the lipid fluidity of the canalicular membranes is significantly less than that of the other fractions. The foregoing response to calcium, on the other hand, is observed only in the more fluid sinusoidal and contiguous membranes.

Subfractions of rat hepatocyte plasma membranes were prepared by three methods. A procedure [11] based on the rate-zonal centrifugation method of Wisher and Evans [12] yielded three fractions, characterized as described previously [11]: zonal light (highly enriched for canalicular membranes; enzyme specific activity ratios [purified membrane]/[homogenate]: 5′-nucleotidase, 61.4; bilirubin glucuronoside glucuronosyltransferase, 36.5; succinate dehydrogenase, < 0.01; glucose-6-phosphatase, 0.02); zonal heavy A and zonal heavy B (mixtures of contiguous and sinusoidal membranes as judged by enzyme markers and the occurrence of desmosomes and gap junctions [11,12]; specific activity ratios for fractions A and B, respectively: 5′-nucleotidase, 7.0 and 3.3; bilirubin glucuronoside glucurono-

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** A discussion of our use of the term 'lipid fluidity' as applied to anisotropic bilayer membranes is given in Refs. 16 and 18. Briefly, it is used here to express the relative motional freedom of the lipid molecules or substituents thereof, combining in the one term concepts of both rates of movement and extent of movement.

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate.

TABLE I

ANISOTROPY PARAMETERS OF DIPHENYLHEXATRIENE AND 12-ANTHROYLOXYSTEARATE IN SUBFRACTIONS OF RAT HEPATOCYTE PLASMA MEMBRANES

Estimations were at 24°C and values are means ± S.E. Values for the Wisher and Evans fractions are for five separate membrane preparations and those for the remaining fractions are for three membrane preparations. *P* values for differences from the canalicular fractions were calculated by Student's *t*-test for paired comparisons.

Preparation method	Membrane subfraction	Diphenylhexatriene		12-Anthroyloxystearate	
		$[(r_o/r)-1]^{-1}$	*P*	$[(r_o/r)-1]^{-1}$	*P*
Wisher and Evans [11,12]	canalicular	3.78 ± 0.40		0.49 ± 0.05	
	contiguous and sinusoidal, A	2.92 ± 0.35	< 0.001	0.35 ± 0.05	< 0.01
	contiguous and sinusoidal, B	2.84 ± 0.34	< 0.005	0.28 ± 0.02	< 0.025
Inoue et al. [13]	canalicular	2.43 ± 0.07			
Inoue et al. [14]	sinusoidal	1.32 ± 0.03	< 0.001		

syltransferase, 21.4 and 10.0; succinate dehydrogenase, < 0.01 each; glucose-6-phosphatase, 0.02 each). The second method [13] employed nitrogen cavitation and calcium treatment to isolate highly purified canalicular membranes (specific activity ratios: alkaline phosphatase, 54.8; γ-glutamyltransferase, 54.9; aminopeptidase M, 56.6; $(Na^+ + K^+)$-dependent adenosine triphosphatase, 2.5; succinate dehydrogenase, 0.1). Lastly, sinusoidal plasma membranes were isolated [14] by Ficoll-sucrose density gradient centrifugation of post-mitochondrial supernatants (specific activity ratios: $(Na^+ + K^+)$-dependent adenosine triphosphatase, 20.0; alkaline phosphatase, 1.6; γ-glutamyltransferase, 3.0; succinate dehydrogenase, 0.05; substantially free of desmosomes and gap junctions).

Lipid fluidity was assessed by the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene and DL-12-(9-anthroyloxy)stearate (Molecular Probes, Junction City, OR), as previously described [15,16]. The fluorescence anisotropy, *r*, was estimated at 24°C, using membrane suspensions equivalent to 50 μg of protein per ml of 5 mM Tris buffer of pH 7.4 containing 146 mM NaCl plus 4 mM KCl. Results are expressed as the anisotropy parameter, $[(r_o/r)-1]^{-1}$, where r_o is the maximal limiting anisotropy, taken as 0.365 for diphenylhexatriene [17] and 0.285 for 12-anthroyloxystearate [15]. Within the limitations discussed previously [18] the anisotropy parameter varies directly with the apparent rotational relaxation time of the probe [19] and thus inversely with the fluidity. Total fluorescence intensity ($2I_\perp + I_\parallel$, where $I_\perp$ and $I_\parallel$ are the intensities of the emitted light oriented, respectively, perpendicular and parallel to the plane of the exciting beam) was quantified to monitor possible changes in fluorescence lifetime. Corrections for light scattering (membrane suspensions lacking fluorescent probe) and fluorescence in the ambient medium (quantified by pelleting the membranes after each estimation) were made routinely. Light scattering corrections were always < 5% of the total signal and did not differ significantly in various preparations.

The results summarized in Table I indicate that the diphenylhexatriene anisotropy parameter is approx. 29–33% greater ($P < 0.005$) in the canalicular as compared to the contiguous plus sinusoidal fractions prepared by zonal centrifugation. The corresponding 12-anthroyloxystearate values of the canalicular membranes are greater by 39% ($P < 0.01$) and 77% ($P < 0.025$), as compared to the zonal heavy fractions A and B, respectively. Membrane fractions isolated by the other procedures yielded somewhat lower diphenylhexatriene $[(r_o/r)-1]^{-1}$ values as compared to the Wisher and Evans method * (Table I). Here again,

* The lower anisotropy values may be due to the somewhat higher content of intracellular organellar contaminants, as judged, for example by the higher specific activity ratios of succinate dehydrogenase (mitochondrial marker) listed above.

however, the canalicular membrane value exceeded that of the sinusoidal fraction by approx. 84% ($P < 0.001$). It is noteworthy that the total fluorescence intensity observed in the canalicular preparations equaled or exceeded that of the other fractions in all the foregoing experiments. Hence the greater canalicular $[(r_o/r) - 1]^{-1}$ values signify decreased fluidity rather than decreased fluorescence lifetimes of the probes.

To explore the effects of calcium, membrane suspensions were incubated with diphenylhexatriene [7] in the isotonic buffer described above modified to contain 0.1 mM ethylene glycol bis-(β-aminoethyl ether)-*N*,*N'*-tetraacetate (EGTA) of pH 7.4 and either no $CaCl_2$ or 4 mM $CaCl_2$. After shaking at 37°C for 2 h, EGTA (pH 7.4) was added to all samples to a final concentration of 8 mM prior to estimating the fluorescence anisotropy. As noted previously [7], chelation of Ca^{2+} after the incubation eliminates possible effects of the cation owing to direct binding, but does not reverse the decrease in fluidity owing to metabolic alterations of the lipids. The results summarized in Table II demonstrate that calcium increased the diphenylhexatriene $[(r_o/r) - 1]^{-1}$, i.e., decreased the fluidity, of the zonal heavy (sinusoidal plus contiguous membrane) fractions A and B by approx. 21% ($P < 0.05$) and 41% ($P < 0.05$), respectively. Similarly, the cation increased the diphenylhexatriene anisotropy parameter of the sinusoidal fraction prepared by the alternative procedure by approx. 23% ($P < 0.05$). Canalicular membranes prepared by either procedure did not show this effect of calcium.

The increased lipid fluidity of the contiguous and sinusoidal membranes as compared to the canalicular preparations is consistent with the observations [20] that the zonal heavy fractions A and B have lower cholesterol/phospholipid molar ratios and lower content of total cholesterol and sphingomyelin. Whether other factors, e.g., saturation or length of the fatty acid side chains or the influence of protein-lipid interactions, also play a role is unknown. The functional significance of the lower fluidity of the canalicular membranes is similarly unknown, although an interesting possibility is that it protects the organelle from the detergent action of bile salts. Erythrocyte membranes of low fluidity are reported to be more resistant to destabilization by detergents [21].

The foregoing effects of calcium on the fluidity of sinusoidal and contiguous, but not canalicular, membranes points to the existence in the former fractions of Ca^{2+}-modulated enzymes capable of altering the lipid composition and fluidity and, thereby, of regulating intrinsic membrane activities. Among the intrinsic activities known to be localized in sinusoidal and contiguous membranes, to be inhibited by ambient Ca^{2+} and to be sensitive to lipid fluidity are $(Na^+ + K^+)$-dependent

TABLE II

EFFECTS OF INCUBATION WITH CALCIUM ON THE ANISOTROPY PARAMETER OF DIPHENYLHEXATRIENE IN RAT HEPATOCYTE PLASMA MEMBRANE SUBFRACTIONS

Fluorescence anisotropy was estimated at 24°C. Values are means ± S.E. for four determinations (Wisher and Evans fractions) or three determinations (other fractions) and two sets of membrane preparations each. Membranes were treated with 8 mM EGTA prior to estimation of fluorescence anisotropy. *P* values for effects of Ca^{2+} were calculated by Student's *t*-test for paired comparisons. n.s., not significant.

Preparation method	Membrane subfraction	Diphenylhexatriene $[(r_o/r)-1]^{-1}$		
		No Ca^{2+}	4 mM Ca^{2+}	*P*
Wisher and Evans [11,12]	canalicular	4.28 ± 0.66	4.41 ± 0.70	n.s.
	contiguous and sinusoidal, A	3.25 ± 0.58	3.95 ± 0.69	< 0.05
	contiguous and sinusoidal, B	3.03 ± 0.51	4.26 ± 0.84	< 0.05
Inoue et al. [13]	canalicular	2.48 ± 0.12	2.51 ± 0.16	n.s.
Inoue et al. [14]	sinusoidal	1.34 ± 0.05	1.65 ± 0.10	< 0.05

adenosine triphosphatase [22–26], adenylate cyclase [1,2,12,27] and K^+ permeability [28–30].

Lastly, it is interesting to compare the functional and structural differentiation of the plasma membranes of hepatocytes, small intestinal enterocytes and renal tubular cells. In both the enterocyte [19] and renal tubular cell [31] the luminal, microvillus membrane is considerably less fluid as compared to the contraluminal, basolateral membrane; and the more fluid contraluminal region contains the $(Na^+ + K^+)$-dependent adenosine triphosphatase. By analogy, the canalicular region of the hepatocyte membrane represents the less fluid region of the cell membrane, whereas the sinusoidal and contiguous membranes are more fluid and contain the $(Na^+ + K^+)$-dependent adenosine triphosphatase.

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