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Medium Viscosity Regulates the Activity of Membrane-Bound and Soluble Phospholipase A_2[†]

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ABSTRACT: Medium viscosity is a regulator of very low density lipoprotein production by cultured hepatocytes; their secretion and synthesis are inversely proportional to the extracellular fluid viscosity. The possibility that the mechanism of this extracellular effect on cell function involves modulation of cell membrane component(s) was considered. Along with this assumption, we studied the effect of medium viscosity on the activity of phospholipase A_2 (PLA_2), an enzyme present in the cell surface membrane, and the activity has been correlated with cellular secretion. We have found that culture medium viscosity inhibits the activity of PLA_2 in the plasma membrane of cultured liver cells, concomitantly with the inhibition of lysosomal enzyme and lipoprotein secretion. It was also found that the degradation of liposomal phosphatidylcholine by soluble snake venom PLA_2 is inversely proportional to the solvent viscosity. The possibility that the effect of medium viscosity on the enzymatic reaction involves the modulation of dynamic properties of membrane phospholipids was then considered. This hypothesis was examined by monitoring the fluorescence depolarization of fluorophores incorporated into phospholipid vesicles. No significant effect of the solvent viscosity on the phospholipid bilayer was observed. It is proposed that the regulation of cellular secretion by extracellular fluid viscosity involves modulation of the cell membrane PLA_2 activity.

The viscosity of blood and plasma is elevated in numerous pathological conditions and is considered a risk factor for coronary heart disease (Lowe et al., 1981). It has been studied mainly in relation to circulation and hemodynamics. In recent years, more attention has been paid to the role of fluid viscosity in biochemical and cellular processes, as it has been shown that solvent viscosity is an important determinant in protein dynamics and enzyme–substrate interaction (Gavish & Werber, 1979; Breece, et al., 1980; Mckinnie & Olson, 1981; Sawicki & Khaleque, 1983). The relevance of extracellular fluid viscosity in cell function has been demonstrated by Yedgar et al., who showed that viscosity is a regulator of lipoprotein metabolism, both in vivo and in cultured hepatocytes (Yedgar et al., 1982, 1985). Increasing the plasma viscosity of hyperlipidemic rats markedly reduced plasma triglyceride and cholesterol levels (Yedgar et al., 1985). In hepatocyte cultures, the viscosity of the extracellular fluid has been shown to be a regulator of very low density lipoprotein (VLDL)[1] production (Yedgar et al., 1982). Increasing medium viscosity linearly inhibited secretion and synthesis of protein and lipid VLDL components, while their cellular levels remained unaltered (Yedgar et al., 1982). Concordant with these findings, we observed that medium viscosity had an immediate inhibitory effect on the secretion of lysosomal enzymes from cultured liver cells (Yedgar et al., 1986b), indicating that the viscosity affects primarily exocytosis prior to its effect on synthesis. In these studies, medium viscosity was modulated by the addition

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[1] Abbreviations: PLA_2, phospholipase A_2; PC, phosphatidylcholine; C_6-NBD-PC, 1-acyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl]phosphatidylcholine; C_6-NBD-FA, C_6-NBD fatty acid; DOPC, dioleoylphosphatidylcholine; HMEM, HEPES-buffered minimum essential medium; DME, Dulbecco-modified Eagle's medium; Dex, Dextran T-500; Xa, xanthan gum; MeC, methylcellulose; DPH, 1,6-diphenyl-1,3,5-hexatriene; ANS, 1-anilinonaphthalene-8-sulfonic acid; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; VLDL, very low density lipoprotein; 4MU, 4-methylumbelliferone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TLC, thin-layer chromatography.

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of various cell-impermeable macromolecules, thus exerting an extracellular effect on cell function.

In searching for the mechanism of this phenomenon, we considered the possibility that extracellular fluid viscosity modulates the activity of enzymes that are located at the plasma membrane and are involved in secretion. Such an enzyme is phospholipase A_2 (PLA_2), present in the plasma membrane of liver as well as other cells (Van den Bosch, 1982), and its activity has been correlated with cellular secretion capacity (Vogt, 1978; Martin & Lagunoff, 1982; Blackwell & Flower, 1983; Grandison, 1984).

Plasma membrane enzymes are likely to be influenced by dynamic properties of the cell surface membrane which, in turn, depend on the topological state of the cell. These properties are altered when the cell is detached from its substratum or when the membrane is isolated (Russel & Esser, 1979). It was desirable, therefore, to study membrane PLA_2 activity in the intact cell in culture. For this purpose, we employed a method described previously (Yedgar et al., 1986a) which was further elaborated in the present study. This method was used to examine the effect of medium viscosity on the activity of liver cell membrane PLA_2 with respect to regulation of cellular secretion.

Materials and Methods

Materials. The following materials were purchased as indicated: Dulbecco-modified Eagle's medium (DME) and minimum essential medium supplemented with 20 mM HEPES (HMEM) from Gibco Laboratories; collagenase CLS II from Worthington; Dextran T-500 (Dex) from Pharmacia; xanthan gum (Xa) from Kelco (division of Merck, Inc.): Methylcellulose (MeC) MX880 from Matheson Coleman and Bell; 1-acyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl]phosphatidylcholine (C_6-NBD-PC), *N*-NBD-phosphatidylethanolamine (*N*-NBD-PE), and dioleoylphosphatidylcholine (DOPC) from Avanti Biochemical; egg phosphatidylcholine (egg PC) from Lipid Products; 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-anilinonaphthalene-8-sulfonic acid (ANS) from Molecular Probes; *Naja mussambica* phospholipase A_2, pentenoic acid, 4MU-β-D-galactoside, and 4MU-β-D-glucosaminide from Sigma.

Hepatocyte Culture. Rat liver cells were isolated and cultivated as primary monolayers on fibronectin-coated plates in serum-free DME, as described previously. The cells were cultivated for 24 h, to obtain confluence, prior to use (Yedgar et al., 1982).

Medium viscosity was raised by the addition of Dex, MeC, or Xa. As previously discussed (Yedgar et al., 1982), the use of macromolecules which differ in chemical characteristics and in their capacity to increase solution viscosity enables one to differentiate viscosity from other variables, such as osmolarity or chemical interaction. For example, the molecular weight of xanthan is 2 million (Dintzis et al., 1970) (that of Dex being 500 000). Its capacity to increase viscosity is about 200 times that of Dex. Thus, elevation of medium viscosity to 4 cP requires only 0.02% Xa (less than 0.1 μM), as compared with 0.2% MeC (about 30 μM) or 4% Dex (about 200 μM) (Yedgar et al., 1982). These materials are not toxic and are widely used in the food and pharmaceutical industries. Relative viscosities were determined by measuring the flow times of the media through a capillary viscometer (Cannon Instrument Co., State College, PA) at 37 °C (Yedgar et al., 1982).

Unilamellar vesicles of DOPC and C_6-NBD-PC, at a molar ratio of 3 to 2, were prepared by injection of an ethanolic solution of the lipids into Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (Kremer et al., 1977) and dialyzed against HMEM prior to use.

Lipid Extraction and Analysis. Following incubation with lipid vesicles, the cells and the media were collected, either separately or together (using a rubber policeman), and acidified with HCl to pH 2.0. Methanol and chloroform were added for lipid extraction according to Bligh and Dyer (1959). Lipid extracts were chromatographed on a silica gel LK6 thin-layer plate (Quantum, NJ) in chloroform/methanol/ammonium hydroxide (65:35:5). The fluorescent lipids were extracted from the silica in chloroform/methanol/10 mM HCl in 0.9% NaCl (50:50:5), and their fluorescence intensities were determined by using a Perkin-elmer LS-5 fluorospectrophotometer.

Determination of PLA_2 activity was performed by measurement of C_6-NBD-FA released from C_6-NBD-PC applied to cultured hepatocytes. The same extraction and analytical procedures were applied following incubation of the lipid vesicles with the soluble snake venom PLA_2 (Yedgar et al., 1986a).

β-D-Galactosidase and β-D-N-acetylglucosaminidase (β-hexosaminidase) activities were determined by measurement of the formation of 4-methylumbelliferone (4MU) from 4MU-D-galactoside and 4MU-*N*-acetyl-D-glucosaminide, respectively (Wenger et al., 1975), as previously described (Yedgar et al., 1986b).

Liposomes of Egg PC. Fifty milligrams of egg PC was dried under nitrogen and suspended by using a vortex cyclomixer in 4.5 mL of Tris-HCl-buffered saline at pH 7.4. The suspension was tip sonicated at 100 W for 15 min in melting ice. The suspension was centrifuged in an Ependorf Microfuge, and the transparent supernatant was collected. Mixed liposomes of egg PC and a fluorescent analogue of phospholipids (C_6-NBD-PC or *N*-NBD-PE) were prepared by mixing the adequate amounts of lipids dissolved in chloroform/methanol, codrying under nitrogen, and suspending in buffer as described above. This ensures the mixing of all the lipids in the lipid bilayer. When DPH or ANS was used, a hexane solution of 200-fold the final concentration of the probe was injected into the egg PC lipsome suspensions. The final molar ratio of the probes to the liposome lipids did not exceed 1:500, so that the dynamic properties of the liposomes were not significantly affected. These lipophilic probes incorporate rapidly into the lipid bilayer, as final fluorescence intensities were obtained immediately upon the introduction of the probes.

Measurement of Fluorescence Anisotropy in Viscous Medium. Liposome suspensions were mixed with concentrated solutions of viscous macromolecules to the desired viscosity, mixed gently to homogeneity, and centrifuged for 5 min at 1500*g* to remove air bubbles. Each sample contained 3 μmol of egg PC in 2.5 mL of medium. The molar ratio of the fluorophores to egg PC was 2.5%, 0.5%, and 0.1% for *N*-NBD-PE, DPH, and ANS, respectively. Fluorescence measurements were carried out in an ordinary 1 × 1 cm quartz cuvette. The fluorescence was excited by a polarized 365-nm vertical beam illuminated through the bottom of the cuvette (the *y* axis). The emission was measured by two perpendicular photomultipliers (aligned with the *x* and *z* axes) after it passed through cutoff filters (400 nm) and mutually perpendicular polarization analyzers. This arrangement enables simultaneous monitoring of the fluorescence emission polarized in parallel ($I_\parallel$) and perpendicular ($I_\perp$) planes with respect to the polarization plane of the excitation beam (Yuli et al., 1981).

Assessment of the Bilayer Microviscosity. The intensities of the perpendicular ($I_\perp$) emission and the ratio of the parallel

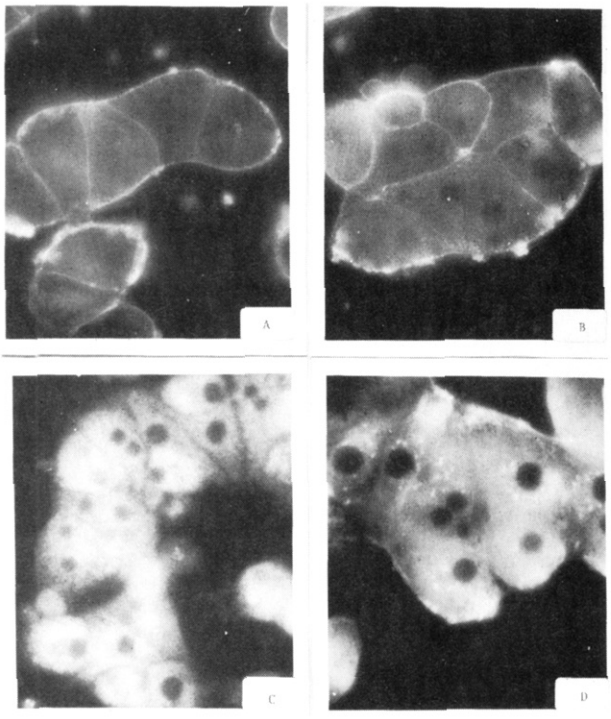

FIGURE 1: NBD lipid analogues in cultured rat hepatocytes. In panels A and B, cells were incubated with 50 μM lipid vesicles containing 40 mol % C_6-NBD-PC and 60 mol % DOPC, for 1 h at 2 °C (A) or 37 °C (B). In panel C, cells were incubated with 10 μM C_6-NBD-FA dissolved in HMEM for 1 h at 37 °C. In panel D, cells were incubated with lipid vesicles composed of 12 mol % C_6-NBD-ceramide and 88 mol % DOPC for 1 h at 37 °C. Following the incubation with the fluorescent lipid, the cells were washed 3 times with cold HMEM and examined under a Ziess inverted microscope.

to perpendicular intensities, $R = I_\parallel/I_\perp$, were recorded. The anisotropy, r, was calculated according to the equation (Weber, 1952):

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} = \frac{R-1}{R+2}$$

The fluorescence intensity, F, was calculated according to the equation:

$$F = I_\perp(R+2)$$

When liposomes were labeled with DPH or ANS, where partitioning of the dyes into the viscous medium is possible, the net anisotropy of the liposome-bound probe (r_L) was calculated according to the anisotropy additivity law (Shinitzky & Barenholz, 1976):

$$r_L = \frac{r_tF_t - r_cF_c}{F_t - F_c} = \frac{(I_\perp)_t(R_t-1) - (I_\perp)_c(R_c-1)}{(I_\perp)(R_t+2) - (I_\perp)_c(R_c+2)}$$

where t (total) and c (control) denote the values obtained in the presence of liposomes or in liposome-free medium, respectively.

Results

Determination of the Surface Membrane PLA_2 Activity of Intact Liver Cells. (A) Microscopic Examination of the Fate of C_6-NBD-PC in Cultured Hepatocytes. Figure 1A,B presents micrographs of cultured liver cells incubated with C_6-NBD-PC at either 2 °C (A) or 37 °C (B). In both treatments, the fluorescent lipid was accumulated in the plasma membrane and did not appear in intracellular membranes. In contrast, when the cells were incubated with C_6-NBD-FA (Figure 1C), this hydrophilic fatty acid defused into the cell and did not appear in the plasma or intracellular membrane.

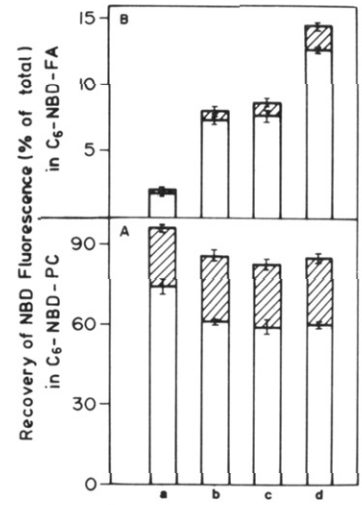

FIGURE 2: Distribution of C_6-NBD-PC and C_6-NBD-FA in cells or medium of hepatocyte culture. Cells were incubated in 50 μM lipid vesicles composed of DOPC/C_6-NBD-PC at a molar ratio of 2:1 for 2 h. The medium was then collected, the cells were washed with cold HMEM, and the wash medium was added to the culture medium. The cells, harvested in HMEM, and the media were separately subjected to lipid extraction and TLC. C_6-NBD-PC and C_6-NBD-FA were extracted from the silica, and their fluorescence intensities were measured. For 100% recovery, an equivalent amount of C_6-NBD-PC in HMEM was incubated without cells for the same time of the experiment, then mixed with an equivalent amount of cells suspended in acidic methanol, and subjected to the lipid extraction procedure. Panels A and B represent C_6-NBD-PC and C_6-NBD-FA, respectively, extracted from the medium (open bars) or cells (hatched bars): (a) incubation at 2 °C; (b) incubation at 37 °C; (c) incubation at 37 °C with endocytosis inhibitors, 5 mM sodium azide and 50 mM 2-deoxyglucose; (d) incubation at 37 °C with 0.2 mM pentenoic acid. The fluorescence of C_6-NBD-FA was measured in enlarged scale and normalized to that of C_6-NBD-PC. The fluorescence quantum yield was the same for the two fluorescent lipids. Each datum represents the mean ● SD for five separate dishes of cultured hepatocytes.

C_6-NBD-FA released from C_6-NBD-PC in the cell membrane does not incorporate into other lipids (see below), and its presence accounts for the fluorescence observed in the cytoplasma in Figure 1A,B. To ensure that NBD fluorescence in intracellular organelles can be visualized in our cell system, the liver cells were incubated with C_6-NBD-FA-ceramide which has been reported to accumulate in the Golgi apparatus (Lipsky & Pagano, 1983). Figure 1D shows that this lipid analogue is indeed visualized in intracellular organelles, presumably the Golgi apparatus, of cultured liver cells. This further suggests that the lack of fluorescence in intracellular organelles in Figure 1A,B is indicative of the absence of C_6-NBD-PC in these loci. The conclusion that C_6-NBD-PC is localized predominantly in the plasma membrane was further supported by a "back-exchange" experiment (Sleight & Pagano, 1984) in which the fluorescent lipid was removed from the plasma membrane by liposomes of nonfluorescent egg PC. In this experiment, liver cells were incubated at 37 °C for 1 h with C_6-NBD-PC, then washed with HMEM, and incubated twice with HMEM containing 0.2 mM egg PC for 20 min. The media and the cells were collected separately, and their C_6-NBD-PC content was determined. Over 90% of the cell-associated C_6-NBD-PC was extracted by the back-exchange procedure. Very weak fluorescence along the cell surface membrane was observed microscopically following this treatment (not shown).

(B) Degradation of C_6-NBD-PC by Cultured Hepatocytes. Following the observation that C_6-NBD-PC incorporates into the cell surface membrane, the degradation of this lipid in the plasma membrane was explored. Cultured liver cells in con-

Table I: Recovery of C_6-NBD-FA in Cultured Hepatocytes[a]

incubation condition	fluorescence recovery (FU)[b] in culture medium	in cells	% recovery
2 °C	791 ± 9	52 ± 2	101
37 °C	532 ± 17	35 ± 3	68
37 °C + 0.2 mM pentenoic acid	752 ± 3	71 ± 5	98

[a] Cultured liver cells were incubated for 1 h with 10 μM C_6-NBD-FA dissolved in HMEM. The medium and the cells were separately collected and subjected to lipid extraction and TLC. C_6-NBD-FA was extracted from the silica, and its fluorescence intensity was determined. For 100% recovery, an equivalent amount of C_6-NBD-FA was incubated without cells for the same time and then mixed with the cells suspension in acidic methanol for lipid extraction. Each datum is the mean ± SD for five separate culture dishes of hepatocytes. [b] FU, arbitrary fluorescence units.

fluency were thoroughly washed with HMEM to remove broken cells and debris and incubated with DOPC/C_6-NBD-PC vesicles under various conditions as described in the legend to Figure 2. The lipids from the cells and medium were then separately extracted and chromatographed on a thin-layer plate. In all cases, fluorescence was observed only in C_6-NBD-FA or C_6-NBD-PC, showing that the fluorescent fatty acid released from the phospholipid did not incorporate into other lipids. Determination of the fluorescence intensity of the lipids extracted from the thin layer is presented in Figure 2. At 2 °C, the phospholipid degradation, as measured by the decrease in C_6-NBD-PC fluorescence, was very small although about 25% of the substrate was associated with the cells. This amount corresponds to 5 nmol/mg of cell protein which is about 3% of the cell phospholipids (Yedgar et al., 1982). The same amount was associated with the cells at 37 °C after various incubation times, although 15% (3 nmol) of C_6-NBD-PC was degraded during 2 h (Figure 2A). It seems, therefore, that C_6-NBD-PC in the medium serves as a reservoir to replace the utilized substrate. The same results were obtained in the presence of endocytosis inhibitors, sodium azide and 2-deoxyglucose (Furuichi et al., 1986; Sleight & Pagano, 1984). These results, together with the localization of C_6-NBD-PC in the plasma membrane (Figure 1B), suggest that the degradation of this substrate occurs at the plasma membrane. However, the total fluorescence recovery was incomplete (less than 90%) after incubation at 37 °C. This was not due to quenching of C_6-NBD-PC, as discussed in the following.

(*C*) *Determination of PLA_2 Activity by Measurement of the Reaction Product.* The results of the thin-layer chromatography, showing NBD fluorescence associated only with C_6-NBD-PC and C_6-NBD-FA, indicate that the fluorescent substrate is acted upon by PLA_2. The activity of this enzyme might be determined by the decrease in C_6-NBD-PC content during its interaction with the cells. Yet, when considering initial rate measurements, especially in short-time experiments, it is, of course, desirable to degrade a small portion of the substrate. Determination of the changes in the substrate concentration would yield unsatisfactory accuracy, and determination of the product is therefore desirable. However, Figure 2 shows that the recovery of the fluorescence at 37 °C was incomplete, suggesting that the fluorescent fatty acid, presumably produced by PLA_2, was further metabolized. This possibility was examined in the experiments outlined in Table I. Cells were incubated with C_6-NBD-FA at 37 °C, and then the cells and medium were collected and subjected to lipid extraction and TLC. Fluorescence was detected only in one band, that of C_6-NBD-FA. This again suggests that this fatty acid analogue does not incorporate into other lipids, as already noted above. However, only about 65% of the fluorescence was recovered after 1 h of incubation at 37 °C (Table I), but the disappearance of the fluorescence was prevented when pentenoic acid, a β-oxidation inhibitor (Schulz & Fong, 1981), was added to the incubation medium (Table I). It may therefore be concluded that the incomplete recovery of the NBD fluorescence is a consequence of the fatty acid metabolism. Prevention of fatty acid oxidation yields full recovery of the NBD fluorescence (Figure 2B and Table I). It is also shown that pentenoic acid did not affect the lipid distribution between the cell and the culture medium. Henceforth, determination of PLA_2 activity at the liver cell membrane was done by incubation of the cells with C_6-NBD-PC in the presence of pentenoic acid. Under these conditions, the hydrolysis of C_6-NBD-PC, as determined by the formation of C_6-NBD-FA, was linear for at least 2 h, when almost 3 nmol of the substrate is hydrolyzed by 1 mg of cell protein.

Table II: Effect of Medium Viscosity on Hydrolysis of C_6-NBD-PC by Cultured Hepatocytes[a]

overnight incubation condition	C_6-NBD-PC incubation condition	C_6-NBD-PC hydrolyzed [nmol (mg of cell protein)$^{-1}$ h^{-1}]
	Experiment A	
(1) DME	HMEM (η_r = 1.08)	1.52 ± 0.04
(2) DME	HMEM + 4% Dex (η_r = 4.1)	0.83 ± 0.02
(3) DME	HMEM + 0.2% MeC (η_r = 4.2)	0.76 ± 0.03
(4) DME	HMEM + 0.02% Xa (η_r = 3.9)	0.80 ± 0.06
(5) DME + 4% Dex	HMEM	1.50 ± 0.03
(6) DME + 0.02 Xa	HMEM	1.54 ± 0.05
	Experiment B	
(1) DME	C_6-NBD-PC at 2 °C replaced by HMEM	0.42 ± 0.04
(2) DME	C_6-NBD-PC at 2 °C replaced by HMEM + 4% Dex at 37 °C	0.22 ± 0.05
(3) DME	C_6-NBD-PC at 2 °C replaced by HMEM + 0.02% Xa at 37 °C	0.19 ± 0.02

[a] 10^6 cultured hepatocytes were incubated in 1 mL of HMEM containing 50 nmol of lipid vesicles composed of 40 mol % C_6-NBD-PC and 60 mol % DOPC. In experiment A, the cells were incubated with the lipid vesicles for 60 min at 37 °C. In experiment B, the cells were first incubated with the lipids on ice for 30 min and then for 60 min in lipid-free HMEM, at 37 °C. PC hydrolysis was determined by the NBD-hexanoic acid produced, as described under Materials and Methods. The fluorescent substrate and product had the same quantum yield in the extraction solvent. Each datum is the mean ± SD for five separate dishes of cultured hepatocytes.

Effect of Extracellular Fluid Viscosity on Liver Cell Membrane PLA_2. The medium viscosity was increased up to about 4-fold of the viscosity of water (at 37 °C), which is in the range of changes in plasma viscosity occurring in physiological and pathological states (Lowe et al., 1981).

Vesicles of DOPC/C_6-NBD-PC were prepared in control HMEM and mixed with viscous media to the desired proportions. Liver cells, cultivated overnight in control DME, were incubated with the lipid vesicles in control or viscous HMEM for 1 h at 37 °C, and then the lipids were extracted and determined. The results, presented in Table IIA (rows 1–4), clearly show that the hydrolysis of C_6-NBD-PC by PLA_2 was inhibited as the extracellular fluid viscosity was increased.

To examine whether the viscosity produces a persistent effect on the PLA_2, the cells were cultivated overnight in either control or viscous DME, following which they were interacted with C_6-NBD-PC in control HMEM for 1 h at 37 °C. The

Table III: Effect of Medium Viscosity on Secretion of β-Galactosidase and β-Hexosaminidase by Cultured Hepatocytes[a]

incubation condition	activity secreted to culture medium [nmol (mg of cell protein)$^{-1}$ h^{-1}]	
	β-galactosidase	β-hexosaminidase
control HMEM (η_r = 1.08)	1.20 ± 0.08	3.24 ± 0.11
HMEM + 4% Dex (η_r = 4.1)	0.55 ± 0.07	1.57 ± 0.09
HMEM + 0.2% MeC (η_r = 4.2)	0.67 ± 0.06	1.73 ± 0.13

[a] Cultured hepatocytes were incubated in control or viscous medium for 1 h at 37 °C. The medium was then collected, acidified to pH 4.4, and incubated with 4MU-galactoside or 4MU-*N*-acetylhexosamine for 1 h at 37 °C. The fluorescence of 4MU produced was measured, and its concentration was determined by reference to a standard curve of known amounts of the fluorophore, in the same medium.

overnight treatment did not affect PC degradation (Table IIA, rows 1, 5, and 6). Only when the cells are in the viscous fluid is PLA_2 activity affected.

The possibility that the viscosity inhibition of PC degradation by the medium viscosity is due to impeded diffusion of the substrate toward the cell membrane or due to alteration of the vesicle structure were tested in the following experiment: Cultured hepatocytes which had been cultivated overnight in control DME were incubated with C_6-NBD-PC on ice for 30 min to allow incorporation of the fluorescent lipid into the cell membrane without significant degradation. The medium was then discarded, and the cells were washed to remove excess C_6-NBD-PC. The cells were then incubated in either control or viscous HMEM (*free* of C_6-NBD-PC) for 1 h at 37 °C. During this period, the incorporated C_6-NBD-PC was subjected to degradation by the membrane PLA_2. As shown in Table IIB, the hydrolysis of the PC incorporated into the cell membrane was decreased as the medium viscosity was increased, indicating that the inhibition of the enzymatic activity is not due to an effect on the accesibility of the substrate to the cells.

To test whether the viscosity influences the cellular fate of the fluorescent phospholipid, the cells, incubated with C_6-NBD-PC in either control or viscous medium were examined by fluorescence microscopy, at 10-min intervals, concurrently with the biochemical assays. No effect of the medium viscosity on the distribution of the fluorescence in the cell could be observed. Under all treatments, the fluorescence accumulated in the plasma membrane and was not visualized in intracellular organelles.

Effect of Viscosity on Secretion of Lysosomal Enzymes. In addition to the effect of medium viscosity on VLDL secretion reported previously (Yedgar et al., 1982), we have observed that increased viscosity has an immediate inhibitory effect on secretion of lysosomal enzymes by cultured liver cells (Yedgar et al., 1986b). Subsequently, we have examined the viscosity effect on secretion of lysosomal enzymes concurrently with studying the effect on the cell membrane PLA_2. For this purpose, we have followed the secretion of β-galactosidase and β-hexosaminidase, as previously described (Yedgar et al., 1986b). The results, presented in Table III, clearly demonstrate that increased medium viscosity decreased the level of the secreted enzymes, concomitantly with the inhibition of PLA_2 activity.

Effect of Medium Viscosity on PC Hydrolysis by Soluble PLA_2. To further assess the role of medium viscosity in the action of PLA_2, we examined the effect of solvent viscosity on the hdyrolysis of PC by PLA_2, by interacting DOPC/C_6-NBD-PC vesicles with soluble snake venom PLA_2. In this

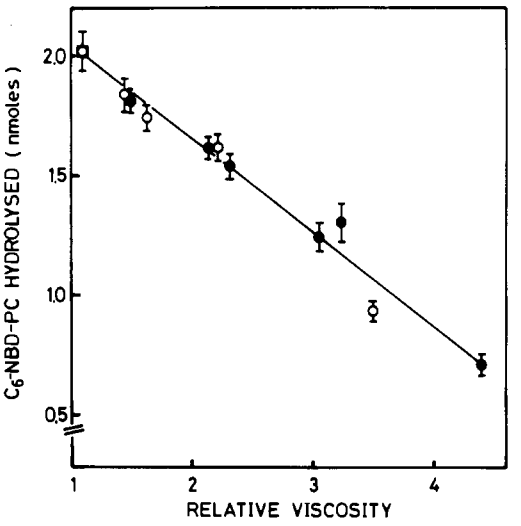

FIGURE 3: Hydrolysis of C_6-NBD-PC by snake venom PLA_2 as a function of solvent viscosity. Lipid vesicles at a concentration of 50 μM composed of 40 mol % C_6-NBD-PC and 60 mol % DOPC were incubated with 10 μg/mL PLA_2 for 20 min at 37 °C in Tris buffer (20 mM) containing 2 mM $CaCl_2$ (□). The solvent viscosity was raised by the addition of up to 4% Dex (●) or 0.02% Xa (○). C_6-NBD-PC hydrolysis was determined by the NBD-hexanoic acid produced, as described under Materials and Methods. The amount of fluorescent lipids was determined by reference to standard curves of known amounts of C_6-NBD-PC or C_6-NBD-FA, which had the same fluorescence quantum yield in the extraction medium. Each point represents the mean ± SD for five replications. Correlation coefficient, r = 0.983.

model system, the soluble enzyme acts on the substrate at the lipid interface, unlike the cultured cells where the enzyme and the substrate (C_6-NBD-PC) are in the same compartment. The results of this experiment (Figure 3) reveal that the inhibitory effect of the viscosity is manifested in this system as well; an inverse linear correlation exists between the degradation of liposomal PC by soluble PLA_2 and the solvent viscosity.

Fluorescence Depolarization of Lipids in Phospholipid Liposomes. To explore the possibility that the effect of medium viscosity is mediated by modulation of the dynamic properties of the phospholipid bilayer, we monitored the fluorescence anisotropy in egg PC liposomes of three probes, each of which incorporates by partitioning into a different part of the lipid bilayer. DPH was used to monitor the midst of the bilayer, as it partitions preferably into the most hydrophobic region. ANS, an amphipathic molecule, was employed to monitor the interface between the hydrophobic tail and the polar head group of the phospholipids in the bilayer. In *N*-NBD-PE, the fluorophore is bound to the amine of the polar head group and thus reports dynamic properties of the bilayer surface. Mixed liposomes of egg PC and the desired probe were prepared and mixed with viscous medium as described under Materials and Methods. The viscosity of the medium was elevated up to about 60 cP by the addition of either Dex (up to 15%) or MeC (up to 0.75%). The results, presented in Table IV, clearly show that increasing medium viscosity of the liposome suspension had no significant effect on the net fluorescent anisotropy of any of the probes examined, especially in the physiological range of viscosity. This suggests that medium viscosity does not affect the microviscosity of the lipid bilayers or the mobility of the phospholipids.

Discussion

Determination of Cell Surface PLA_2 Activity. To attain a method for determination of PLA_2 activity in the surface membrane of intact cells, it is necessary to establish that this

Table IV: Net Anisotropy (r_L) of Fluorescent Probes Associated with Egg PC Vesicles[a]

viscosity agent	medium viscosity (cP)	net anisotropy DPH	ANS	*N*-NBD-PE
none (control)	1.0	0.115	0.135	0.083
Dextran, 3.5%	3.1	0.116	0.130	0.085
Dextran, 7.5%	3.8	0.116	0.110	0.080
Dextran, 12.0%	25.1	0.117	0.110	0.083
Dextran, 15%	41.3		0.110	0.080
MeC, 0.10%	1.71		0.135	
MeC, 0.25%	3.4	0.108	0.135	
MeC, 0.50%	19.3	0.109	0.135	
MeC, 0.75%	60.1	0.109	0.133	

[a] Mixed liposomes of egg PC and the desired fluorescent probe were prepared as described under Materials and Methods and mixed with viscous buffer to obtain the indicated viscosity. Fluorescence anisotropy of the probes in liposomes and in liposome-free buffer was measured, and net anisotropy was calculated as described under Materials and Methods.

substrate is hydrolyzed in the plasma membrane, by phospholipase A_2, and that the hydrolysis product is not metabolized and can be fully recovered.

Our results demonstrate that C_6-NBD-PC incorporates into the plasma membrane of cultured hepatocytes and does not appear in intracellular membrane as observed under the microscope. The substrate is hydrolyzed to produce one fluorescent product, C_6-NBD-FA, and endocytosis inhibitors did not alter the fate of this substrate, as examined biochemically and microscopically. These findings suggest that C_6-NBD-PC in hepatocyte culture is hydrolyzed by PLA_2 in plasma membrane, similar to the suggestion of Sleight and Pagano (1984), who studied the fate of C_6-NBD-PC in fibroblasts. This conclusion is further supported by our findings that the hydrolysis of the fluorescent phospholipid is blocked by the cell-impermeable inhibitor of PLA_2, as described previously (Yedgar et al., 1986a). Our data provide direct evidence that this fluorescent PC analogue is hydrolyzed predominantly (practically exclusively) in the cell surface membrane of cultured hepatocytes. Sleight and Pagano (1984) have also suggested that in cultured fibroblasts C_6-NBD-PC inserted into the plasma membrane is internalized into the Golgi apparatus and recycled to the plasma membrane. In the liver cell culture used here, no accumulation of C_6-NBD-PC was observed in intracellular organelles. This might suggest that in liver cells this process, if it occurs, is too fast or too little to produce detectable accumulation in the Golgi apparatus or that the fate of C_6-NBD-PC is different in liver cells.

As noted above, incubation of the fluorescent substrate C_6-NBD-PC with the cells yielded only one fluorescent product, C_6-NBD-FA. This might be a result of direct action of PLA_2 or alternatively one of the two other pathways known to occur in mammalian cells: the action of phospholipase A_1 followed by lysophospholipase or that of phospholipase C followed by diglyceride lipase (Bell et al., 1979; Billah et al., 1980). As already noted, fluorescent lysoPC was not obtained by incubation of C_6-NBD-PC with the cells. This might suggest that this substrate is not utilized signficantly by phospholipase A_1 at the cell membrane or that the lysoPC, if formed, is further utilized by lysophospholipase. However, it is well accepted that lysophospholipase exists intracellularly and not in the plasma membrane of liver cells (Van den Bosch, 1982; Martin & Lagunoff, 1982). Since the hydrolysis of C_6-NBD-PC occurs at the cell surface membrane, this pathway does not account for the formation of C_6-NBD-FA. The other pathway is even less likely to occur since phospholipase C found in mammalian cells is specific for phosphatidylinositol and PC is not utilized by this enzyme (Shukla, 1982; Lapetaina & Siess, 1983; Van den Bosch, 1982). It may be concluded, therefore, that the formation of C_6-NBD-FA from NBD-PC, reported here, is a result of direct action of phospholipase A_2.

As required for product determination, the fluorescent product C_6-NBD-FA does not incorporate into other lipids and is fully recovered when the substrate is applied in the presence of pentenoic acid. Under these conditions, the hydrolysis of C_6-NBD-PC by PLA_2, measured by the determination of the product, is linear within 2 h at least, when about 3 nmol (about 15%) of the substrate is hydrolyzed by 1 mg of cell protein. These findings make a suitable method for the initial rate determination of the PLA_2 activity.

Effect of Medium Viscosity on PLA_2 Activity. Our data demonstrate that fluid viscosity is a regulator of phosphatidylcholine hydrolysis by PLA_2. The degradation of PC by both membranal and soluble PLA_2 is inhibited by increasing viscosity of the aqueous environment. This effect correlates well with the inhibited secretion of VLDL (Yedgar et al., 1982) and lysosomal enzymes by cultured hepatocytes.

The mechanism of phospholipid degradation by PLA_2 is rather complex and is not fully understood (Slotboom et al., 1982; Verger & Pattus, 1982; Hendrikson & Dennis, 1984). A number of factors have been proposed to be involved in this interaction. Generally, they can be categorized into substrate-related or enzyme-related characteristics. Among them is the aggregation state of the substrate, and its dynamic properties, or the conformational state of the enzyme. Theoretically, each of these factors might be affected by hydrodynamic properties of the aqueous environment.

Our findings that viscosity influences hydrolysis of PC molecules that are either aggregated in vesicles or incorporated into the cell membrane indicate that this effect is not due to reduction of the reactant diffusion rate in the solution. The same data suggest that the viscosity is independent of the aggregation states of the substrate (i.e., in cell membrane or liposomes.). Furthermore, it can be concluded that medium viscosity does not affect dynamic properties of the lipid bilayer, as measured by fluorescence anisotropy (Table IV). These conclusions are in accord with reports of NMR studies which suggest that solvent viscosity does not influence the segmental motion of phospholipids in vesicles (Horwitz et al., 1973; Lichtenberg et al., 1975). It is therefore plausible to conclude that the inhibition of PC hydrolysis exerted by the medium viscosity is not mediated by an effect on membrane phospholipid dynamics.

On the other hand, it is well accepted that solvent viscosity plays an important role in protein dynamics. Gavish and Werber (1979) have shown that the catalysis rate of carboxypeptidase A is regulated by solvent viscosity. The same phenomenon was reported for the interaction of heme proteins with either CO or O_2 (Breece et al., 1980; McKinnie & Olson, 1981; Sawicki & Khaleque, 1983) and for some steps in the bacteriorhodopsin photocycle (Breece et al., 1981). These studies suggest that the catalytic activity is regulated by the rate of protein conformational change, which is, in turn, inversely proportional to solvent viscosity (Gavish & Werber, 1979; Sawicki & Khaleque, 1983). Our data demonstrate for the first time a similar regulatory effect of solvent viscosity on the interaction of either membrane-bound or soluble PLA_2 with a lipid substrate. It is reasonable to assume, therefore, that the regulation of PLA_2 activity and cellular secretion by medium viscosity is mediated by an effect on the cell membrane PLA_2 itself.

The involvement of membrane PLA_2 in exocytosis has been previously suggested in various cell types, e.g., mast cells (Martin & Lagunoff, 1982), adrenal medullary cells (Frye & Holz, 1983), anterior pituitary cells (Grandison, 1984), and other (Vogt, 1978). Our data, which demonstrate the correlation between the inhibition of PLA_2 and of secretory activities exerted by the extracellular fluid viscosity, are further evidence as to the linkage between this enzyme and exocytosis.

As already noted above, the viscosity values applied in this study are in the range of changes in plasma viscosity that occur in physiological and pathological states (Lowe et al., 1981). The involvement of extracellular fluid viscosity in secretory processes, demonstrated in this study, might be pertinent to transmembrane processes in general and to pathological states associated with elevated viscosity of body fluids.

Registry No. Phospholipase A_2, 9001-84-7; β-galactosidase, 9031-11-2; β-hexosaminidase, 9027-52-5.

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