Regulation of Rat Hepatic Lipase by the Composition of Monomolecular Films of Lipid†

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ABSTRACT: The regulation of hepatic lipase (HL) by the lipid composition of monomolecular substrate films was examined using a monolayer technique at constant surface pressure. HL-catalyzed hydrolysis of triacylglycerol, a poor substrate for HL in pure monomolecular films, was activated by diradylglycerol and its phosphorylated derivatives in mixed films containing 10 mol % triacylglycerol. When triacylglycerol was progressively diluted with dialkylglycerol, triacylglycerol hydrolysis by HL was maximal between 90 and 98 mol % dialkylglycerol. The best activators, dialkylphosphatidic acid and dialkylphosphatidylethanolamine, increased triacylglycerol hydrolysis 13–14-fold, and the enhancement of HL-catalyzed triacylglycerol hydrolysis by the activator lipids was inversely related to the average mean molecular area of the mixed films. The hydrolysis of 5 mol % triacylglycerol in mixed films that also contained phosphatidylcholine and 0–20 mol % cholesterol was inhibited approximately 80% when the concentration of cholesterol was 10–13 mol %. Interestingly, between 15 and 17 mol % cholesterol the hydrolysis rate was restored to about 50% of the uninhibited rate, but at 20 mol % cholesterol this value decreased back to 80% inhibition of hydrolysis. The hydrolysis of phosphatidylethanolamine in mixed films with 0–20 mol % cholesterol decreased approximately 30% in films containing 10–12 mol % cholesterol. However, at 15 mol % cholesterol the hydrolysis rate was restored to the same level observed for a pure phosphatidylethanolamine film. This enhancement of HL activity occurred at about the same cholesterol concentration as the restoration of triacylglycerol hydrolysis observed for the triacylglycerol/phosphatidylcholine/cholesterol films. Restoration of HL-catalyzed triacylglycerol or phosphatidylethanolamine hydrolysis in films with 13–15 mol % cholesterol directly corresponded to deviations from the condensation of the film measured as a decrease in the average mean molecular area of the lipid in these mixed films. HL adsorption to the monomolecular substrate films tested was constant and therefore did not account for differences observed in hydrolysis rates. These data indicate that the lipid composition of the monomolecular substrate film may regulate HL activity through factors such as substrate conformational and/or packing changes and by direct interaction with a binding site (or sites) on HL. Therefore, these same factors may regulate HL during the hydrolysis of high-density lipoproteins, chylomicron remnants, and intermediate-density lipoproteins, the apolipoprotein E-containing particles previously postulated to be physiologic substrates for HL (Thuren et al., 1991, 1992).

Hepatic lipase (E.C. 3.1.1.32; HL)[1] is located on the surface of endothelial cells lining the vascular lumen of the liver (Kuusi et al., 1979), where it hydrolyzes phospholipids and neutral glycerides present in the surface monomolecular coat surrounding the inner hydrophobic core of lipoprotein particles. It has been proposed that one function of HL is to hydrolyze the dietary triacylglycerol (TG) of chylomicron remnants (Nilsson et al., 1987; Sultan et al, 1990) and the TG of intermediate-density lipoprotein (IDL) as it is converted to low-density lipoprotein (LDL) in the VLDL–IDL–LDL cascade (Murase & Itakura, 1981). Also, the phospholipase activity of HL is thought to play a key role in high-density lipoprotein (HDL) interconversion (Shirai et al., 1981; Patsch et al., 1987; Groot et al., 1981; van't Hooft et al., 1981). HL is in the same gene family with lipoprotein lipase (LPL) and pancreatic lipase (PL) (Komaromy & Schotz, 1987; Ben-Zeev et al., 1987), and of these three enzymes, the physiological activity of HL is least understood. The substrate specificity of this enzyme in model systems has provided some understanding of the mechanisms that regulate its action. When phospholipids and neutral glycerides were assayed in a relatively uniform physical state as mixed micelles with Triton X-100, both lipid classes were hydrolyzed readily by HL (Wilcox et al., 1991; Kucera et al., 1988; Thuren et al., 1990).

When the Verger–de Haas zero-order trough is used (Lairon et al., 1980), the physicochemical state of the substrate interface remains constant throughout the course of each reaction. We recently used monomolecular films of lipid in the Verger–de Haas system (Lairon et al., 1980) to show that the activity of HL is regulated both by the lipid composition of the monomolecular film and by apolipoproteins (Thuren

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[1] Abbreviations: dialkyl-G, 1,2-*O*-didodecyl-*sn*-glycerol; dialkyl-PA, 1,2-*O*-didodecyl-*sn*-glycero-3-phosphate; dialkyl-PC, 1,2-*O*-didodecyl-*sn*-glycero-3-phosphocholine; dialkyl-PE, 1,2-*O*-didodecyl-*sn*-glycero-3-phosphoethanolamine; HDL, high-density lipoprotein; HL, hepatic lipase; IDL, intermediate-density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoprotein; LPL, lipoprotein lipase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, pancreatic lipase; TG, triacylglycerol; VLDL, very low density lipoprotein.

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et al., 1991). For the 1,2-didodecanoyl species of lipid, phosphatidylethanolamine (PE) was readily hydrolyzed while phosphatidylcholine (PC) was not hydrolyzed by HL. LPL and PL also do not hydrolyze monomolecular films containing this species of PC (Vainio et al., 1983a; Pieroni & Verger, 1979). HL did, however, hydrolyze 1,2-dioleoyl-*sn*-glycero-3-phosphocholine at a rate 15-fold less than the rate observed for 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine. We also demonstrated that pure trioctanoylglycerol was a poor substrate for HL unless PC was persent (Thuren et al., 1991). In this case PC was present as a nonhydrolyzable film component. This activation is an intriguing aspect of the mechanism of HL activity which is shared by the other enzymes from this gene family (Vainio et al., 1983a; Pieroni & Verger, 1979). Laboda et al. (1986) observed the same substrate preference for 1 mol % of lipid substrate in an inert film of sphingomyelin/cholesterol; triolein and dioleoylphosphatidylethanolamine hydrolysis were equal and both were much greater than dioleoylphosphatidycholine hydrolysis. Consistent with these specificity studies, PE and TG were better substrates than PC during the hydrolysis of HDL by HL (Azema et al., 1990; Landin et al., 1984; Thuren et al., 1992). However, the contribution of PC toward total hydrolysis of lipoprotein lipid by HL should not be underestimated, since the lipid mass of PC in the coat film of the lipoprotein is greater than the mass of PE or TG.

Both LPL and PL require a protein cofactor for optimal activity and we have shown that apolipoproteins have significant effects on substrate hydrolysis by HL as well. At low surface pressures we found that apolipoprotein E stimulated phospholipid hydrolysis 2–3-fold but did not influence TG hydrolysis (Thuren et al., 1991). Specifically we found that apolipoproteins A-I, A-II, C-I, C-II, and C-III either had no effect on or inhibited HL activity. From these data we postulated that apolipoprotein E-rich HDL particles were preferred substrates for HL, a point verified in our recent studies (Thuren et al., 1992).

In the present study we examine the effect of lipid film composition on the neutral lipid hydrolase and phospholipase activities of HL. We address the question of whether or not the activation of TG hydrolysis is specific for PC by determining the minimal chemical requirement at the *sn*-3 position of the activator lipid component in mixed films with TG. We also examine the effect of cholesterol on the hydrolysis of phospholipid films and of TG in mixed films that contain TG, PC, and cholesterol since approximately 5–20 mol % cholesterol has been estimated to exist in the surface coat of lipoprotein particles (Miller & Small, 1983). If the lipid composition and the presence of cholesterol affect HL activity in monomolecular films, then these factors could also regulate HL activity physiologically since these substrates are an excellent model of the surface lipid film of lipoproteins.

EXPERIMENTAL PROCEDURES

Lipids. Trioctanoylglycerol, 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine, 1,2-didodecanoyl-*sn*-glycero-3-phosphoethanolamine, and cholesterol were obtained from Sigma Chemical Co. Dialkyl lipids were synthesized according to previously published procedures (Abdelmageed et al., 1989). Briefly, 3-*O*-benzyl-*sn*-glycerol was alkylated with 1-bromododecane. The resulting 3-*O*-benzyl-1,2-*O*-didodecyl-*sn*-glycerol was hydrogenated to form 1,2-*O*-didodecyl-*sn*-glycerol. 1,2-*O*-Didodecyl-*sn*-glycerol was converted to 1,2-*O*-didodecyl-*sn*-glycero-3-phosphoethanolamine by treatment with phosphorus oxychloride followed by 2-aminoethanol and ring opening of the phosphobase with acetic acid. 1,2-*O*-Didodecyl-*sn*-glycero-3-phosphocholine was then prepared from choline and the corresponding chemically synthesized phosphatidylethanolamine by the transphosphatidylation reaction of phospholipase D from cabbage (Boehringer Mannheim) (Comfurious & Zwaal, 1977). 1,2-*O*-Didodecyl-*sn*-glycero-3-phosphate was prepared by cleavage of 1,2-*O*-didodecyl-*sn*-glycero-3-phosphoethanolamine by the same enzyme (Comfurious & Zwaal, 1977). The lipids were purified either by silicic acid column chromatography (70–230 mesh, EM Science), thin-layer chromatography (silica gel G, 1000 μm, Analtech), or a combination of the two methods. The identity of 1,2-*O*-didodecyl-*sn*-glycerol and 1,2-*O*-didodecyl-*sn*-glycero-3-phosphoethanolamine was confirmed by mass and NMR spectrometry. All other lipids were resolved by thin-layer chromatography and identified by their migration with authentic diacyl standards (Serdary Research Laboratories, Inc.) which were visualized by charring.

Purification and Iodination of Rat Hepatic Lipase. Rat HL was purified by a modification of the method of Jensen and Bensadoun (1981; Waite et al., 1991) from liver perfusate. HL was iodinated with sodium [^{125}I]iodide (17 mCi/μg, Amersham Corp.) by a method similar to the one used for LPL (Slotboom et al., 1978; Jackson et al., 1980; Vainio et al., 1983b) as previously reported (Thuren et al., 1991). The iodinated enzyme preparation had a molar ratio of iodide to protein of 0.7 with a radiospecific activity of 20 000 cpm/μg of protein. The specific activity of the iodinated enzyme for a monomolecular film of phosphatidylethanolamine was about 20% less than that observed for the unlabeled enzyme which had been subjected to similar treatment without radiolabel.

Enzyme Assay Using Monomolecular Films of Lipid as Substrate. The surface barostat method (Verger & de Haas, 1976) was used to determine the rate of hydrolysis of lipid monomolecular films at constant surface pressure. The surface pressure was measured by the Wilhelmy plate method using a platinum plate. The monolayer apparatus, optimal conditions for HL hydrolysis, and the technique for using monomolecular films composed of more than one lipid component were described previously (Thuren et al., 1991). The aqueous subphase was prepared with deionized and filtered water (Pure-Flow, Inc., Mebane, NC) and consisted of 20 mM Tris-HCl, 0.5 M NaCl, and 5 mM $CaCl_2$, pH 8.4. A key-type Verger–de Haas zero-order trough (Lairon et al., 1980) with a reaction compartment (total volume 35 mL, total surface area 23.3 cm^2), a surface balance compartment for the platinum Wilhelmy plate, and a film reservoir (20 × 155 mm) was used for kinetic measurements. Experiments were started by injection of 0.75 μg of either nonradiolabeled HL or ^{125}I-labeled HL (15 000 cpm, 1.3×10^{-5} μmol of protein) into the reaction compartment and each film was maintained at a constant surface pressure of 15 mN/m. The rates were calculated under steady-state conditions of hydrolysis. After each reaction, when appropriate, films were recovered and radioactivity was counted according to the method of Rietsch et al. (1977) for determination of the amount of HL adsorbed to the monomolecular film. Standard deviations were calculated from three identical measurements. For each data set the amount of surface-adsorbed HL was relatively constant.

Each hydrolysis rate reported in the study of TG hydrolysis by HL in the presence of the nonhydrolyzable analogs represents the mean and standard deviation calculated from four identical experiments. Other hydrolysis experiments were conducted three times with similar observations and a

representative experiment is shown with standard deviations calculated from three identical measurements. Surface pressure–mean molecular area isotherms for the monomolecular films studied in the hydrolysis reactions were obtained between 0 and 20 mN/m. The average mean molecular area at 15 mN/m (the surface pressure of interest since all reactions were conducted at this pressure) for each film hydrolyzed is shown as the mean and standard deviation obtained from three identical isotherms. The surface pressure–mean molecular area isotherms were measured at the air–water interface in a rectangular trough (150 × 575 mm). The subphase in these experiments was the same as for the kinetic experiments. The lateral compression rate was 10 mm/min. Hydrolysis reactions and surface pressure–mean molecular area isotherms were conducted at 24.5 °C.

RESULTS

Previous studies conducted in our laboratory demonstrated that the HL-catalyzed hydrolysis of TG in a monomolecular film could be increased dramatically by addition of 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine (Thuren et al., 1991a). This stimulation was found in the range of surface TG concentrations, 2–5 mol % of total surface lipid, reported for lipoprotein coat lipid (Miller & Small, 1980; Hamilton & Small, 1981). In an effort to better understand the nature of this activation, we determined the minimal chemical requirement at the *sn*-3 position of the second lipid component in mixed films with substrate TG. In previous studies we used mixed films composed of TG and 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine in which PC was not hydrolyzed by HL under standard assay conditions, a requisite for these studies (Thuren et al., 1991a). However, the lipid classes of interest here are substrates for HL, and therefore the nonhydrolyzable ether lipids dialkyl-G, dialkyl-PE, dialkyl-PA and dialkyl-PC were used. In order to determine the effect this substitution may have had on our results, we demonstrated that the activation of TG by dialkyl-PC was only slightly lower (25%) than that observed in the presence of diacyl-PC (data not shown). On the basis of this result, we concluded that dialkyl lipids were suitable compounds for comparing the ability of different lipid classes to activate TG hydrolysis even though the activation observed may have been somewhat lower than that expected for the corresponding diacyl-linked species. The mixed films studied contained 10 mol % TG and 90 mol % dialkyl lipid. The data in Figure 1A demonstrate that HL-catalyzed TG hydrolysis was greater for each of the mixed films than for the hydrolysis of a pure TG monomolecular film. In the presence of dialkyl-G, TG hydrolysis was activated 6-fold. Therefore, the minimal chemical requirement at the *sn*-3 position for HL activation was a hydroxyl group. Addition of dialkyl-PA, dialkyl-PE, or dialkyl-PC to TG in the monomolecular film resulted in 13-, 14-, and 5-fold activation of TG hydrolysis, respectively, over the hydrolysis observed for a pure TG film. The activating effects of dialkyl-PC and dialkyl-PE did not seem to be additive. Addition of dialkyl-PE did not enhance dialkyl-PC-induced activation of HL-catalyzed TG hydrolysis when 10 mol % dialkyl-PE was added to 60 mol % dialkyl-PC, the concentration known to cause maximal activation of HL. These results indicated that the activation of HL could be influenced by a spectrum of lipids and was not restricted to PC, the major phospholipid of lipoprotein particles.

We measured lateral compression isotherms for these TG phospholipid and DG mixtures at the air–water interface under the conditions of hydrolysis (Figure 2). The isotherms were continuous up to a surface pressure of at least 20 mN/m without any evidence of transition or nonideal mixing of lipid components. The mean molecular area of each of the lipid mixtures hydrolyzed above was determined at 15 mN/m (Figure 1B) from these surface pressure–mean molecular area isotherms (Figure 2). The mean molecular area of pure TG was 106 Å^2 and that decreased to 64 Å^2 when dialkyl-PE was added. These decreases mainly reflect characteristics of the phospholipid since the film contained 90% of the activator lipid. Similar effects on surface area changes would be expected with the diacyl lipids (Paltauf et al., 1971). These data demonstrate that TG hydrolysis in these mixed films was inversely related to the mean molecular area of the film; TG hydrolysis was activated to the greatest extent in films with the smallest average mean molecular areas.

Data in Figure 3A show the dialkyl-G concentration dependency of HL activation. The rate of TG hydrolysis in the mixed TG/dialkyl-G films remained at the relatively constant and low level observed for a pure TG film until the concentration of dialkyl-G was increased to about 70 mol %. At this point enzyme activity began to increase until it reached a maximum between 90 and 98 mol % dialkyl-G. This suggested that in mixed TG/dialkyl-G films the hydrolysis of

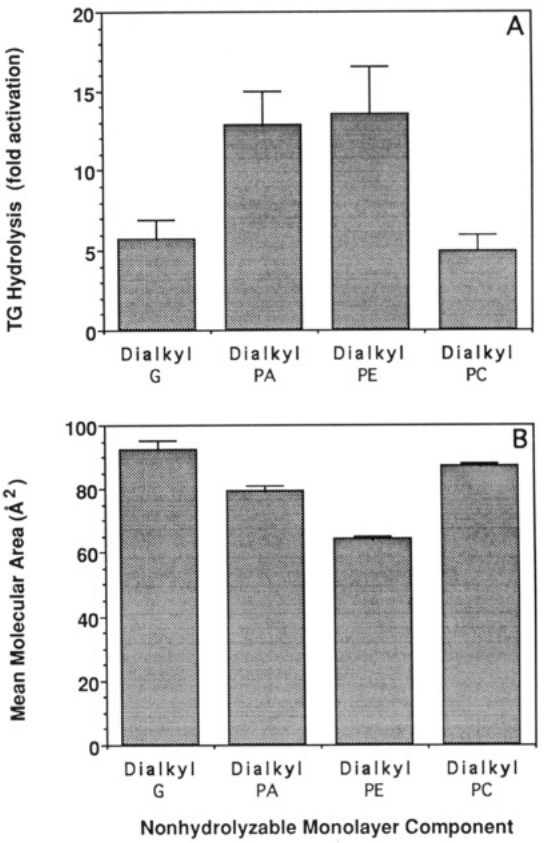

FIGURE 1: Activation of hepatic lipase-catalyzed triacylglycerol hydrolysis in mixed monomolecular films. (A) Trioctanoylglycerol hydrolysis by HL in mixed monomolecular films with dialkyl-G (1,2-*O*-didodecyl-*sn*-glycerol), dialkyl-PA (1,2-*O*-didodecyl-*sn*-glycero-3-phosphate), dialkyl-PE (1,2-*O*-didodecyl-*sn*-glycero-3-phosphoethanolamine), or dialkyl-PC (1,2-*O*-didodecyl-*sn*-glycero-3-phosphocholine) as the nonhydrolyzable component. The hydrolysis rate observed for a pure TG film was 0.019 nmol/min. The hydrolysis values reported for the mixed films represent the fold activation over hydrolysis of pure TG. Mixed films contained 10 mol % TG and 90 mol % of the nonhydrolyzable lipid component. Assay conditions were as described in Experimental Procedures. (B) The average mean molecular area at 15 mN/m (the surface pressure maintained during hydrolysis) for each of the monomolecular films was determined from surface pressure–mean molecular area isotherms.

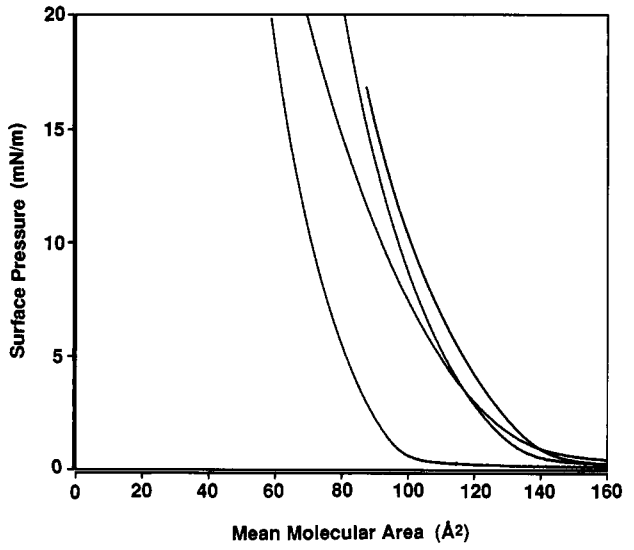

FIGURE 2: Surface pressure–mean molecular area isotherms for TG/dialkyl lipid mixtures (10:90 mol/mol). Conditions were as described in Experimental Procedures. From left to right the mixtures contained dialkyl-PE, dialkyl-PA, dialkyl-PC, and dialkyl G.

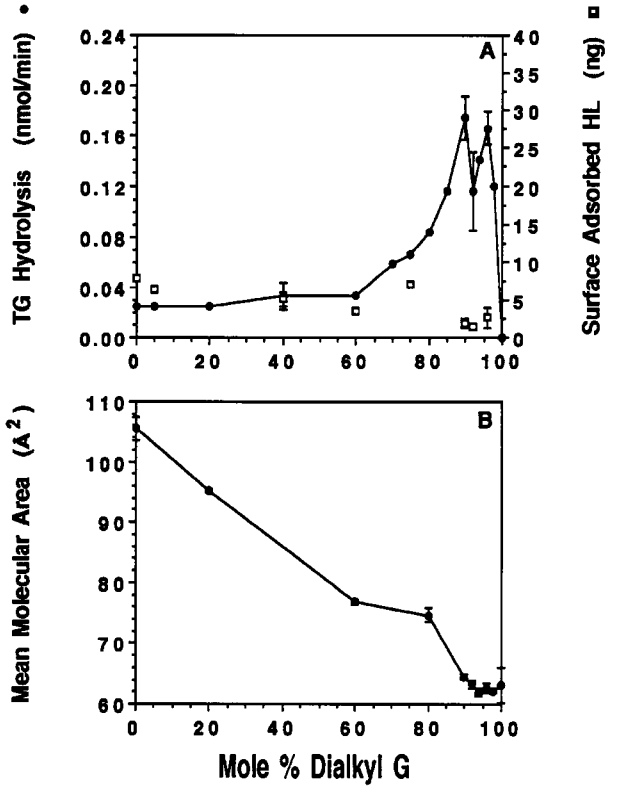

FIGURE 3: Activation of hepatic lipase-catalyzed triacylglycerol hydrolysis in mixed monomolecular films as a function of the dialkylglycerol concentration. (A) Enzyme activity is expressed as nanomoles of trioctanoylglycerol hydrolyzed per minute as a function of the mole percent of dialkyl-G in the mixed film (closed circles). The amount of HL adsorbed to the surface of the films is also shown (open squares). Assay conditions were as described in Experimental Procedures. (B) The average mean molecular area at 15 mN/m for the monomolecular films was determined from surface pressure–mean molecular area isotherms. The bars indicate standard deviations.

TG by HL was independent of the surface concentration of the substrate. The concentration of dialkyl-G required for optimal activation of HL was higher than that previously found for 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine (Thuren et al., 1991). In that case 50–60 mol % PC produced maximal

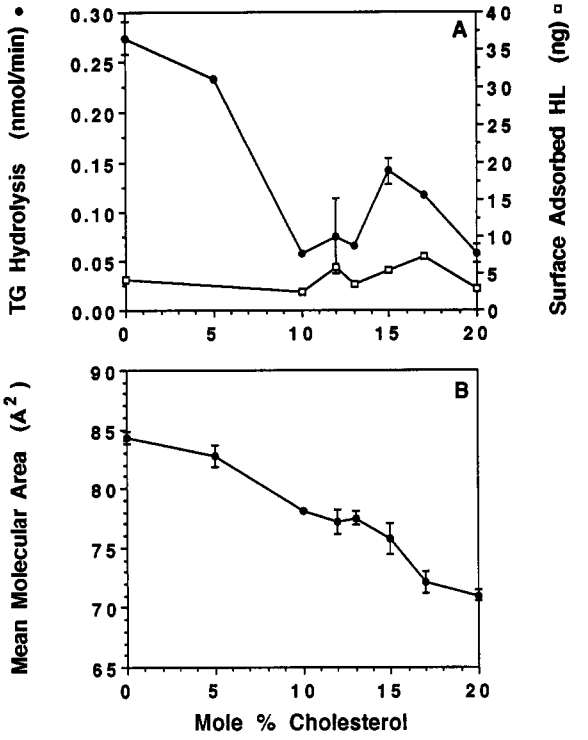

FIGURE 4: Effect of cholesterol on hepatic lipase-catalyzed triacylglycerol hydrolysis in mixed monomolecular films containing triacylglycerol, phosphatidylcholine, and cholesterol. (A) Enzyme activity is expressed as nanomoles of trioctanoylglycerol hydrolyzed per minute as a function of the mole percent of cholesterol in the mixed film (closed circles). The concentration of TG was constant at each point (5 mol %). The amount of HL adsorbed to the surface of the film is also shown (open squares). Assay conditions were as described in Experimental Procedures. (B) The average mean molecular area at 15 mN/m for each of the monomolecular films was determined from surface pressure–mean molecular area isotherms. The bars indicate standard deviations.

activation and activation was seen with as little as 30–40 mol % PC.

In comparing the two graphs in Figure 3, a relationship is seen between hydrolysis and the mean molecular area obtained from surface pressure–molecular area isotherms (data not shown) at 15 mN/m (Figure 3B). The activation of TG hydrolysis was observed to be concomitant with a deviation from the linear decrease in mean molecular area of lipid mixtures containing greater than 60 mol % dialkyl G. When the mean molecular area of the TG/dialkyl-G mixtures reached about 75 Å^2, HL hydrolysis was activated, apparently as the result of a change in the physicochemical properties of the substrate brought about by these concentrations of dialkylglycerol. The measured mixed lipid isotherms were continuous up to a surface pressure of 20 mN/m without evidence of nonideal mixing of components as was the case with dialkyl phospholipid/TG films.

Since these data showed that HL could be activated by a nonhydrolyzable lipid, it was of interest to determine the effect of cholesterol on TG hydrolysis, especially since the surface coat of lipoprotein particles is estimated to contain 5–20 mol % cholesterol (Miller & Small, 1980). In order to study this effect we used lipid films composed of 0–20 mol % cholesterol, 95–75 mol % PC, and a fixed concentration of TG (5 mol %) as substrate for HL (Figure 4A). TG was hydrolyzed maximally at a rate of 0.27 nmol/min in the PC/TG (95:5 mol %/mol %) mixed film that contained no cholesterol. The addition of 5 mol % cholesterol to the monomolecular film decreased the hydrolysis rate of TG by 15%. However, when

the concentration of cholesterol in the film was 10–13 mol %, the hydrolysis rate was decreased about 80%. Interestingly, between 15 and 17 mol % cholesterol the rate of hydrolysis was restored to about 50% of that seen in the absence of cholesterol. The hydrolysis rate decreased when the cholesterol content exceeded 15 mol % and was only 20% of the uninhibited rate when the film contained 20 mol % cholesterol. The decrease in the percentage of PC in the film from 95% to 75% was not responsible for the decreased hydrolysis since optimal phospholipid activation of TG hydrolysis by HL was previously seen with as little as 50 mol % PC in the film (Thuren et al., 1991a). We also measured lateral compression isotherms of these lipid mixtures at the air–water interface (data not shown). The isotherms were continuous up to a surface pressure of 20 mN/m and without any evidence of a deviation from expected compression behavior. The mean molecular area of the TG/PC/cholesterol mixtures at 15 mN/m (Figure 4B) was determined from surface pressure–mean molecular area isotherms. As in Figure 3, a relationship between hydrolysis and the average mean molecular area of the substrate film was observed (Figure 4). Deviation from the linear decrease in average mean molecular area at about 12–15 mol % cholesterol, corresponding to a mean area of approximately 75 Å^2, appeared to be associated with the partial restoration of hydrolysis observed with 15 mol % of this component.

On the basis of the observation that the presence of cholesterol in mixed TG/PC/cholesterol films affected the hydrolysis of TG, it was important to study the effect of this component on phospholipid hydrolysis to determine if the regulation of HL by cholesterol was general or specific for TG. To address this question, the hydrolysis of PE was studied in mixed monomolecular films that contained 0–20 mol % cholesterol (Figure 5A). 1,2-Didodecanoyl-*sn*-glycero-3-phosphoethanolamine was chosen as the model phospholipid since this substrate was hydrolyzed readily under the conditions employed with the monomolecular film (Thuren et al., 1991a). Another reason that PE was an appropriate choice is that this substrate was shown to be preferentially hydrolyzed by HL, compared to PC, during the hydrolysis of lipoprotein particles (Azema et al., 1990) and various artificial substrates (Kucera et al., 1988; Laboda et al., 1986; Landin et al., 1984; Ehnholm et al., 1975). The rate of hydrolysis of a pure PE film was 0.56 nmol/min, and that decreased 10% with the addition of 5 mol % cholesterol. When mixed films containing 10–12 mol % cholesterol were tested, PE hydrolysis was decreased about 30% when compared with pure PE. However, when the PE/cholesterol mixture containing 13 mol % cholesterol was assayed, PE hydrolysis was restored to the same level as observed with a pure PE film. This enhancement of HL activity occurred at about the same cholesterol concentration as the restoration of TG hydrolysis observed for the TG/PC/cholesterol films (cf. Figure 4A). Concentrations of cholesterol between 15 and 20 mol % decreased hydrolysis to a level comparable to that observed for films containing 10 and 12 mol % cholesterol. These results demonstrated that the effect of cholesterol in films with PE was mixed and that a narrow concentration range of cholesterol existed in which phospholipid hydrolysis was enhanced. Lateral compression isotherms for cholesterol/PE mixtures at the air–water interface were measured, and mean molecular areas were determined from the isotherms. The isotherms were continuous up to a surface pressure of 20 mN/m (Figure 6) and had essentially the same shape as isotherms for PC/TG/cholesterol mixtures. The mean molecular area of each PE/cholesterol mixture at a surface pressure of 15 mN/m obtained from

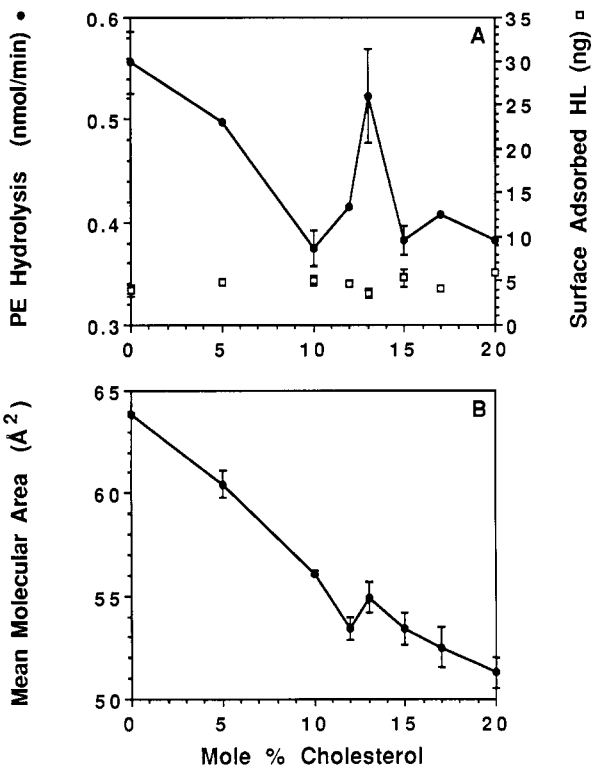

FIGURE 5: Effect of cholesterol on hepatic lipase-catalyzed phosphatidylethanolamine hydrolysis in mixed monomolecular films. (A) Enzyme activity is expressed as nanomoles of 1,2-didodecanoyl-*sn*-glycero-3-phosphoethanolamine hydrolyzed per minute as a function of the mole percent of cholesterol in the mixed film (closed circles). The amount of HL adsorbed to the surface of the film is also shown (open squares). Assay conditions were as described in Experimental Procedures. (B) The average mean molecular area at 15 mN/m for each of the monomolecular films was determined from surface pressure–mean molecular area isotherms. The bars indicate standard deviations.

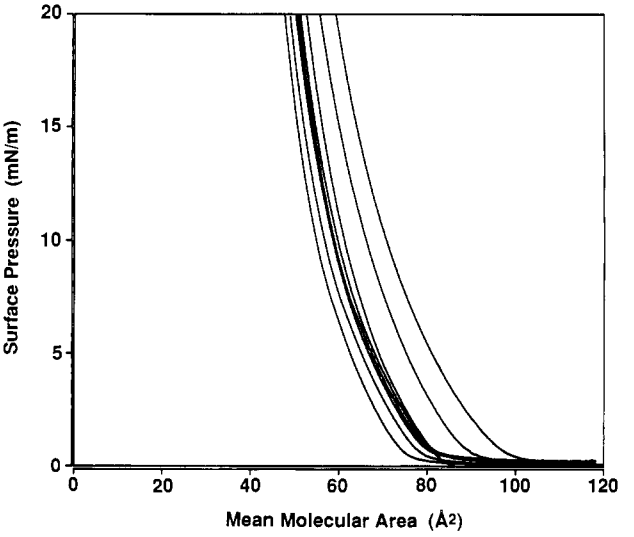

FIGURE 6: Surface pressure–mean molecular area isotherms for PE/cholesterol mixtures. Conditions were as described in Experimental Procedures. From left to right the mixtures had PE/cholesterol molar ratios of 80:20, 83:17, 88:12, 85:15, 87:13, 90:10, 95:5, and 100:0 (mol %:mol %).

surface pressure–mean molecular area isotherms (Figure 6) is shown in Figure 5B. Again, deviation from the linear decrease in average mean molecular area of PE and cholesterol at about 13 mol % cholesterol corresponded to the observed enhancement of HL-catalyzed PE hydrolysis (Figure 5). However, the mean molecular area at which the activation

occurred for PE/cholesterol was significantly lower than that seen with activation of TG hydrolysis in TG/dialkyl-G (Figure 3) and TG/PC/cholesterol films (Figure 4), approximately 55 Å^2 vs 75 Å^2, respectively.

DISCUSSION

Here we demonstrate that both the molecular composition and physical properties of the lipid substrate interface regulate activity of HL. We postulate also, on the basis of these and previous results (Kucera et al., 1988), that HL activity on substrate lipid is regulated by the presence of a second modulatory lipid molecule such as the dialkyl lipid used here. Other studies using different reaction conditions and substrate films have also demonstrated that the lipid composition of monomolecular films influences HL activity (Thuren et al., 1991a; Jackson et al., 1986; Laboda et al., 1988), and substrate conformation has been shown to be an important factor in the mechanism of other enzymes that catalyze interfacial lipid hydrolysis (Thuren et al., 1984, 1987; Thuren, 1988; Barlow et al., 1988; Scott et al., 1991). Earlier we proposed that HL may bind more than one lipid molecule in or close to its active site (Kucera et al., 1988). Related to this there could be a direct activation of HL by the binding of a relatively small number of activator lipid molecules to the enzyme. This number could be as low as one if a specific binding site existed on HL. Further, the observed activation of HL could be related to the observed decrease in the mean molecular area that occurs in the presence of the activator molecule found in these experiments.

The lipid composition of the films did not appear to affect the interfacial concentration of HL, indicating that the changes in activity observed for various lipid mixtures were not related to the amount of enzyme associated with the film. Lipid binding properties of pancreatic carboxyl ester lipase were regulated in similar types of mixed films by lipid miscibility changes (Tsujita et al., 1987). Nonideal mixing of lipids in the lipid film resulted in increased lipid binding and activation of porcine pancreatic phospholipase A2 and pancreatic lipase–colipase (Cunningham et al., 1989). However, our isotherm experiments demonstrated that the mixed lipid films used herein did not undergo changes in lipid miscibility at the surface pressure range used in this study, 0–20 mN/m. This is not surprising since we used low surface pressure and short- to medium-chain lipids, in contrast to high surface pressure above 40 mN/m and long-chain lipids used with the pancreatic lipase and phospholipase A2 (Tsujita et al., 1987; Cunningham et al., 1989). Further, we have shown earlier using Triton X-100/phospholipid mixed micelles that different phospholipid species can activate HL-catalyzed hydrolysis of a second phospholipid molecule when both lipids are present in the same mixed micelle (Thuren et al., 1990). In this substrate system, lipid mixing is considered to be a complete and rapid phenomenon (Kucera et al., 1988). Therefore, that study serves as an example that HL activity can be regulated by a second lipid molecule and that this activation is not dependent on changes in lipid mixing. The changes in lipid miscibility may result in changes in stability of HL protein, causing an activation. However, our reaction rates were linear with time up to 90 min when using selected mixed lipid monolayers as substrates for HL, suggesting that HL protein stability was not affected by the lipid composition of the films. This was also true in the Triton X-100 micellar system in which 40–50% of the substrate present was hydrolyzed in a near linear fashion.

Data presented in this study suggest that the action of HL is related to subtle changes in the molecular conformation of the substrate in monomolecular films. The relationship between the film composition and HL activity was demonstrated by the enhancement of TG hydrolysis in mixed films that contained 10 mol % TG with activator dialkyl-G, dialkyl-PA, dialkyl-PE, or dialkyl-PC, compared to the hydrolysis of a pure TG film at a constant surface pressure. Mixtures that contained dialkyl-PA and dialkyl-PE and had the smallest mean molecular areas (79 and 64 A^2, respectively) were hydrolyzed at higher rates than the other substrates tested.

In addition to the activation by lipid packing, direct activation of HL by the binding of activator lipid molecule(s) may occur. This is supported by the observation that among the phospholipids the capacity to activate is directly related to the relative rates of hydrolysis of these phospholipids by HL in mixed Triton X-100 micelles (Kucera et al., 1988) and monomolecular films (Thuren et al., 1991a). The interpretation that the activator molecule may bind directly to HL implies that two lipid binding sites exist on HL, a possibility previously suggested on the basis of studies with micellar substrates (Kucera et al., 1988). In addition, the lipids activating HL-catalyzed triacylglycerol hydrolysis are capable of hydrogen bonding, and therefore, it is possible that these activator lipids may alter the orientation of HL bound to the lipid film, which may result in activation of HL. At present, insufficient information is available on the three-dimensional structure of HL to support any of these possibilities.

We favor the interpretation that cholesterol produces alterations in the organization of the film that regulate HL, even though this is still quite speculative. While a direct interaction of cholesterol with HL may occur, we believe this to be unlikely. If the effect of cholesterol on catalysis were to be mediated at a specific site on HL, we would expect the effect on TG and PE hydrolysis to be roughly equal; this is not the case. Ten mole percent cholesterol in the TG/PE film (Figure 4) caused on 80% inhibition. On the other hand, the same percentage of cholesterol in the PE film (Figure 5) produced only a 15% reduction in hydrolysis. A second argument against direct binding of cholesterol is that cholesterol does not appear to be involved in the transacylation reaction catalyzed by HL, whereas a variety of phospholipids and neutral lipids are (Waite & Sisson, 1974).

It is possible that the effect of cholesterol on TG hydrolysis in the tertiary system of PC/TG/cholesterol (Figure 3) could be the same as that seen with the binary systems that measured PE hydrolysis (Figure 5). It is known that cholesterol causes a condensation of the film when mixed with phospholipids (Ibdah & Phillips, 1988). It is interesting to note that the activation of HL occurs in films at an approximate ratio of one cholesterol to six phospholipid molecules. Also, at this ratio there is a diversion from the condensing effect of cholesterol. It has been demonstrated that interactions between cholesterol and PC occur at specific mole ratios including a cholesterol mole fraction of 0.33 (cholesterol to acyl chain) (Lundberg, 1982). At this point hexagonal arrangements exist in which the mobility of the acyl chains was thought to occur. It would follow then that the effect of cholesterol on TG hydrolysis in the TG/PC mixed films is primarily mediated through cholesterol–PC interactions. The dramatic inhibition of TG hydrolysis by cholesterol seen in Figure 4, therefore, should be mediated through a sequestration of activator PC by cholesterol, making PC unavailable for its activation effects. This sequella is consistent with the dramatic 30–40-fold activation of TG hydrolysis by PC (Thuren, 1991). The partial relief of hydrolysis seen at 14–17 mol % cholesterol in Figure 3 would imply that either the PC in the hexagonal

patches is free to interact with HL or that this hexagonal patch itself is an activator, either directly or through the formation of a favored film structure for catalysis. The same phenomenon appears to exist for PE/cholesterol mixtures except that cholesterol would sequester the substrate rather than the activator phospholipid molecule.

Laboda et al. (1988) found that cholesterol activated HL action on TG in an inert matrix. In general they used higher cholesterol percentages and there is only one point of comparable cholesterol concentration used, 14 mol %. This is a concentration of cholesterol that gave a partial restoration of TG hydrolysis in our studies (Figure 4). Since a number of conditions that we used differed from those of these workers, we can only concur that cholesterol regulates HL in a complex manner, probably through the quality of the film.

The activation of HL by lipid is significantly greater than the 3-fold activation of HL-catalyzed HDL phospholipid hydrolysis by apolipoprotein E (Thuren et al., 1991, 1992). This high degree of activation suggests that, physiologically, the lipid composition of the outer monomolecular film of lipoprotein particles may influence the packing of TG or phospholipid and, consequently, the accessibility of these substrates to the active site of HL. These results suggest that HL is efficiently able to catalyze hydrolysis of surface lipid film triacylglycerol of lipoprotein particles. Activity of HL seems to be targeted toward surface lipid film triacylglycerol of lipoproteins rather than lipoprotein core triacylglycerol. The results presented clearly demonstrate that surface cholesterol, in addition to apoproteins, will regulate HL *in situ*. Although very little is known about distribution of free cholesterol between the core and surface of lipoproteins, it has been suggested that at least in different-sized chylomicron particles the amount of free cholesterol in the surface varies (Gotto et al., 1986). This further suggests that it is possible that the concentration of free cholesterol in the surface of lipoproteins is a physiologically important regulatory factor for the activity of HL.

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