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BINDING OF INSULIN TO THE EXTERNAL SURFACE OF LIPOSOMES

EFFECT OF SURFACE CURVATURE, TEMPERATURE, AND LIPID COMPOSITION

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The binding of insulin to the external surface of phosphatidylcholine liposomes as a function of the temperature, the surface curvature, and the composition of lipids was studied. The amount of the saturated binding of insulin to liposomes was assessed by gel-filtration chromatography. The binding of insulin to small unilamellar vesicles was highly dependent upon the temperature, favoring low temperatures. As the temperature increased, there was a distinct temperature range where the binding of insulin to small unilamellar vesicles decreased. The temperature ranges for dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) small unilamellar vesicles were found to be 10–20°C and 21–37°C, respectively. These temperature ranges were quite different from the reported ranges of the gel→liquid crystalline phase transition temperatures (T_c) for DMPC or DPPC small unilamellar vesicles. In contrast to other proteins, the amount of insulin bound to DMPC and DPPC small unilamellar vesicles was negligible at or above the upper limit of the above temperature ranges, and increased steadily to 6–7 μmol of insulin per mmol of phospholipid as the temperature decreased to or below the lower limit of these temperature ranges. On the other hand, the binding of insulin to the large multilamellar liposomes cannot be detected at all temperatures tested. The affinity of insulin to neutral phosphatidylcholine small unilamellar vesicles appeared to be related to the surface curvature of the liposomes, favoring the liposomes with a high surface curvature. Furthermore, the amount of insulin bound to small unilamellar vesicles decreased as the content of the cholesterol increased. The presence of 10% molar fraction of phosphatidic acid did not appear to affect the binding of insulin to small unilamellar vesicles. However, the presence of 5% molar fraction of stearylamine in DPPC small unilamellar vesicles increased the amount of bound insulin as well as the extent of aggregation of liposomes. The results of the present study suggest that the interstitial regions of the acyl chains of phospholipids between the faceted planes of small unilamellar vesicles below T_c may be responsible for the hydrophobic interaction of insulin and small unilamellar vesicles. The tight binding of insulin to certain small unilamellar liposomes could lead to an overestimation of the true amount of insulin encapsulated in liposomes, if care is not taken to eliminate the bound insulin during the procedure of encapsulating insulin in liposomes.

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Abbreviations: T_c, gel→liquid crystalline phase transition temperature; DLPC, L-α-dilauroylphosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; DPPA, L-α-dipalmitoylphosphatidic acid; DSPC, L-α-distearoylphosphatidylcholine; DOPC, L-α-dioleoylphosphatidylcholine.

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Introduction

Recently there has been an increasing interest in the potential application of liposomes as a delivery system for pharmacological agents and macromolecules [1,2]. One important factor which may influence the bioavailability of a liposome-associated agent and its pharmacological actions is the location of the agent in the liposome. Generally, aqueous-soluble drugs and macromolecules are encapsulated in the internal water compartment of the liposome. However, some aqueous-soluble amphipathic substances may be incorporated directly into the lipid bilayer of a liposome in addition to being entrapped in the internal water compartment [3]. Furthermore, the binding of amphipathic substances to the liposomal surface could also occur. It has been shown that some amphipathic water-soluble polypeptides, such as immunoglobulins [4] and serum albumin [5], remain tightly bound to the external surface of liposomes even after separation by gel-filtration chromatography. Presumably, enzymes or peptide hormones exposed to the external aqueous compartment may have different modes of interaction with target cells or tissues from those entrapped in the interior of liposomes.

The molecular surface of insulin is comprised of both polar and nonpolar areas [6]. It is quite possible that insulin can bind to the surface of liposomes through either or both of these surface areas. The consideration of the potential association of insulin to the external surface of a liposome is important in evaluating the interaction of liposome-encapsulated insulin with cells in vitro and in vivo. Insulin encapsulated in liposomes has been used to facilitate the transport of insulin across intestinal epithelia in vivo [7–10]. In this type of study, one of the most important factors in evaluating the hypoglycemic effect of various preparations of liposome-encapsulated insulin after oral administration to diabetic animals is the determination of the true amount of insulin entrapped in the liposomes. It is crucial to differentiate the insulin bound to the external surface of liposomes from the insulin entrapped in the interior of the liposomes, since neither insulin alone nor insulin situated outside the liposomes was found to have a significant hypoglycemic effect after oral administration to diabetic animals. The present study is undertaken to investigate whether and how insulin may bind to the external surface of a liposome as a function of the temperature, surface curvature, and lipid composition of the liposome.

Materials and Methods

L-α-Dilauroylphosphatidylcholine (DLPC), L-α-dimyristoylphosphatidylcholine (DMPC), L-α-dipalmitoylphosphatidylcholine (DPPC), L-α-distearoylphosphatidylcholine (DSPC), L-α-dioleoyl phosphatidylcholine (DOPC), cholesterol and bovine brain sphingomyelin were purchased from Sigma Chemical Co. Egg phosphatidylcholine and hydrogenated egg phosphatidylcholine were obtained from P-L Biochemicals, Inc. $^{111}InCl_3$ was obtained from Medi-Physics and purified as described previously [11]. Other chemicals were of reagent grade.

Zn-free insulin was prepared from regular bovine Zn-insulin (Sigma Chemical Co.) according to the method of Carpenter [12]. Proinsulin-free insulin was prepared by passage of Zn-insulin over a column (2.5 × 110 cm) of Sephadex G-50F (Pharmacia) equilibrated with 1 M acetic acid according to the procedure of Steiner et al. [13]. ^{125}I-labeled insulin was prepared by the method of Greenwood and Hunter [14] according to the procedure of Freychet et al. [15], except the molar equivalent of insulin:iodine:chloramine T was 1:1:20. The ^{125}I-labeled insulin was 96% precipitable by trichloroacetic acid and 90% precipitable by guinea pig anti-porcine immunoglobulins (Miles Laboratories, Inc.) using poly(ethylene glycol) as the precipitating agent of insulin-immunoglobulin complex. Biologically active tyrosine A14 mono[^{125}I]iodoinsulin was prepared according to the procedure of Gliemann et al. [16], and kindly provided by Dr. Thomas Paquette at the Diabetes Research Center of the University of Washington.

Unilamellar liposomes with a high curvature and a small diameter were prepared by sonicating a thin film of a lipid mixture or the dry powder of a pure synthetic phospholipid in 0.9% NaCl, 7 mM Tris-HCl buffer, pH 7.4 as described previously [17]. Briefly, a suspension of unilamellar liposomes was prepared by sonicating the lipid mixture with

a Branson sonifier (Model 350) at an output of 50 W for 15 min at 45°C, using a titanium microtip. The small unilamellar liposomes were then centrifuged at $10000 \times g$ for 3 min to remove the titanium particles and large aggregates. The average size of DPPC small unilamellar liposomes was estimated to be 206 ± 54 Å from negative-stain electron micrographs of the liposomes using potassium phosphotungstate as the stain. Multilamellar liposomes were prepared by sonicating a thin film of lipid in a bath sonifier for 10 min at room temperature.

The binding of insulin with liposomes was performed by incubating various types of liposomes containing 5–6 μmol/ml of lipids with $2.7 \cdot 10^{-4}$ M of bovine Zn-insulin or Zn-free insulin in the presence of absence of $(1.4–4.3) \cdot 10^{-10}$ M of ^{125}I-labeled insulin or tyrosine A14 mono[^{125}I]iodoinsulin at various temperatures for varying periods. The incubation mixture was in 0.154 M NaCl, 7 mM Tris-HCl, pH 7.4. For studying the effect of ionic strength, 1.58 M NaCl, 7 mM Tris-HCl, pH 7.4 was used. To determine the amount of insulin needed for the saturated binding of insulin to liposomes, the concentration of Zn-insulin in the above incubation mixture was varied from $1.7 \cdot 10^{-5}$ M to $2.7 \cdot 10^{-4}$ M. The unbound insulin and the insulin bound to liposomes were separated by passage of 500 μl of the incubation mixture over a column (0.9×76 cm) of Sepharose 4B or Sepharose CL4B (Pharmacia) or a column (0.9×40 cm) of Bio-Gel A 1.5 M (Bio-Rad) equilibrated with the same buffer solution of Tris-saline. In some cases, the column was equilibrated with 7 mM Tris-HCl and 1.58 M NaCl for investigating the effect of ionic strength on the binding of insulin to liposomes. Unless it is specified, the gel-filtration chromatography was performed at the same temperature as the binding condition by means of a water-jacketed column.

To investigate the effect of insulin on the permeability of ions to the membrane of the liposomes, liposomes entrapping $^{111}In^{3+}$ were used. The encapsulation of $^{111}In^{3+}$ by liposomes was performed by sonicating the thin film of lipid in 1 mM nitrilotriacetic acid, 106 mM isotonic sodium phosphate, pH 7.4 containing 15–20 μCi $^{111}InCl_3$. The entrapped $^{111}InCl_3$ was removed by passage of the liposome suspension over a small column (0.6×7 cm) of AGlX8 (phosphate form, Bio-Rad) as described previously [17]. The isotonic phosphate buffer in the liposome suspension was changed back to the buffer solution of Tris-saline by passage of the liposomes over a 0.9×35 cm column of Sephadex G-50 (Pharmacia) equilibrated with 0.154 M NaCl, 7 mM Tris-HCl, pH 7.4. The release of $^{111}In^{3+}$ from liposomes as a result of the binding of insulin was assessed by the technique of gel-filtration chromatography, as described above for the binding of insulin to liposomes.

Radioactivity of ^{125}I and ^{111}In in column fractions was monitored by a well-type gamma counter. The concentration of liposomes in each fraction was determined by phosphate analysis [18,19] after perchloric acid ashing of the samples or by the fluorescence method using diphenylhexatriene [20]. The concentration of insulin was measured either by radioimmunoassay, using poly(ethylene glycol) as the precipitating agent of the insulin-antibody complex [21] or by the method of Lowry et al. [22], using bovine Zn-insulin as a standard and 0.1% sodium dodecyl sulfate (SDS) to solubilize the lipid.

Results

Binding of insulin to small unilamellar DPPC liposomes

The typical patterns of the gel-filtration chromatography of the mixture of regular insulin and tyrosine A14 mono[^{125}I]iodoinsulin or the small unilamellar DPPC liposomes alone, are shown in Figs. 1A and 1B. The binding of insulin to liposomes was indicated by the migration of regular insulin and/or tyrosine A14 mono[^{125}I]iodoinsulin with the small unilamellar DPPC liposomes (Fig. 1C), when they were incubated together and applied to the same Sepharose 4B column.

In Fig. 1C the amount of insulin in each fraction eluted from a Sepharose 4B column was determined by two methods. The first method was based on the radioactivity of tyrosine A14 mono[^{125}I]iodoinsulin in each fraction. The second method was based on the Lowry protein assay. The amount of insulin bound to liposomes, as determined by the method based on tyrosine A14 mono[^{125}I]iodoinsulin, was 3-times higher than that

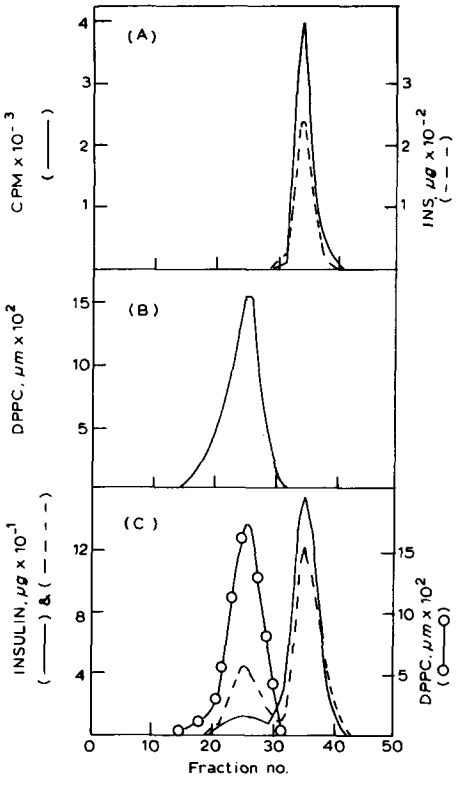

Fig. 1. Binding of tyrosine A14 mono[^{125}I]iodoinsulin to small unilamellar DPPC liposomes. The elution profile of the gel filtration chromatography of (A) the mixture of tyrosine A14 mono[^{125}I]iodoinsulin and Zn-insulin, (B) small unilamellar DPPC liposomes, and (C) the mixture of (A) and (B) after an incubation time of 5 min at 21°C, from a Sepharose 4B column. The concentration of Zn-insulin (————), as determined using the Lowry protein assay, is plotted against that of the estimated amount of Zn-insulin (- - - - -) based on the cpm of tyrosine A14 mono[^{125}I]iodoinsulin in each fraction and the specific radioactivity of the insulin.

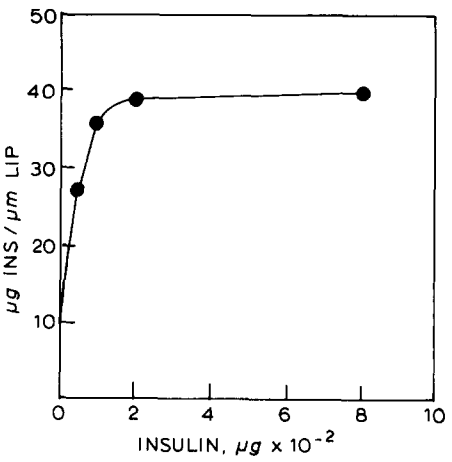

Fig. 2. Saturation curve of the binding of Zn-insulin to the small unilamellar DPPC liposomes. The specific binding of Zn-insulin (μg insulin per μmol lipid) to small unilamellar DPPC liposomes was determined by incubating 2 mg of DPPC liposomes with various amounts of Zn-insulin in 0.5 ml of buffer at 21°C for 5 min followed by gel filtration chromatography in an A 1.5 m column and analysis of the column fractions for lipid and protein. The liposomes and bound insulin eluted at the void volume in advance of the residual unbound insulin. From the amounts of Zn-insulin in DPPC in the liposome fractions, the specific binding of Zn-insulin to DPPC liposomes was calculated.

estimated by the method of Lowry et al. (40 μg insulin/ μmol DPPC). This suggests that the affinity of A14 mono[^{125}]iodoinsulin to the small unilamellar DPPC liposomes was higher than that of the regular Zn-insulin to the same liposomes. Similar results were also observed when ^{125}I-labeled insulin (see Materials and Methods) instead of tyrosine A14 mono[^{125}I]iodoinsulin was used (data not shown). The discrepancy between iodinated insulins and the regular Zn-insulin cannot be attributed to the radioactivity of the degraded products of iodinated insulin, since more than 95% of the ^{125}I radioactivity bound to liposomes was precipitable by 5% trichloroacetic acid. Furthermore, the discrepancy was not a result of the underestimation by Lowry's procedure because of the presence of lipid, since in the presence of 0.1% SDS the amount of lipid in the column fractions did not interfere with the color formation in Lowry's protein assay. By contrast, about the same amount of liposome-bound insulin (48–50 μg insulin/μmol DPPC) was found by both methods, when the bovine Zn-insulin was substituted with the same amount of non-radioactive [^{127}I]iodinated insulin in the binding experiment. This indicates that ^{125}I-labeled insulin is displaced differently by regular Zn-insulin and non-radioactive iodinated Zn-insulin. The binding of insulin to DPPC small unilamellar liposomes was found to be saturable (Fig. 2) and rapid. The saturated amount of insulin bound to small unilamellar DPPC liposomes after 5 min and 1 h incubation time was virtually identical.

In investigating whether or not other forms of insulin may bind to small unilamellar liposomes differently, our results indicated that the amount of Zn-free insulin bound to the small unilamellar

DPPC liposomes (44 μg insulin/μmol phospholipid at 21°C) was quite similar to that of the liposome-bound Zn-insulin. Furthermore, the saturated amount of bound proinsulin-free insulin was virtually identical to that of the bound Zn-insulin under the same binding conditions, suggesting that insulin, rather than proinsulin, is responsible for the observed phenomenon of the binding of insulin to liposomes.

In studying the nature of the binding of insulin to liposomes, our results indicated that hydrophobic interaction between insulin and small unilamellar DPPC liposomes could be important, since the saturated amount of Zn-insulin bound to DPPC small unilamellar liposomes was not affected significantly by increasing the ionic strength of the incubation mixture from 0.154 M NaCl to 1.58 M NaCl. It was also found that the binding of insulin to DPPC small unilamellar liposomes tended to enhance the amount of the release of encapsulated $^{111}In^{3+}$ from liposomes (Fig. 3). The amount of $^{111}In^{3+}$ released from liposomes incubating with insulin for 5 min or 1 h was virtually identical. It was noted that only about 70% of the encapsulated $^{111}In^{3+}$ was released by the binding of insulin. The size distribution of the liposomes remained unchanged after the release of $^{111}In^{3+}$. This suggested that insulin did not cause a significant structural reorganization of the liposomes.

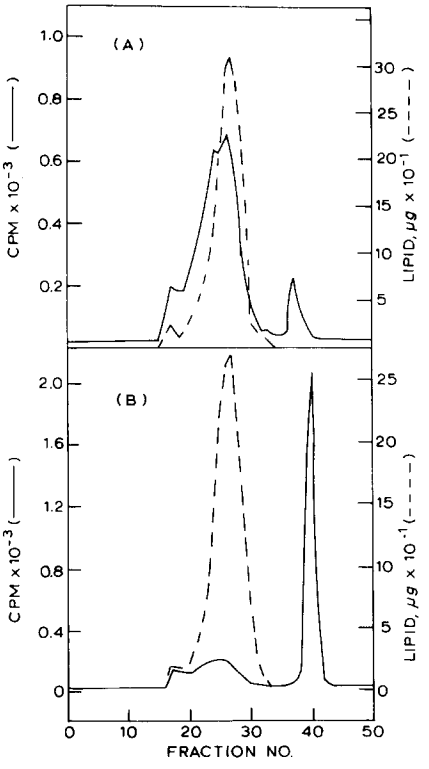

Fig. 3. Leakage of $^{111}In^{3+}$ from small unilamellar DPPC liposomes in the absence (A) and the presence (B) of Zn-insulin. (A) DPPC liposomes (5.1 mg/ml) encapsulating $^{111}In^{3+}$ were incubated with 1 mM ethylenediaminetetraacetic acid (EDTA) at 21°C for 1 h prior to the separation of liposome-encapsulated $^{111}In^{3+}$ (fraction No. 16 to No. 33) from the released $^{111}In^{3+}$ bound to EDTA (fraction No. 36 to No. 42) in a Sepharose 4B column. (B) Zn-insulin (1.19 mg/ml) was included in the incubation mixture of (A) and all the experimental conditions were the same as that of (A). The same DPPC liposomes was used for (A) and (B), and Sepharose 4B columns similar in dimensions were run at the same time.

Effect of temperature, surface curvature and composition of liposomes on the binding of insulin to liposomes

The amount of Zn-insulin bound to small unilamellar DPPC or DMPC liposomes was markedly affected by temperature (Fig. 4). A similar temperature-dependence of the binding of ^{125}I-labeled insulin to DPPC or DMPC small unilamellar liposomes was also observed (data not shown). The effect of temperature on the binding of insulin to

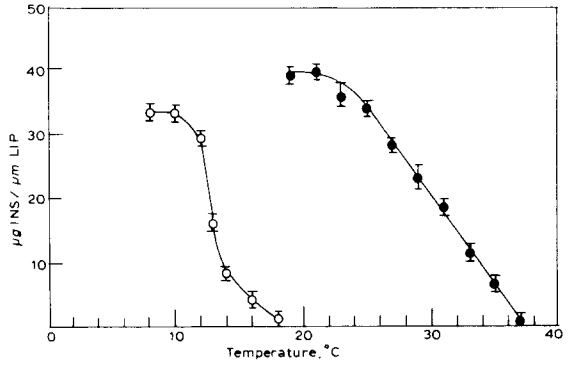

Fig. 4. Effect of temperature on the binding of Zn-insulin to small unilamellar DMPC and DPPC liposomes. The saturated amount of Zn-insulin bound to DMPC (○——○) and DPPC (●——●) liposomes was assessed by incubating 1.6 mg/ml Zn-insulin with 4 mg/ml lipid followed by column chromatography at various temperatures in three A 1.5 m columns of the same dimensions to separate the liposome-bound from the unbound insulin. The concentrations of insulin and lipid in the liposomal fractions were determined by protein assay and phosphate assay, respectively. Each point on the graph is an average of three measurements from three different columns.

liposomes was reversible, as indicated by the fact that the extent of the binding of insulin to small unilamellar liposomes was not affected by prior incubation of the mixture of liposomes and insulin at temperatures which were different from the final incubating temperature. Our results indicated that at 21°C, ^{125}I-labeled insulin did not bind to small unilamellar liposomes with low gel → liquid crystalline phase transition temperature (T_c), such as liposomes made from DLPC, DOPC and egg phosphatidylcholine, with or without charged lipids, such as 5–10% stearylamine or dipalmitoylphosphatidic acid. However, ^{125}I-labeled insulin bound tightly to liposomes with high T_c, such as small unilamellar liposomes made from DSPC, bovine brain sphingomyelin and hydrogenated egg phosphatidylcholine (data not shown).

Bath-sonicated large multilamellar DPPC liposomes did not bind to regular Zn-insulin (Fig. 5) or ^{125}I-labeled insulin (data not shown), even at temperatures where a maximal binding of insulin to DPPC small unilamellar liposomes occurred. The presence of an increasing amount of

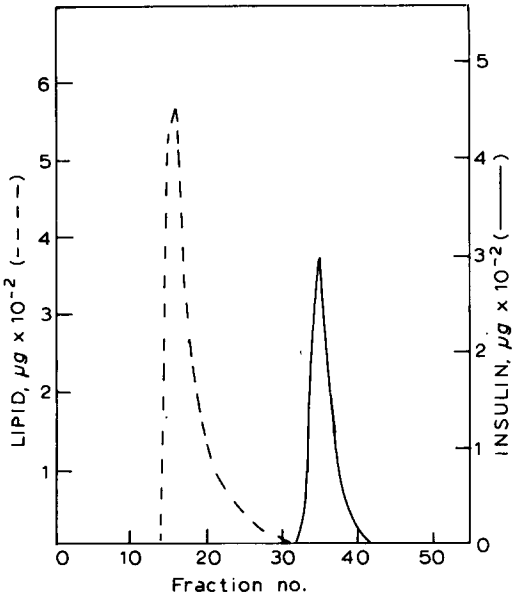

Fig. 5. Lack of binding of Zn-insulin to large multilamellar DPPC liposomes. Multilamellar DPPC liposomes (4 mg/ml) were incubated with Zn-insulin (1.6 mg/ml) at 21°C for 5 min and chromatographed in a Sepharose CL4B column. The amount of Zn-insulin and lipid in the column fractions was determined by protein assay and phosphate assay, respectively.

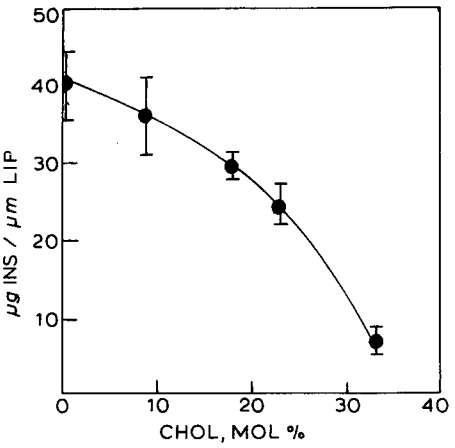

Fig. 6. Effect of the content of cholesterol on the binding of insulin to DPPC small unilamellar liposomes. The saturated amount of Zn-insulin bound to DPPC liposomes containing varying amounts of cholesterol was assessed at 21°C by the method described in Fig. 5. Each point was an average of two independent measurements.

cholesterol in DPPC small unilamellar liposomes tended to decrease the saturated amount of bound Zn-insulin (Fig. 6). The presence of 10% molar fraction of the negatively charged DPPA in DPPC small unilamellar liposomes did not appear to affect the saturated amount of bound Zn-insulin (44.8 ± 2.5 μg insulin/μmol phospholipid at 21°C) significantly. However, the presence of 5% molar fraction of the positively charged stearylamine in DPPC small unilamellar liposomes increased the saturated amount of bound Zn-insulin to 64.7 ± 7.0 μg insulin μmol phospholipid at 21°C. Furthermore, Zn-insulin tended to induce extensive aggregation of stearylamine containing DPPC small unilamellar liposomes at room temperature, resulting in a very low recovery of lipid from the column.

Discussion

Two lines of evidence suggest that the association of insulin with the liposomal fractions after gel-filtration chromatography is a phenomenon of a tight binding of insulin to the external surface of liposomes, rather than the internalization or encapsulation of insulin into the internal aqueous compartment of small unilamellar liposomes. As

shown in Fig. 2, at low insulin concentrations, 90% or more of the total insulin present is associated with the liposomes, while the encapsulating volume of the small unilamellar liposomes is on the order of 2–5% of the total volume. Furthermore, if the insulin was being internalized, as the insulin concentration increased the amount of insulin in association with the liposomes would have continued to increase proportionately. These findings preclude the possibility of insulin being encapsulated in the internal aqueous compartment of the liposomes.

The results of the present study indicate that, under some conditions, insulin can bind tightly to the surface of certain liposomes. The amount of insulin bound to liposomes can be as high as 40–60 μg per μmol of phospholipid. The lack of binding of insulin to the large multilamellar liposomes at all temperatures tested implies that the sites that are responsible for the binding of insulin to the small unilamellar liposomes of the same composition of lipid are inaccessible to insulin in the large multilamellar liposomes. This suggests that the choline group of the phospholipid molecule is probably not involved significantly in the binding of insulin to the small unilamellar liposomes.

The effect of temperature on the binding of insulin to DMPC and DPPC small unilamellar liposomes at two characteristic regions of temperature (10–20°C for DMPC and 21–38°C for DPPC) suggests that the change of the saturated amount of insulin bound to liposomes with the temperature shown in Fig. 4 is a reflection of the change in the surface of the small unilamellar liposomes rather than of the change of the conformation of insulin with temperature. The effect of temperature on liposomes was further supported by the study of the binding of insulin to other liposomes with different composition.

It is interesting to point out that the temperature ranges where the change of the binding of insulin to DMPC and DPPC small unilamellar liposomes occur are lower than the temperature ranges where the changes of various physical parameters accompanying the gel → liquid crystalline phase transition of the liposomes occur. Van Dijck et al. [23] reported that the thermotropic behavior and the pattern of the light scattering of small unilamellar DMPC liposomes goes through a broad transition in the temperature ranges of 18–25°C and 16–28°C, respectively. Similarly, Lentz et al. [24] showed that the depolarization of the fluorescence of 1,6-diphenyl-1,3,5-hexatriene imbedded in the bilayer of small unilamellar DMPC and DPPC liposomes also has a broad transition in the temperature ranges of 14.3–27.4°C and 29.7–40.6°C, respectively. These temperature ranges appear to be significantly higher than the temperature ranges for DMPC and DPPC small unilamellar liposomes in binding to insulin. Thus, it is likely that the molecular mechanism responsible for the binding of insulin to the small unilamellar, and not the large multilamellar, liposomes is not merely the phenomenon of motion of the acyl chain of the phospholipid molecule in the lipid bilayer. Perhaps the packing structure of the phospholipid molecules on the surface of the highly-curved small unilamellar liposomes at different temperatures plays an important role in the binding of insulin.

One possible explanation for the effects of size of liposomes and temperature on the binding of insulin to the surface of liposomes may be derived from the polygonal structure of the highly curved DPPC unilamellar liposomes below T_c recently proposed by Blaurock and Gamble [25]. According to the polygonal model of DPPC small unilamellar liposomes below the T_c, one may speculate that in or near the interstitial, 'edge', regions of the polygonal liposomes, the acyl chains of phospholipids are packed very irregularly in such a way as to become quite accessible for hydrophobic interactions with insulin. Thus, the amount of insulin binding to a lipid bilayer will depend on the degree of the polygonal character on the surface of liposomes. The temperature ranges (Fig. 4) where the transition of the binding of insulin to liposomes occur could be the temperature ranges of the transition from the polygonal character at a low temperture to the spherical structure of the liposomes at a high temperature. Conceivably, the decrease of the binding of insulin to liposomes with the increase of the content of cholesterol (Fig. 6) may well be a consequence of the interference of cholesterol in the formation of the polygonal structure of liposomes.

There are two possible explanations for the

difference between the binding affinity of iodinated insulins and regular Zn-insulin to the small unilamellar liposomes, when the small unilamellar liposomes are incubated with a trace amount of iodinated insulin and a large excess of regular insulin above the aggregating concentration of 10^{-7} M [26]. The first explanation, a possible change in the tertiary structure of insulin after iodination, is not very likely in view of the full biological activity of tyrosine A14 mono[^{125}I] iodinated insulin [16]. An alternative explanation is that the regular Zn-insulin may not form dimers or polymers with iodinated insulin as easily as with regular Zn-insulin. It has been shown by Blundell et al. [6] that the nonpolar interaction plays an important role in dimer association of insulin. Conceivably, some of the hydrophobic surface of insulin will be masked by the dimerization of insulin, leaving less nonpolar regions in the insulin for other hydrophobic interactions. Therefore, under the binding condition where the concentration of regular insulin was above the critical concentration of dimer and polymer formation at 10^{-7} M [27], the amount of regular Zn-insulin bound to liposomes was decreased by the competing process of the self-association of the regular Zn-insulin, whereas the trace amount of the iodinated insulins (10^{-10} M) was not so much affected by self-association. This is supported by the fact that when the regular Zn-insulin was replaced by the same concentration of non-radioactive iodinated Zn-insulin in the binding experiment, the phenomenon of the preferential binding of ^{125}I-labeled insulin to small unilamellar liposomes was abolished.

One immediate consequence of the tight binding of insulin to the external surface of small unilamellar liposomes is the interference of the externally bound insulin in estimating the true amount of insulin encapsulated in liposomes. Depending upon the composition of lipids and the method of preparing liposomes, the fraction of small unilamellar liposomes existing in a suspension of liposomes may vary. If the procedure of separating liposome-entrapped insulin and untrapped insulin is carried out at a temperature where the small unilamellar liposomes can bind to insulin tightly, the estimated amount of insulin encapsulated in liposomes may be higher than the true amount of entrapped insulin. When insulin is encapsulated in liposomes with a low T_c, such as egg phosphatidylcholine with or without charged lipids at room temperature, the amount of insulin associated with the liposomes is the true encapsulation value. Using 10 mg egg phosphatidylcholine containing 10% (mol) DPPA or 20 mg DMPC/cholesterol/DPPA (7:2:1, mol/mol), the encapsulation efficiency of insulin in 1 ml aqueous solution was on the order of 2–5%. However, when insulin is encapsulated in liposomes with a high T_c, such as DPPC or DSPC, the true encapsulation value is obtained only if the non-encapsulated insulin is separated completely from the liposomes at a temperature at which insulin does not bind to the liposomes.

It has been shown that insulin situated on the outer surface of liposomes has little effect on the blood-glucose level after oral administration [7]. The difference in the hypoglycemic activity of various liposome-entrapped insulin observed in some previous studies may in part be explained by the difference in the amount of insulin bound to the external surfaces of these liposomes. Furthermore, the finding from the present study suggests that results from the studies of the interaction of liposome-encapsulated insulin with cells in vitro should be interpreted with great caution.

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