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THE ROLE OF THE PHOSPHOLIPID PHASE TRANSITION IN THE REGULATION OF GLUCAGON BINDING TO LECITHIN

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Summary

Glucagon can interact rapidly with multilamellar vesicles of dimyristoyl glycerophosphocholine over a narrow temperature range around or above the phase transition temperature of the pure phospholipid. The temperature dependence of the rates arises, in large part, from glucagon-induced alterations in the phase transition properties of the phospholipid. Similar effects are observed with dilauryl glycerophosphocholine but the rate of reaction of glucagon with multilamellar dipalmitoyl glycerophosphocholine is too slow to measure.

The rate of reaction of glucagon with equimolar mixtures of two phospholipid molecules has also been studied. Mixtures of dilauryl glycerophosphocholine and distearoyl glycerophosphocholine are known to exhibit lateral phase separation in the gel state. The presence of distearoyl glycerophosphocholine has no effect on the rate of reaction with glucagon, despite the increased number of phase boundaries present. In the case of mixtures of dilauryl glycerophosphocholine and dimyristoyl glycerophosphocholine, glucagon appears to induce some lateral phase separation. This is demonstrated by the ability of glucagon to react rapidly with this lipid mixture, even at temperatures well below the phase transition temperature of the mixture and by differential scanning calorimetry.

The thermodynamics of the binding of glucagon to dimyristoyl glycerophosphocholine and dilauryl glycerophosphocholine were analyzed with Scatchard plots calculated from measurements of the fluorescence enhancement caused

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Abbreviations: DLPC, 1,2-dilauroyl-*sn*-3-glycerophosphocholine; DMPC, 1,2-dimyristoly-*sn*-3-glycerophosphocholine; DSPC, 1,2-distearoyl-*sn*-3-glycerophosphocholine; Pipes, 1,4-piperazine-diethanesulfonic acid.

by lipids. Equilibrium binding constants of glucagon to dimyristoyl glycerophosphocholine and dilauryl glycerophosphocholine are $1 \cdot 10^5$ and $5 \cdot 10^4$ M^{-1}, respectively. These values are relatively insensitive to temperature, indicating that the equilibrium being measured is between lipid-bound glucagon and free lipid which has had its phase transition properties altered. The number of moles of lipid bound per mole of glucagon decreases markedly above the phase transition temperature. In the water-soluble complex formed between glucagon and dimyristoyl glycerophosphocholine, the peptide binds directly to only 40% of the lipid molecules but, nevertheless, is able to modify the phase transition properties of all of the lipid in the particle.

Introduction

Glucagon interacts with 1,2-dimyristoyl-*sn*-3-glycerophosphocholine (DMPC) to form water-soluble lipoprotein particles [1] which are kinetically stable only below the phase transition temperature of the phospholipid [2]. In the resulting complex, the intensity of the fluorescence emission from glucagon is markedly enhanced and shifted to lower wavelengths [3]. In this work, we have utilized this effect to study the thermodynamics and kinetics of the interaction of glucagon with lecithin molecules containing saturated fatty acids of varying chain length. We demonstrate that the apparent equilibrium constant for the binding of glucagon to lecithin is relatively insensitive to temperature, but that the amount of lipid bound decreases around the temperature of the phospholipid phase transition. A model to explain these results as well as the marked effects of temperature on the rate of reaction between glucagon and lipid is presented. In addition, the interaction of glucagon with mixtures is phospholipid molecules is evaluated. Evidence is presented indicating that glucagon can induce partial lateral phase separation of mixtures of disaturated lecithins.

Experimental Procedures

Materials. Crystalline bovine-porcine glucagon was purchased from the Elanco Corp. and used without further purification. Synthetic phospholipids were purchased from Calbiochem. Co. and Sigma Chem. Co.

Methods. Unless otherwise indicated, all experiments were performed using the following buffer: 20 mM Pipes, 1 mM EDTA, 150 mM NaCl and 0.02 mg/ml NaN_3, pH 7.40. Glucagon was dissolved by briefly warming a suspension of the peptide in buffer above room temperature. The resulting solution was centrifuged at 27 000 × *g* for 15 min in a Sorvall centrifuge. The glucagon concentration in the supernatant was determined from its absorbance at 278 nm using the absorption coefficient of 2.38 $l \cdot g^{-1} \cdot cm^{-1}$ [4]. Weighed samples of phospholipid were directly dispersed into buffer by vortex mixing for 1 min above the phase transition temperature. In the case of phospholipid mixtures, these were dissolved in $CHCl_3$, the solvent evaporated under N_2, depositing the lipid as a film and final traces of solvent were removed by drying for at least 1 h under high vacuum with a liquid N_2 trap. The resulting

lipid film was dispersed by vortex mixing with buffer above the phase transition temperature of the higher melting lipid. Lipid concentrations were determined by using the method of Bartlett [5] after perchloric acid ashing of the samples.

Fluorescence. Fluorescence emission intensities were recorded in the ratio mode on a Perkin-Elmer MPF-44 fluorimeter using an excitation wavelength of 295 nm where only tryptophan absorbs and an emission wavelength of 340 nm to maximize the difference between the fluorescence emission of the free and lipid-bound peptide. The fluorescence emission maximum for free glucagon is 350 nm while for all of the lipids used in the study, with which glucagon interacts, the emission maximum for the glucagon-lipid complex is 338 nm. Slit widths were set at 4 nm and the response time at 0.3 s. The glucagon solution was about 0.1 mg/ml and had an absorbance below 0.1 at the excitation wavelength. The contents of the 1 cm cuvette were continually stirred. The lipid suspension, at a temperature close to that of the cuvette, was vortex mixed and a sample rapidly transferred with the SMI positive displacement pipette to the fluorimeter cuvette.

Analysis of lipid binding. 10- or 20-μl aliquots of lipid suspension (10 mg/ml), were added to 3 ml of a glucagon solution (0.1 mg/ml) maintained at constant temperature. After each addition of lipid, the fluorescence intensity was monitored until no further change occurred. Fluorescence intensities were corrected for blank measurements from suspension of lipid in buffer and for dilution effects. Each of these corrections amounted to less than 10% of the total value. No correction was made for dissipation of the excitation beam caused by turbidity. This effect is thought to be small in general, as no turbidity could be visualized after solubilization of the lipid by glucagon. However, at temperatures above the phase transition, a slow decrease in fluorescence intensity was observed over several hours. This apparent decrease in fluorescence intensity was correlated in time with the onset of increased turbidity and is due to glucagon-induced lipid aggregation occurring above the phase transition temperature [2]. As this phenomenon is relatively slow, under the conditions of these experiments, measurements could be made at times short enough to minimize the effect of turbidity on the observed fluorescence intensity. The limiting fluorescence intensity was obtained by a linear regression analysis of a plot of the reciprocal of the corrected fluorescence intensity vs. the reciprocal of the lipid concentration [6]. Since these plots were linear, it is not likely that the small amount of turbidity observed only at high lipid-to-glucagon ratios led to large inaccuracies.

It was assumed that the fraction of protein bound to lipid was equal to the fractional increase in fluorescence intensity. The data were analyzed by means of Scatchard plots (v/[P] vs. v, where v is the average mole ratio of bound protein to lipid molecules and [P] is the concentration of free protein in solution). The intercept of the linear regression line of these plots gave v which is reported as its reciprocal, i.e., the average number of lipid molecules bound per glucagon molecule, and the reciprocal of the slope gave the binding constant. This analysis assumes that the system is at equilibrium and that the binding sites are equal and independent. The resulting Scatchard plots gave good fits to a straight line with correlation coefficients generally greater than

0.98. There was a tendency for the correlation coefficient to vary with temperature, having a maximum value greater than 0.99 at the temperature at which the rate of reaction was maximal but was lower below this temperature where the rate was slow as well as above this temperature where the slow increase in turbidity was observed.

Rate of reaction of lipid and glucagon. To 1 ml of a solution of glucagon (0.1 mg/ml), equilibrated at the desired temperature, 100 μl of lipid suspension (10 mg/ml) were added and the fluorescence emission was monitored as a function of time while the suspension was continuously stirred. The kinetics of the reaction were complex, as expected for multilamellar vesicles having lipid with varying degrees of exposure to the external environment. The reaction was characterized by an initial small decrease in fluorescence intensity, perhaps due to effects of turbidity, followed by a rapid rise and finally a slower rise to the final state. The process could not be fitted to any simple rate expression. In order to compare qualitatively the rates of reaction, we have reported the time required to reach 50% of the final value. This occurs during the rapid phase of the reaction. The measured $t_{1/2}$ values show more deviation than would be expected from the precision of the fluorescence measurements. Factors such as the non-uniformity of size and the state of aggregation of the multilamellar lipid suspensions, errors in temperature measurement, particularly for the DMPC system, which was very sensitive to small temperature changes occurring, for example, if the added lipid was not at precisely the same temperature as the reaction cuvette and the finite time required to start the reactions, many of which had $t_{1/2}$ values of less than 6 s, may all contribute to this error.

Analysis of the composition of glucagon-solubilized lipid. When suspensions of equimolar mixtures of 1,2-dilauroyl-*sn*-3-glycerophosphocholine (DLPC) with either DMPC or 1,2-distearoyl-*sn*-3-glycerophosphocholine (DSPC) were added to a solution of glucagon at 0°C to give a final molar ratio of lipid-to-protein of about 40:1, not all of the lipid was solubilized. In order to determine whether the two lipids of the mixture was solubilized in equal proportions, the resulting suspension was immediately centrifuged in an Eppendorf microcentrifuge at 4°C. Both supernatant and precipitate fractions were analyzed for phosphate content and for fatty acid composition by gas-liquid chromatography (GLC). Methyl esters of the fatty acids were prepared according to the method of Morrison and Smith [7] using 14% BF_3 in CH_3OH and an internal standard of methyl palmitate.

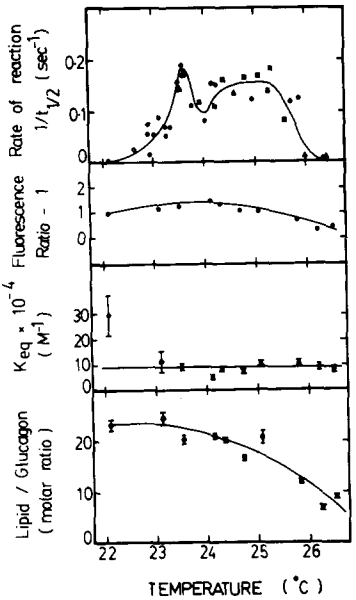
Differential scanning calorimetry. These experiments were performed with a Perkin-Elmer DSC-2 scanning calorimeter. In order to achieve the required sensitivity, glucagon-lipid samples were concentrated by lyophilization. To 75 ml of a solution of glucagon (0.1 mg/ml) in 0.1 M ammonium acetate buffer, pH 7.4, were added 5 ml of a suspension of DLPC (10 mg/ml), both solutions having been cooled in an ice bath. The resulting turbid solution was clarified by centrifugation in a Sorvall refrigerated centrifuge for 10 min at 37 000 × *g* at 0°C. The supernatant was lyophilized and contained DLPC and glucagon at a 15:1 molar ratio. For the sample containing glucagon, which was reacted with an equimolar mixture of DMPC and DLPC, the lipid suspension (10 mg/ml) was added in aliquots at about 10°C, until the solution

became turbid. Upon standing at 0°C, the solution completely clarified and was lyophilized. The final preparation contained lipid and glucagon at a 30:1 molar ratio. A pellet obtained by centrifugation of the same lipid mixture was used for the scan in the absence of glucagon. It thus had a ratio of DMPC:DLPC identical to the glucagon-containing sample. Samples for differential scanning calorimetry were prepared in a cold room at 4°C by redissolving the lyophilized sample in the ammonium acetate buffer at a concentration of about 50 mg/ml and transferring about 10—15 μl of the resulting solution to aluminum sample pans. Lyophilization, required to prepare a sample of sufficiently high concentration for the calorimeter used, could have caused an alteration in the distribution of lipids. However, since the entire lipid-glucagon sample was dissolved in buffer before being transferred to the calorimeter, the lipids would be able to redistribute before the calorimetry scans were performed. Because of the relatively small amounts of lipid transferred to the pan, rapid heating rates between 1.25 and 5°C/min were employed using the most sensitive detector range (0.1 mcal·s^{-1}). More rapid heating rates caused a slight delay in the onset of the transition but no gross change in the shape or temperature range over which the transition occurred. Cooling scans at the more rapid scan rates were displaced 3—5°C to lower temperatures but otherwise were mirror images of heating scans. There was no change in the traces upon repeated scanning. In the case of DLPC, because of the freezing of water, a good baseline could not be established on heating before the lipid transition occurred. However, in cooling scans, the lipid transition could be observed before the freezing of the buffer. No cryoprotectant was added to avoid possible alteration of the glucagon-lipid interaction.

Results

The rate of reaction of glucagon with phospholipids is highly dependent on temperature and phospholipid structure. The reaction of glucagon with DMPC occurs at a rapid rate over only a 2 or 3°C temperature range. Rapid rates of reaction were also observed for DLPC below 20°C but no reaction between glucagon and dipalmitoyl glycerophosphocholine or the distearoyl homolog was observed at any temperature. Under certain conditions, however, some reaction between glucagon and dipalmitoyl glycerophosphocholine has been observed by injecting the lipid into water from a solution of methanol [3] or by adding a glucagon suspension to a thin lipid film [8]. At temperatures where the rate of reaction is rapid, the binding of lipid to glucagon could be analyzed (Figs. 1 and 2). The results indicate that the equilibrium constant for lipid binding is relatively insensitive to changes in temperature, while the number of lipid molecules bound per molecule of glucagon drops markedly at higher temperatures, coinciding with a decrease in the rate of reaction and with a decreased fluorescence enhancement.

The interaction of glucagon with DLPC in equimolar mixed lipid systems was also analyzed. Mixtures of DLPC with DSPC have been shown to exhibit lateral phase separation in the gel state [9] while saturated phosphatidylcholines differing by only two carbon atoms in the acyl group do not exhibit this phenomenon [10]. The temperature profile for the rate of reaction of



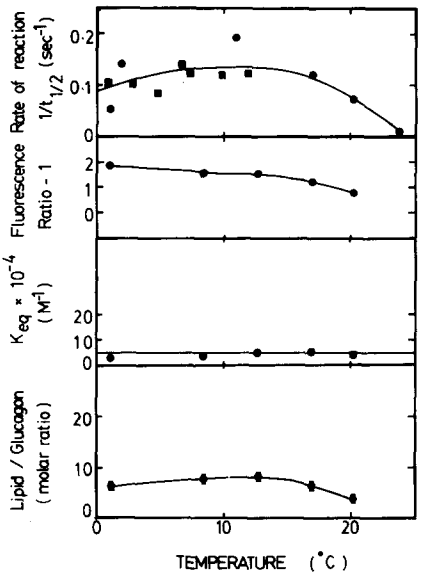
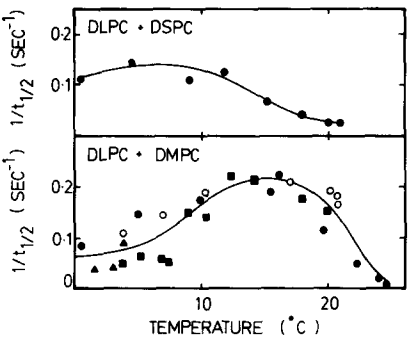


Fig. 1. Effect of temperature on the binding of glucagon to DMPC. All experiments performed with 20 mM Pipes, 1 mM EDTA, 150 mM NaCl and 0.02 mg/ml NaN_3, pH 7.40, and a glucagon concentration of about 35 μM. The rate of reaction is reported as the reciprocal of the time required to reach 50% of the total enhancement of fluorescence with 1.25 mM lipid. Different points correspond to experiments performed with different solutions of glucagon and different lipid suspensions. The fluorescence ratio is calculated from the maximum fluorescence at lipid saturation, obtained from a plot of (fluorescence intenity)$^{-1}$ vs. (lipid concentration)$^{-1}$, divided by the fluorescence intensity of glucagon in the absence of lipid. For zero to correspond to no fluorescence enhancement, one is subtracted from this ratio, K_{eq} and lipid:glucagon molar ratios are obtained from the slope and intercept, respectively, of Scatchard plots. Error bars represent twice the standard deviation of these values and in some cases fall within the points.

Fig. 2. Same as Fig. 1 for DLPC.

glucagon with DLPC/DSPC mixtures (Fig. 3) resembles that for DLPC alone. However, when DLPC is mixed with DMPC, the profile changes with the rate increasing as the temperature is raised from 0 to 14°C and the rate remains rapid above 20°C.

We investigated, for the mixed lipid systems, whether the composition of the lipid which was solubilized by interaction with glucagon was identical to the starting mixture. In the case of the DLPC/DMPC mixture, about 85% of the total lipid was solubilized under the conditions used (see Experimental Procedure). The fraction of DLPC in the starting material and in the supernatant and precipitate fractions were identical to within 3%. Therefore, no phase separation of these two lipids occurred as a result of preferential solubilization of one of the components. In the case of the DLPC/DSPC mixture, approx. 50% of the total lipid was solubilized. In this case, also the compositions of the starting material and the supernatant and precipitate fractions were identical to within 3%. Therefore, although it is concluded that glucagon interacts with only the DLPC portion of this lipid mixture (see Discussion), it is capable of solubilizing DSPC as well, without altering the composition of the solubilized portion of the lipid mixture.



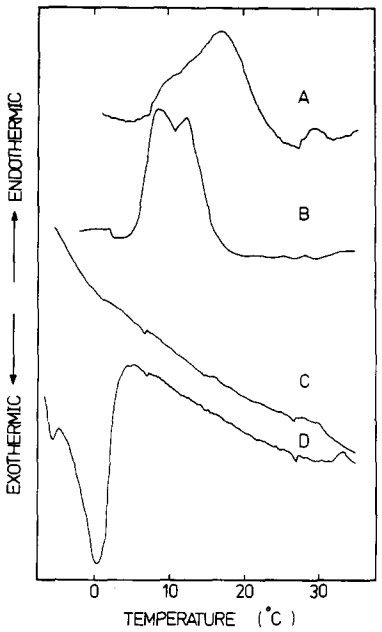


Fig. 3. Effect of temperature on the rate of reaction of glucagon with mixed lipid systems. Equimolar mixture of the two phospholipids with final total lipid concentration equal to 1.25 mM. Other conditions as for Fig. 1.

Fig. 4. Differential scanning calorimetry of glucagon-lipid complexes. Scan rate 5°C/min. A and B, heating scans. C and D, cooling scans. A, Glucagon in solution with an equimolar ratio of DLPC and DMPC. Glucagon-lipid molar ratio 1:30. B, The same equimolar mixture of DLPC and DMPC alone. C, Glucagon in solution with DLPC. Glucagon-DLPC molar ratio 1:15. D, DLPC alone.

In order to evaluate the effect of temperature on the interaction of glucagon with phospholipids, the phase transition of DLPC and of DLPC/DMPC mixtures was measured in the presence and absence of glucagon by differential scanning calorimetry. Similar experiments have already been reported for DMPC and glucagon [11]. The effect of glucagon on DMPC or on DLPC/DMPC mixtures is to broaden the phase transition and to shift it to somewhat higher temperatures (Fig. 4). The phase transition of DLPC can no longer be detected in the presence of glucagon, possibly because of its breadth and its proximity to the ice-water transition. In the case of the equimolar mixtures of DLPC and DMPC, an additional peak at about 25—30°C is observed in the presence of glucagon but not in its absence. This peak corresponds closely to the transition previously observed for pure DMPC in the presence of glucagon [11] and has an area equal to 6% of the large peak.

Discussion

The phase transition temperature of pure DMPC is 23.9°C [9]. In the presence of glucagon, the transition occurs over a range of approx. 24—30°C [11]. Similar effects on transition properties of phospholipids have also been observed with serum apolipoprotein A-I [12, 13], a protein which can, like

glucagon [1], solubilize phospholipid in the form of discoidal particles. As a consequence of this shift in the phase transition characteristics of the lipid, when glucagon or apolipoprotein A-I interacts with DMPC at or slightly above the phase transition temperature, it will convert a large fraction of the phospholipid from the liquid-crystalline to the gel state. For example, at 24.5°C, pure DMPC would be in the liquid-crystalline state while in the presence of glucagon this phospholipid would be largely below its phase transition temperature. As the conversion of phospholipid from the liquid-crystalline to the gel state is exothermic, with an enthalpy change of −5.4 kcal/mol phospholipid [9] and glucagon combines with about 50 mol DMPC to form the lipoprotein complex [1], an enthalpy change of the order of −100 kcal/mol glucagon would be expected for the isothermal mixing of DMPC and glucagon at 25°C. Such large exothermic heats of reaction have been observed for both the interaction of glucagon with DMPC at 25°C (Epand, R.M. and Sturtevant, J.M., unpublished results) as well as for apolipoprotein A-I at 28°C [14, 15]. This exothermic process would contribute to the stabilization of the transition state and could therefore explain the enhancement of the rate of reaction at temperatures slightly above 23.9°C with glucagon (Fig. 1) as well as with apolipoprotein A-I [16]. The slower reaction observed with glucagon at about 23°C may result from the interaction of glucagon with defects in the phospholipid bilayer in the gel state which increase as the phase transition is approached [17]. In the case of DLPC, there is also a rapid rate of reaction with glucagon above the phase transition temperature of the pure phospholipid. It may arise as a result of a shift in the phase transition properties of the lipid. That such a shift in the properties of DLPC occurs in the presence of glucagon is suggested by the fact that calorimetry scans of the lipid are altered by the presence of glucagon (Fig. 4) and the fluorescence properties of glucagon are altered by DLPC over the range 0—20°C (Fig. 2), suggesting that some gel-state lipid is present in this temperature range.

We have compared the effect of temperature on the rate of reaction of glucagon with DLPC mixed with a second phospholipid; either DSPC with which it shows limited miscibility in the solid state or DMPC with which it is expected to be miscible. In the case of DLPC/DSPC mixtures, the observed rate of reaction (Fig. 3) was very similar to that found for DLPC alone (Fig. 2) and was not enhanced by the additional phase boundaries present between domains of DLPC and DSPC, even though both DSPC as well as DLPC were solubilized by glucagon. In comparison, the major effect of glucagon on the phase transition of the equimolar mixture of DLPC and DMPC (Fig. 4A and B) is analogous to that for DMPC alone, i.e., the transition is broadened and shifted to higher temperatures in the presence of the hormone and the maximum rate of reaction occurs at temperatures slightly above the phase transition temperature of the lipid (Fig. 3). However, at low temperatures, below 5°C, where the DLPC/DMPC mixture remains in the gel state both before and after interaction with glucagon, the rate is still very rapid. This suggests that at these low temperatures, glucagon is preferentially interacting with DLPC and is inducing partial lateral phase separation. This is confirmed by the results of differential scanning calorimetry which show an additional transition, at about 28°C (Fig. 4A) corresponding to that of the DMPC-glucagon complex [11].

Therefore, glucagon can induce lateral phase separation in mixed phospholipid bilayers, differing by as little as two carbon atoms in the fatty acid portion. This lateral phase separation does not result from the preferential solubilization of one of the components.

Scatchard analysis of the lipid binding data indicates that the equilibrium constant for the binding of glucagon is relatively invariant with temperature. This would not be the case if the equilibrium were between the glucagon-lipid complex and unpertubed pure lipid multilamellar vesicles. This is because the unperturbed lipid would undergo a change in phase from the liquid-crystalline to the gel state upon interaction isothermally with glucagon only between 24 and 26° C. This glucagon-induced phase change would contribute to an increased relative stability of the glucagon-lipid complex only in this narrow temperature range. Since the association constant for glucagon with DMPC is found to be invariant with temperature, it suggests that glucagon can induce a change in the phase transition characteristics of phospholipids which remains, at least for a short time, even after the glucagon has dissociated. Thus, the equilibrium constant being measured appears to be between a glucagon-lipid complex and free lipid which remains in the same phase as it is in the presence of glucagon. The invariance of the association constant with temperature indicates that the enthalpy of the reaction is very small, not in disagreement with hydrophobic interactions being a major stabilizing force for the complex. The Gibbs energy for binding lipid to glucagon below the phase transition is —6.9 kcal/mol. If it is assumed that the Gibbs energy for the binding of glucagon to unmodified lipid is proportional to the number of lipid molecules bound, then as the number of bound lipid molecules decreases in the region 25—26° C, the van't Hoff enthalpy change is —140 kcal/mol. This large negative value is similar to that found with batch calorimetry for the binding of glucagon to lipid in this temperature region (unpublished results). The ability of glucagon to induce a change in the phase transition characteristics of the phospholipid which remains, at least for some time after the glucagon has dissociated, means that it can act as a catalyst in altering the properties of lipid bilayers. Thus, glucagon can affect the release of heat from more lipid than it is actually bound to at any time. If a similar phenomenon occurred with apolipoprotein A-I, it might explain why a maximal enthalpy change is never reached when it is mixed with increasing amounts of DMPC (Ref. 15 and Sturtevant, J.M., personal communication).

It was previously demonstrated that the lipid-to-peptide molar ratio in isolated, water-soluble DMPC-glucagon complexes is 57:1 [1]. From the results of the present study, we can conclude that only 40% of the lipid in the lipoprotein complex is bound to glucagon. Nevertheless, it is clear from the differential scanning calorimetry results that the remaining 60% of the lipid was not directly bound to glucagon has altered phase transition properties. The unbound lipid corresponds to a discoidal shaped single lipid bilayer with a 93 Å radius, while the remaining 27 Å on the outer rim of the 120 Å radius lipid bilayer [1] is directly bound to the glucagon. This corresponds to the bound lipid being three or four molecules in depth. In the case of DLPC, the amount of lipid bound per glucagon may be less because of smaller lipid-lipid interactions resulting in a thinner layer of bound lipid.

The binding affinity of glucagon to saturated phospholipids is of the order of 10^5. This is much less than the binding affinity of glucagon for specific membrane receptor sites which equals $2.5 \cdot 10^8$ [18]. Therefore, if lipids are directly involved in the binding of glucagon to hormone receptor sites, this interaction may contribute about 60% of the free energy of binding. Other lipid structures will have different affinity binding constants, for example, lysolecithin has been found to bind to glucagon with an affinity constant of $2 \cdot 10^6$ M^{-1} [19]. The number of lipid molecules bound per molecule of glucagon in that study is identical to that found in the present work for DMPC. This ratio is therefore not related to the size of the lipid micelle which is much smaller in the case of lysolecithin. In order to contribute to the binding of glucagon, the lipid region of the membrane would have to be near its phase transition temperature for the interaction to occur rapidly. In addition, if the temperature were at or slightly above the phase transition temperature, then glucagon could promote the conversion of lipid from the liquid-crystalline state to the gel state.

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