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INTERACTION OF GLUCAGON WITH SPHINGOMYELINS

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In the presence of either egg or bovine brain sphingomyelin, the spectral properties of glucagon undergo changes which are similar to those which occur in the presence of synthetic phosphatidylcholines. The fluorescence emission spectra are blue shifted about 10 nm in the presence of lipid and the peptide acquires an increased helical content, determined by circular dichroism. As with phosphatidylcholines, the changes in spectral properties do not occur above the phase transition temperature of the glucagon-lipid mixture. Freeze-fracture electron microscopy indicates that glucagon forms an ellipsoidal complex with bovine brain sphingomyelin, similar to the glucagon-dimyristoylphosphatidylcholine complex. However, the sphingomyelin complexes break down to vesicular structures both above and below the region of the phase transition. These results indicate that the dissociation of glucagon from the lipid at higher temperatures results from changes in the phase of the lipid rather than from a thermal denaturation of glucagon. The effect of glucagon on the phase transition behaviour of palmitoyl sphingosine phosphorylcholine was measured by differential scanning calorimetry. The major effect of glucagon on both this lipid and on dimyristoylphosphatidylcholine is to broaden the phase transition and to shift it to higher temperatures. Similar results are obtained for the effects of glucagon on an equimolar mixture of dimyristoylphosphatidylcholine and palmitoyl sphingosine phosphorylcholine. Glucagon is able to solubilize mixtures of bovine brain sphingomyelin with either dimyristoylphosphatidylcholine or egg lecithin. The lipid composition of the solubilized material is similar to that of the starting lipid film. These results together with those from the differential scanning calorimetry on the synthetic mixtures indicate that glucagon can bind to sphingomyelin-phosphatidylcholine mixtures and that it does not induce extensive lateral phase separation between the components. The maximal stability of the glucagon-lipid complex at the phase transition of the lipids indicates that the glucagon-lipid interaction is highly dependent on the structural organization of the lipid.

Introduction

It has been shown that the 29 amino acid, polypeptide hormone, glucagon can form stable water-soluble complexes with dimyristoylphosphatidylcholine (DMPC) [1]. The rate of formation of these ellipsoidal complexes and their stability is critically dependent on the phase transition properties of the phospholipid [2]. If the interaction of glucagon with phospholipids were to be physiologically relevant for man it would have to occur at 37°C. Sphingomyelins, a major component of biological membranes, have phase transitions in this temperature range [3,4,5,19]. The properties of sphingomyelins have been recently reviewed [6].

Abbreviations: PSM, DL-*erythro*-*N*-palmitoyl sphingosine phosphorylcholine; DMPC, dimyristoyl-L-α-phosphatidylcholine; DSC, differential scanning calorimetry; Pipes, 1,4-piperazinediethanesulfonic acid.

Because of their prevalence and relatively high phase transition temperature it was of interest to extend our studies on glucagon-lipid interactions to sphingomyelins.

Materials and Methods

Materials. Crystalline bovine-porcine glucagon was purchased from the Elanco Corp., dimyristoylphosphatidylcholine from Calbiochem and the naturally occurring phospholipids from Sigma Chem. Co. A sample of the synthetic sphingomyelin, DL-*erythro*-*N*-palmitoyl sphingosine phosphorylcholine (PSM), was generously supplied by Dr. D. Shapiro of the Weizmann Institute of Science. This lipid was twice recrystallized by the method of Estep et al. [7] to give a purified product which had phase transition characteristics indistinguishable from those already reported [5].

Glucagon concentrations. The concentration of glucagon was determined from the absorbance of lipoprotein solutions after correcting for light scattering, using an absorption coefficient of 2.12 $l \cdot g^{-1} \cdot cm^{-1}$ at 278 nm [8] or by the method of Lowry et al. [9] using glucagon as a standard in the presence of 0.5% sodium dodecyl sulfate to dissolve the lipid.

Phospholipid concentrations. Lipid concentrations were determined from total phosphate analysis after perchloric acid ashing of the samples [10].

Buffers. The buffers used in this work were 0.1 M ammonium acetate buffer, pH 7.4, and 20 mM Pipes, 1 mM EDTA, 150 mM NaCl and 0.02 mg/ml NaN_3, pH 7.40.

Preparation of phospholipid complexes. A 1 mg/ml suspension of glucagon was added to a thin lipid film which had been deposited by evaporation of the solvent from a chloroform-methanol (2:1, v/v) solution. The suspension was votexed above the phase transition temperature of the phospholipid and allowed to cool slowly to room temperature. Additional material was solubilized by repeated heating and cooling cycles. The solubilized material was separated as a supernate after centrifugation at 20°C for 30 min at 10000 rpm in an SS-34 rotor of a Sorval centrifuge.

Determination of the nature and amount of phospholipid solubilized by glucagon. The amounts of sphingomyelin and phosphatidylcholine solubilized by glucagon by the procedure described in the above paragraph were determined both before and after separation of the phospholipids by thin-layer chromatography on Kieselgel H run in chloroform/methanol/10% ammonium hydroxide (60:30:8, v/v). The spot corresponding to each lipid, which was visualized with iodine vapor, was scraped off the chromatogram and analyzed for phosphate content. Recovery of lipid from the plates was always greater than 90%. Areas devoid of lipid at similar positions in the chromatogram were used as blanks. In the absence of glucagon only traces of lipid remained in the supernate.

Fluorescence spectroscopy. Fluorescence emission intensities were recorded in the ratio mode on a Perkin-Elmer MPF-44 fluorimeter using an excitation wavelenght of 295 nm where only tryptophan absorbs. Slit widths were set at 2 nm and a 290 nm cut off filter was used in the emission path to reduce scattered light intensity.

For fluorescence titrations with increasing increments of PSM, the lipid was added in a small volume as an aqueous suspension in Pipes buffer. The rate of the reaction at 42°C was quite slow with half of the maximal change in fluorescence occurring in approx. 7 min. To accelerate the rate of attainment of equilibrium, the samples were warmed and cooled several times through the region of the phase transition, followed by incubation at 42°C for 10 min after each addition of lipid. All samples used for fluorescence and circular dichroism (see below) are unfractionated and the lipid/peptide ratios are determined by the amounts of these materials added to the solution.

Circular dichroism. These spectra were recorded on a Cary Model 61 spectropolarimeter calibrated according to the values given by Cassim and Yang [11]. Spectra were recorded for sphingomyelin vesicles, to obtained the contribution from the amide chromophore contained in this phospholipid. The glucagon spectra from the sphingomyelin complexes were corrected for the lipid contribution. This correction was small above 210 nm. A narrow pathlength of 0.1 mm was used to minimize the Duysen's effect [12].

Differential scanning calorimetry (DSC). A scanning microcalorimeter designed by Privalov [13]

was employed, using a scan rate of 0.5 $K \cdot min^{-1}$. Repeated scans of the same sample were almost identical. From these scans a straight baseline could be drawn which connected the DSC curve at temperatures below the phase transition region to the DSC curve at temperatures above the phase transition. The excess heat capacity in the region of the phase transition, C_{ex}, was calculated from the derivation of the observed DSC curve from the constructed baseline. The observed calorimetric enthalpy change for the transition is $\Delta H_{cal} = \int C_{ex} dT$. For each van't Hoff component of the transition

$$C_{ex} = \alpha(1-\alpha)\frac{\Delta H_{cal}\,\Delta H_{VH}}{RT^2}$$

where α is the fractional extent to which the transition has occurred, ΔH_{VH} is the van't Hoff enthalpy and T is the absolute temperature. The observed variation of C_{ex} with T could be fitted, with the aid of a computer program using an iterative procedure, to the best values of ΔH_{cal}, ΔH_{VH} and T_m, the temperature at which $\alpha = 0.5$ for each component of the transition. The cooperative unit is calculated from the ratio of $\Delta H_{VH}/\Delta H_{cal}$.

Freeze-fracture electron microscopy. 0.1 to 0.4 μl aliquots of the solution containing the sphingomyelin-glucagon complex were sandwiched between 75 μm thick copper foils and incubated for 3 min at selected temperatures prior to being plunged into liquid propane. The samples were accelerated into liquid propane by a guillotine device similar to that of Costello and Corless [14]. Our device employs an environmental chamber where the sample can be incubated at 100% humudity and a wide range of temperatures prior to freezing. After freezing the samples were freeze-fractured in a Polaron E-7500 freeze-fracture module at -120°C under a vacuum of $5 \cdot 10^{-7}$ Torr or better. Replicas were cast by resistance evaporation, floated off in 3 M HNO_3 transferred to graded concentrations of up to 100% Clorox, washed in distilled H_2O and mounted on bare 460 mesh Hex grids (Polaron Instruments, Inc. Doylestown, PA). Representative micrographs were taken with a Siemens 101 electron microscope.

Results

The effects of egg and bovine brain sphingomyelins on the spectral properties of glucagon are similar to what has already been reported for the effects of synthetic phosphatidylcholines [1,2,18]. The fluorescence emission of glucagon is shifted from 351 nm to 343 nm in the presence of a 50-fold molar excess of bovine brain sphingomyelin, to 340 nm in the presence of a 40-fold molar excess of egg sphingomyelin and to 345 nm in the presence of 60-fold molar excess of PSM at temperatures below 35°C for the natural sphingomyelins and below 45°C for PSM. Above 45°C there is no shift in the emission maximum caused by the presence of any of the sphingomyelins. The rate of dissociation of glucagon from PSM at 54°C was measured and found to be relatively slow, with a half-time of 2 min. After several half-times the fluorescence emission spectra of the sample at 54°C containing PSM was indistinguishable from that of glucagon alone. There is also an increase in the intensity of fluorescence emission in the region of the phase transition (Fig. 1). This increase is less than that found with DMPC [15]. The enhanced fluorescence is not observed either above 45°C or below 15°C (Fig. 1). This is reminiscent of the behaviour of glucagon in the presence of cholesterol and phosphatidylcholine [16] although

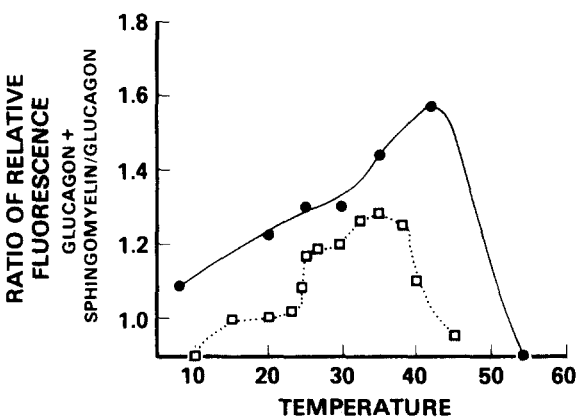


Fig. 1. Effect of temperature on the fluorescence emission intensity of glucagon-sphingomyelin complexes at the wavelength of maximal emission. Excitation wavelength 295 nm. Glucagon (30 μM) in the presence of either 1.8 mM PSM in the Pipes buffer, pH 7.4 (●———●) or 1.5 mM bovine brain sphingomyelin in ammonium acetate, pH 7.4 (□·····□).

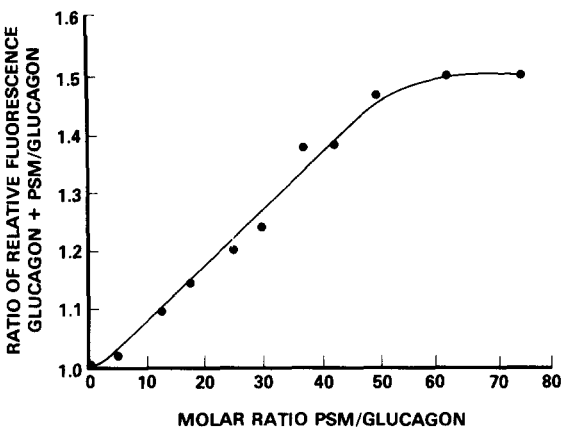


Fig. 2. Effect of PSM concentration on the fluoroescence emission intensity of glucagon at the wavelength of maximal emission. Excitation wavelength 295 nm. Glucagon (30 μM) in the Pipes buffer, pH 7.4. Temperature 42°C.

in the case of sphingomyelin, the lipid-induced blue shift of the emission is observed even at lower temperatures. The effect of increasing concentrations of PSM on the fluorescence properties of glucagon at 42°C was measured (Fig. 2). Although the points were too scattered to calculate an accurate binding constant, perhaps because of the slow rate of reaction of glucagon with this lipid, the fluorescence titration does indicate a high binding affinity with approx. 50 lipid molecules bound per peptide. This is similar to what has been found for the binding of glucagon to DMPC [2].

Circular dichroism studies also indicate the similarity of the interaction of glucagon with sphingomyelin and with phosphatidylcholine. At 27°C and an egg yolk sphingomyelin to glucagon molar ratio of 35 we observed the mean residue ellipticity of glucagon at 222 nm to be -16500 deg $\cdot$ cm^2 $\cdot$ (dmol)$^{-1}$. Thus monomeric glucagon, which had little if any helical content in the absence of lipid [17], acquires about 50% helical content in the presence of either phosphatidylchcoline [8] or sphingomyelin.

Freeze-fracture electron microscopy indicates some similarities between the interaction of glucagon with sphingomyelin and with DMPC [18]. Above the transition temperature, bovine brain sphingomyelin alone tends to form smaller unilamellar vesicles in addition to large multilamellar vesicles [19]. This is also observed in the presence of glucagon Fig. 3d). At the transition region, many complexes were formed which are apparently oblate ellipsoidal in shape. The shape of individual complexes is similar to, but not as distinct as, that of the glucagon-DMPC complexes [18]. The glucagon-sphingomyelin complexes are usually connected together side-by-side to form multilayered structures (Fig. 3c). Unlike multilamellar vesicles, the multilayers of glucagon-sphingomyelin complexes have open ends, indicating the edges of the complexes are hydrophilic. This is also observed in the case of DMPC with glucagon [18]. At slightly lower temperature, some connected units dissociate into individual units, but unlike the case of DMPC-glucagon, the dissociation is incomplete (Fig. 3b). At lower temperatures, most oblate ellipsoids break down, and small unilamellar vesicles of about 50 nm in diameter are formed. Only rarely are the remainder of large aggregates observed, as shown in Fig. 3a. The breakdown of the ellipsoidal structure can be correlated with the decrease in fluorescence enhancement of glucagon observed at low temperatures in the presence of sphingomyelin (Fig. 1), suggesting decreased interaction of the peptide with lipid. The finding is in agreement with the concept that glucagon interacts most strongly with phospholipid in the region of its phase transition [16].

The effect of glucagon on the phase transition of sphingomyelin is best illustrated by the differential scanning calorimetry (DSC) study of PSM. Glucagon caused the phase transition of the pure lipid, which occurs at 41.5°C, to be broadened and shifted to somewhat higher temperature (Fig. 4). The DSC of the pure PSM is much sharper, having a maximal excess heat capacity of 7.5 kcal $\cdot$ K^{-1} $\cdot$ (mol. lipid)$^{-1}$ measured by us for this same preparation [27]. The DSC curve of the glucagon complex is made up of at least two components. The minor one corresponding to 8% of the total enthalpy, is centered at 38.7°C and has a van't Hoff enthalpy of 386 kcal/mol, corresponding to a cooperative unit of 119. The major component, corresponding to 92% of the total enthalpy, is centered at 43.8°C and has a van't Hoff enthalpy of 147 kcal/mol corresponding to a cooperative unit of 45. The total enthalpy of the transition is

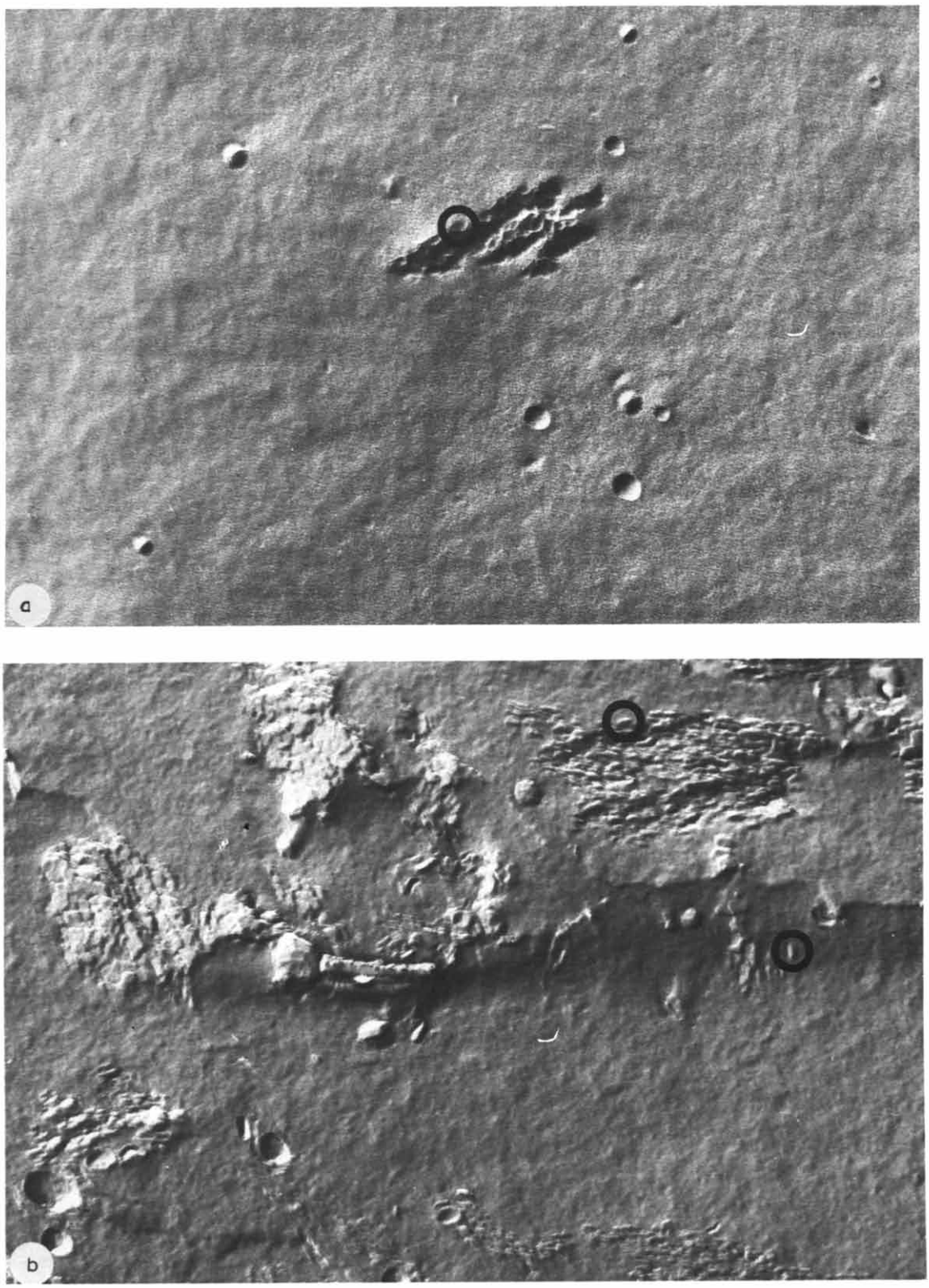
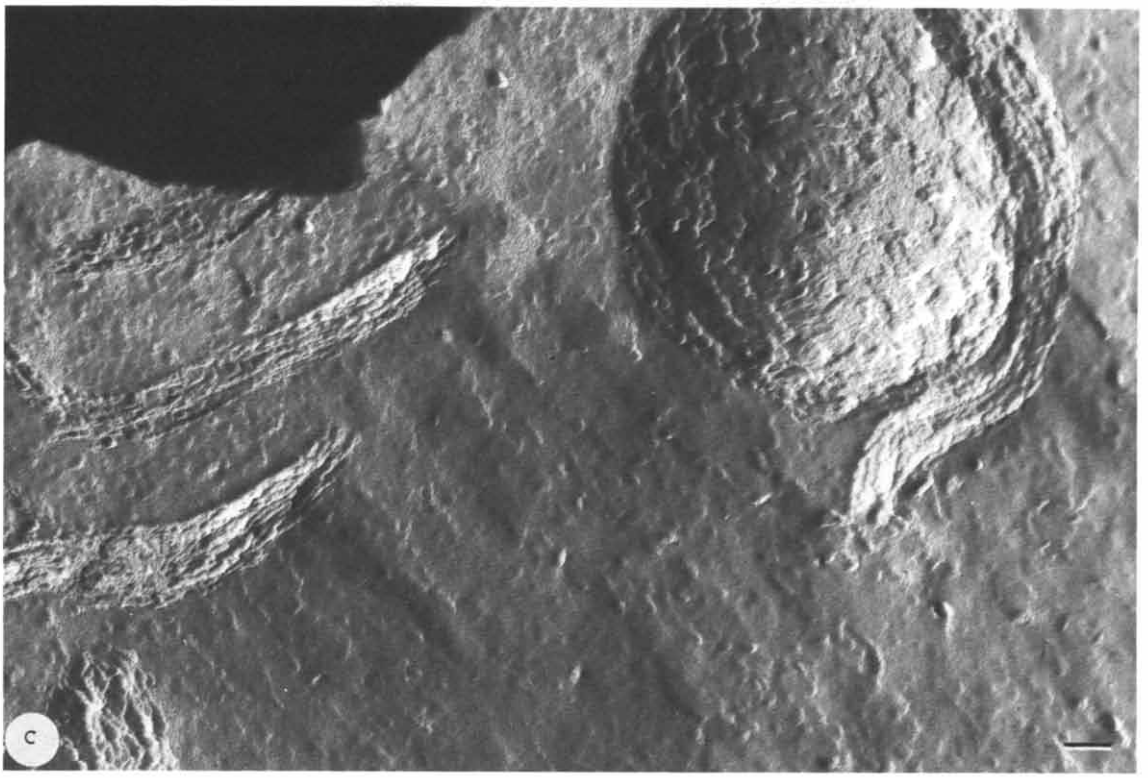
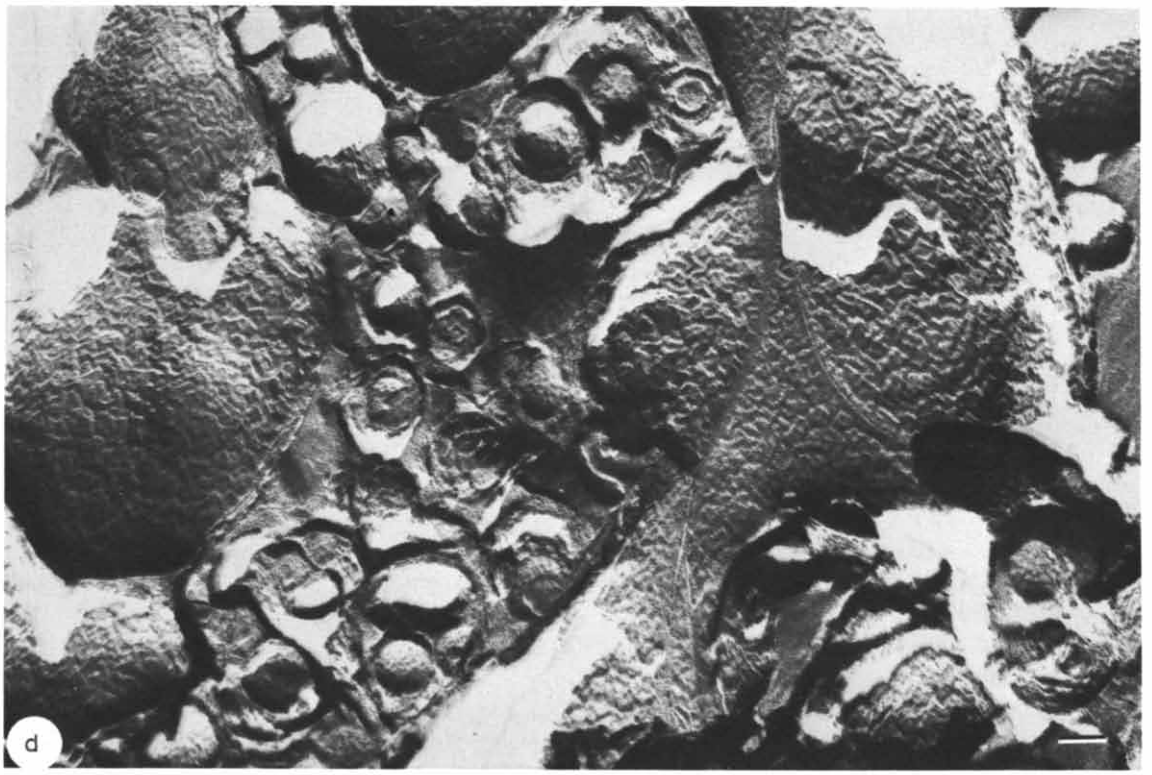


Fig. 3. Freeze-fracture micrographs of glucagon-sphingomyelin complex quenched from (a) 20°C, (b) 30°C, (c) 40°C and (d) 55°C.





Bar = 100 nm. Molar ratio of bovine brain sphingomyelin to glucagon was 30 : 1 in Pipes buffer. Some individual complexes are circled.

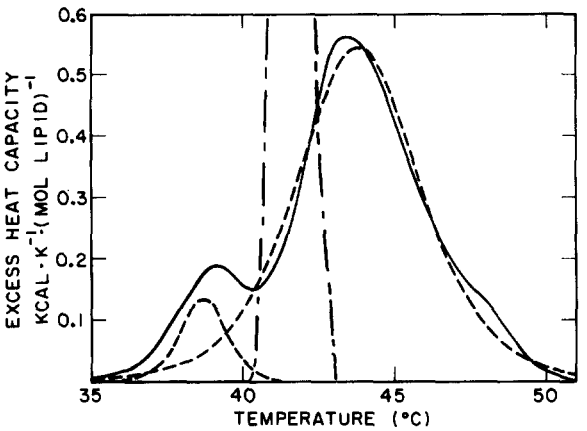


Fig. 4. DSC of the transition region of the glucagon-palmitoyl sphingosine phosphorylcholine complex at a 1 to 6 molar ratio and its resolution into components (dashed curve). Lipid concentration 2 mM. Scan rate 0.5 K·min^{-1}. For comparison a portion of the DSC curve for the pure lipid is shown (-—·—-). The full DSC scan for this preparation is given by us in Ref. 27.

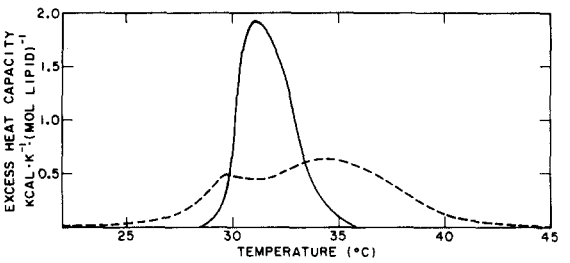


Fig. 5. DSC of the transition region of equimolar mixture of palmitoyl sphingosine phosphorylcholine and dimyristoyl phosphatidylcholine in the presence (------) and absence (———) of glucagon. Total lipid concentration 4.5 mM, glucagon concentration 0.15 mM. Scan rate 0.5 K·min^{-1}.

reduced from 7.0 kcal/mol for the pure phospholipid to 3.2 kcal/mol in the presence of glucagon. These results are similar to what has been found with DMPC [20]. In the case of DMPC it was found that the phase transition properties of the lipid once solubilized by glucagon was not further altered by lowering the lipid to glucagon ratio. In the case of PSM a low lipid to glucagon ratio was used to ensure maximal effect of the hormone on the phase transition properties of the lipid. A similar lowering of transition enthalpy, further broadening of the transition and a shift of part of the transition to higher temperatures was observed for the addition of glucagon to bovine brain sphingomyelin (data not shown).

The phase behaviour of mixtures of sphingomyelin with phosphatidylcholine have been investigated [21,22]. We wished to determine whether glucagon would induce extensive lateral phase separation of these phospholipids by preferentially interacting with one of them. We measured the phase transition of an equimolar mixture of DMPC and PSM using DSC (Fig. 5). Glucagon again had the effect of broadening the phase transition and shifting it to higher temperatures. The DSC curve for the phospholipid mixture in the presence of glucagon can be resolved into two components. A minor component at 29.6°C corresponds to 15% of the total enthalpy and has a van't Hoff enthalpy of 235 kcal/mol and a cooperative unit of 44. The major component occurs at 34.5°C, about 3.5 K higher than the melting temperature of the lipid mixture. It corresponds to 85% of the total enthalpy and has a van't Hoff enthalpy of 96 kcal/mol and a cooperative unit of 18. These two peaks could be interpreted as arising from regions enriched in one or the other of the two lipids of the mixture. We believe this is unlikely, however, since a similar splitting into two peaks is observed for complexes formed between glucagon and a single lipid. Although glucagon may promote some lateral phase separation in this lipid mixture, this separation is not extensive since only a small fraction of the phase transition occurs at 24°C or 41.5°C, the melting temperatures of the pure phospholipid components. The total enthalpy of the transition was lowered from 6.0 to approx. 5.3 kcal/mol by glucagon. Because of the breadth of the transition, extending over 20 K, in the presence of glucagon this transition enthalpy may be less accurate.

The ability of glucagon to solubilize mixtures of bovine brain sphingomyelin with either DMPC or egg lecithin was determined. In all cases, the lipid composition of the solubilized product was within 20% of the initial lipid film. Glucagon was able to solubilize about 80% of either bovine brain sphingomyelin, DMPC, mixtures of these two phospholipids ranging from 80% to 25% sphingomyelin, or mixtures of egg lecithin with 60% or more bovine brain sphingomyelin. Only 4% of a

mixture of egg lecithin with 45% bovine brain sphingomyelin was solubilized and virtually no solubilization occurred with lower amounts of sphingomyelin added to egg lecithin. The phase transition properties of a mixture of 70% bovine brain sphingomyelin and 30% egg lecithin was not drastically altered by the presence of glucagon at a lipid to peptide ratio of 40:1 as measured by DSC. In addition, we have shown that the fluorescene emission maximum of glucagon is shifted from 350 nm to about 340 nm in the presence of any of the solubilized bovine brain sphingomyelin-DMPC mixtures, It therefore appears that if glucagon does induce phase separation, it would be solubilized by the sphingomyelin portion of the mixture. However, the solubility seems to be determined by the phase transition of the mixture rather than by that of pure components, indicating no phase separation occurs. In other words, glucagon binds to sphingomyelin-phosphatidylcholine mixture but it does not induce extensive lateral phase separation between the components.

Discussion

We have studied the interaction between glucagon and three sphingomyelins: bovine brain, egg and the synthetic PSM. The results indicate that glucagon interacts most strongly with sphingomyelins at temperatures at and slightly below the transition temperature. Above the transition temperature the glucagon-lipid complexes dissociate. That the association occurs at the transition temperature of the lipid, and that this applies also to saturated phosphatidylcholine [2,8,15,18] as well as to sphingomyelin, confirms the fact that this is a property of the phospholipid-peptide interaction and is not caused by a thermally-induced conformation change of the peptide.

In the interaction with glucagon, sphingomyelin differs from pure DMPC in that below the transition temperature the phospholipid/glucagon complex is unstable. The complex again dissociates into soluble peptide and small liposomes. The complexes are reformed upon heating the mixture to the vicinity of the transition temperature. This is supported by both fluorescence and electron microscopic evidence. The dissociation of lipid/glucagon complex below as well as above the transition temperature of the phospholipid was also observed in glucagon/DMPC/cholesterol mixtures [16]. However, the mechanisms of dissociation from sphingomyelin at high temperature and at low temperature are morphologically distinct. As the temperature reaches the transition temperature of the lipid, the ellipsoidal complexes are seeen to attach side-by-side to form large sheets or multilayered spheres (Figs. 3b and 3c). At a slightly higher temperature, glucagon is released abruptly, and the lipid is left in the form of large, multilamellar vesicles (Fig. 3d). In contrast, the dissociation of lipid and glucagon is a slow process at low temperature. At temperatures below the transition temperature of the lipid, the ellipsoidal complexes exist individually; the release of glucagon in solution leaves the remaining lipid in the form of small, mostly unilamellar, vesicles although occasional complexes are still observed (Fig. 3a). The release takes place over a wide range of temperature, and seemingly occurs individually, ellipsoid-by-ellipsoid, or in small groups of ellipsoids.

Based on the existing evidence, we may presume that glucagon forms a complex with gel state, saturated phospholipids. The association rate is the highest at the transition temperature of the lipid because of the high fluctuation rate and the maximal amount of interphase domain boundaries at the phase transition [2,16,24–26]. At this high fluctuation condition, glucagon probably interacts with the interphase boundaries. The addition of a small percentage of cholesterol serves to create more structural defects (domain boundary) in the bilayer, thereby increasing the rate of reaction [16,24]. An analogous situation occurs with interaction of serum apolipoprotein-A1 and lipids [24]. At lower temperatures, the ellipsoidal complexes no longer have as many structural defects and the glucagon tends to readily dissociate from complexes containing cholesterol or mixed lipids (such as sphingomyelin). However, complexes containing certain saturated phospholipids (such as DMPC, DPPC) remain stable, perhaps because of more perfect packing of the gel phase [26] leading to a slower rate of dissociation. Thus, the interaction between lipid and glucagon is highly structure-dependent.

The ability of glucagon to interact at physiological temperatures with naturally occurring lipids such as sphingomyelin makes more likely the possibility that these interactions have a role in glucagon storage, secretion, transport or binding to cell membranes. The ability of glucagon to alter phospholipid phase transition properties means that binding of glucagon to a putative lipoprotein receptor could alter the fluidity of the lipid component causing it to become more solid-like. It has been suggested that changes in the properties of sphingolipids could provide a mechanism for the transbilayer transmission of an extracellular signal [23]. Another possible role of the glucagon-induced solidification of phospholipids would be as a source of energy which could be coupled to some energetically unfavourable process in the membrane. This energy would arise from the partial solidification of 50 lipid molecules per glucagon molecule and therefore could reach large values when expressed per mole of glucagon [20].

Acknowledgements

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