Calorimetric and Fluorescence Characterization of Interactions between Enkephalins and Liposomal and Synaptic Plasma Membranes Containing Gangliosides†

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Received January 14, 1985

ABSTRACT: The interactions of the opioid peptide [D-Ala^2]methionine-enkephalinamide with dipalmitoylphosphatidylcholine (DPPC) large unilamellar vesicles containing gangliosides G_{M1}, G_{d1a}, and G_{t1b} and synaptic plasma membranes selectively enriched with dimyristoylphosphatidylcholine (DMPC) and ganglioside G_{d1a} have been investigated by using high-sensitivity differential scanning calorimetry. In the absence of gangliosides, the addition of enkephalinamide in concentrations of up to 10^{-3} M does not induce any appreciable change in the heat capacity function of DPPC. In the presence of gangliosides, however, changes in the heat capacity function were observed with as little as micromolar concentrations of the enkephalinamide; the same is true for DMPC–G_{d1a}-enriched synaptic membranes. The magnitude and the nature of the enkephalinamide effect depend on the type of ganglioside studied. For DPPC vesicles containing ganglioside G_{M1} only a slight broadening in the heat capacity function and a small upward shift in the transition temperature were observed. For DPPC vesicles containing ganglioside G_{d1a} the effect was more dramatic; enkephalinamide concentrations as low as 10^{-5} M caused the appearance of two well-defined peaks in the heat capacity function in contrast to the one peak observed in the absence of enkephalinamide. In the case of DPPC vesicles containing ganglioside G_{t1b} the enkephalinamide effect was seen at concentrations of 10^{-4} M or higher. Synaptic plasma membranes were isolated from bovine brain, selectively enriched with exogenous lipid, and their thermotropic behavior was characterized by steady-state fluorescence spectroscopy and differential scanning calorimetry. This lipid enrichment results in the appearance of a membrane phase transition otherwise absent in the intact membrane preparation. Synaptic plasma membranes enriched with DMPC and 10 mol % ganglioside G_{d1a} also exhibited a similar response to the addition of enkephalinamide, suggesting that enkephalins might be able to induce phase separation processes in both synthetic and natural membranes. These results provide evidence for the existence of high-affinity interactions between specific gangliosides and enkephalins and suggest that gangliosides may play an indirect role in the biological functioning of opiate receptors.

The enkephalins are endogenous opiate-like peptides occurring in the central nervous system and in some peripheral tissues. First described by Hughes (Hughes et al., 1975), these pentapeptide neurotransmitters compete with morphine and morphine analogues for opiate receptor binding sites in the brain (Kosterlitz & Hughes, 1975; Lewis & Stern, 1983). For some years there have been contradictory results regarding the molecular nature of the opiate receptor, specifically whether it is a protein–lipid complex [for a review, see Lee & Smith (1980)]. It has been shown (Loh et al., 1978; Loh & Law, 1980; Jarrell et al., 1980) that enkephalins are able to bind to negatively charged lipids such as phosphatidic acid, phosphatidylserine, and cerebroside sulfate, as well as more neutral phospholipids like egg yolk phosphatidylcholine and lysophosphatidylcholine (Deber & Benham, 1984). However, the binding of enkephalins to these lipids is characterized by low-affinity constants (K_D = 10^{-1}–10^{-3} M) (Loh & Law, 1978; Jarrell et al., 1980; Deber & Benham, 1984) when compared to the affinity for the opiate receptor itself (K_D = 10^{-9} M) (McLawhon et al., 1983). Despite this fact, some speculation has arisen as to whether or not the binding of enkephalins to membrane lipid may play a physiological role in mediating the transfer of the peptide to the receptor [for a review, see Loh & Law (1980)].

The possibility of gangliosides being an integral part of such a protein–lipid receptor has also been examined (McLawhon et al., 1981), and recently, it has been shown that enkephalins affect the synthesis of gangliosides in several cell lines (Dawson et al., 1979, 1980; McLawhon et al., 1983). Thus, there appears to be a correlation between enkephalin concentration and ganglioside behavior even though the molecular origins of such a correlation are still unknown. Gangliosides are particularly abundant in the brain where they comprise approximately 10% of the total lipid of the cortex. Moreover, gangliosides are asymmetrically localized on the outer surface of the neural membranes particularly at the presynaptic nerve endings. Thus, a potential interaction between enkephalins and gangliosides might result in substantial changes in the behavior and properties of the cell membranes containing opiate receptors. To investigate the nature of the interaction between membranes containing gangliosides and enkephalins, we have performed high-sensitivity differential scanning calorimetric studies of the interactions between [D-Ala^2]methionine-enkephalinamide and phospholipid bilayer membranes containing ganglioside G_{M1}, G_{d1a}, and G_{t1b} as well as selectively enriched synaptic plasma membranes. [D-Ala^2]Methionine-enkephalinamide was chosen for these initial studies because it is a biologically active opioid peptide commonly used in many in vivo experiments (Dawson et al., 1980; Behnam & Deber, 1984). The data presented here indicate that enkephalins have a profound effect on phospholipid bilayers containing gang-

† This investigation was supported by Grants NS-20636 and GM-30819 from the National Institutes of Health.

0006-2960/85/0424-4076$01.50/0

lioside, particularly the disialoganglioside G_{d1a}, and that micromolar concentrations of enkephalin are able to induce phase separation processes within the lipid bilayer.

Materials and Methods

Materials. Dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) were obtained from Avanti Biochemicals (Birmingham, AL) and used without further purification. The active opiate peptide [D-Ala^2]methionine-enkephalinamide (Tyr-D-Ala-Gly-Phe-Met-NH_2) was obtained from Sigma Chemicals (St. Louis, MO) and stored at −20 °C as a 5 mg/mL aqueous stock. Gangliosides were isolated and purified from beef brain as previously described (Myers et al., 1984). The purity of all lipid and ganglioside preparations was checked by thin-layer chromatography on silica gel 60 (E. Merck).

Lipid concentrations were estimated by a modified Bartlett phosphate assay as described by Marinetti (1962). Ganglioside concentrations were calculated from total sialic acid determinations by using a resorcinol method similar to that described by Spiro (1966). In this assay, samples and standards in 0.5-mL aqueous solutions were incubated with 0.5 mL of resorcinol solution (2 mL of 2% resorcinol, 16 mL of concentrated HCl, 0.05 mL of 0.1 M $CuSO_4 \cdot 5H_2O$ and 1.95 mL of H_2O) for 15 min in a boiling water bath. After the solutions were cooled, 2 mL of 1-butanol was added, the samples were mixed thoroughly, and absorbance at 585 nm was determined. Standards of 3–30 μg of sialic acid from a 0.1 M stock were used.

Vesicle Preparation. All the vesicle preparations used for these experiments were fused unilamellar vesicles (FUV's) prepared essentially as described by Schullery et al. (1980). DPPC was first dried from a chloroform solution and lyophilized overnight. The dried lipid was suspended in 50 mM KCl containing 0.02% sodium azide to give a concentration of 50 mg/mL. The lipid suspensions were sonicated by using a bath sonicator (Model G112 SP1G, Laboratory Supplies, Hicksville, NY) and then centrifuged at 15000*g* for 60 min above the lipid phase transition temperature to pellet any residual multilamellar vesicles. The sonicated vesicles were then incubated at 4 °C for 1 week before use. This low-temperature incubation triggers a spontaneous fusion process and produces a homogeneous population of single lamellar vesicles of about 900-Å diameter (Wong et al., 1982). The size and homogeneity of the vesicle preparations were checked by negative-staining electron microscopy. The incorporation of ganglioside GM_1, G_{d1a}, or G_{t1b} into the lipid bilayer was achieved by adding the desired amounts of ganglioside, from an aqueous stock containing 20 mg of ganglioside/mL, to diluted aliquots of the vesicle preparations (5 μmol/mL total lipid) followed by incubation at 45 °C for 90 min. This procedure results in the formation of a stable population of lipid vesicles containing ganglioside molecules asymmetrically located in the outer membrane surface (Felgner et al., 1981; Myers et al., 1984).

Isolation of Synaptic Plasma Membranes and Preparation of DMPC Selectively Enriched Synaptic Membranes. Synaptic plasma membranes (SPM's) were isolated essentially as described by Jones & Matus (1974). Brains of freshly slaughtered bovine were removed and placed on ice for transport to the laboratory. The brains were washed with cold distilled water and trimmed of meninges, blood clots and vessels, and white matter. (Unless indicated all procedures were performed at 4 °C.) The cortex was then homogenized in 10% sucrose and the homogenate centrifuged for 20 min at 800*g*. The supernatant was removed and centrifuged 20 min at 9000*g*; the resulting pellet was then washed once in 10% sucrose. Hypotonic lysis to free the synaptosomes from mitochondria, synaptic vesicles, and other cellular organelle contaminants was accomplished by suspending the pellet in 5 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.2, for 30 min at 0 °C followed by six strokes of a hand-operated Potter-Elvehjem tissue grinder. Five milliliters of this lysate was then layered on a discontinuous sucrose gradient consisting of 2 mL of 10% sucrose, 15 mL of 28.5% sucrose, and 15 mL of 35% sucrose. The gradients were centrifuged in a Beckman L8-80 ultracentrifuge using an SW-27 rotor for 2 h at 60000*g*. SPM's appear as a white band at the interface of the 28.5% and 35% layers. After collection, the membranes were then pelleted and washed once in 10 mM Tris, pH 7.4, and then stored in the same buffer at −70 °C at a concentration of 1.5–2.0 mg of protein/mL.

Purity of the preparation was confirmed by electron microscopy which indicated an average size of 0.7 ± 0.2 μm; this is within the limits described by Jones & Matus (1975). Assays for cytochrome oxidase (to rule out mitochondrial contamination) and sialidase activity were also used to assess purity. The procedure of Smith (1955) was followed for determination of cytochrome oxidase activity. The activities in the various fractions of the preparations were similar to those reported by Jones & Matus (1974). Sialidase activity of the synaptosomal preparations was determined by using the substrate (4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUF-NeuNAc) following a protocol similar to that of Potier et al. (1979) and Scheel et al. (1982). The activity of this membrane-bound enzyme was 0.6 nmol of MUF-NeuNAc hydrolyzed/mg of protein at 37 °C and is comparable to that seen by Scheel et al. (1982).

In order to assess the effects of lipid composition and membrane physical parameters on the functional properties of synaptic plasma membranes, intact SPM's were selectively enriched with DMPC up to a molar fraction of 0.8 of the total lipid. These DMPC selectively enriched synaptic membranes (DMPC–SESM) were prepared in the following manner. An aliquot of synaptic membranes, usually 1–2 mg, were first solubilized by addition of 1.7% cholate followed by mild sonication for 1 min using a Laboratory Supplies bath sonicator. The solubilized membranes were then placed in number 2 Spectra/Por membrane tubing and dialyzed for 18 h at 4 °C against 10 mM Tris, pH 7.4. Negative-stain electron micrographs of the sample made after this initial dialysis revealed that essentially no closed vesicular forms of the membranes were present. At this point, 2.5 mg of DMPC/mg of SPM protein was added to the sample and the mixture sonicated 30 s. The sample was then dialyzed overnight again in the same buffer to remove remaining cholate and allow formation of the SESM's. A study utilizing [^{3}H]cholate (New England Nuclear) indicated a final cholate:lipid ratio of 1:400. The integrity of the DMPC–SESM's was assured by electron microscopy which revealed closed vesicles of an average size of 0.65 ± 0.15 μm. Phosphate analysis and thin-layer chromatography of the samples indicated DMPC comprised 80 ± 5% of the total phospholipid. Integrity of the DMPC–SESM's was further assessed by high-sensitivity differential scanning calorimetry and determination of the sialidase activity of the SESM's (0.3 nmol of MUF-NeuNAc hydrolyzed/mg of protein).

All protein determinations were made by using the procedure of Lowry et al. (1951) with bovine serum albumin as a standard.

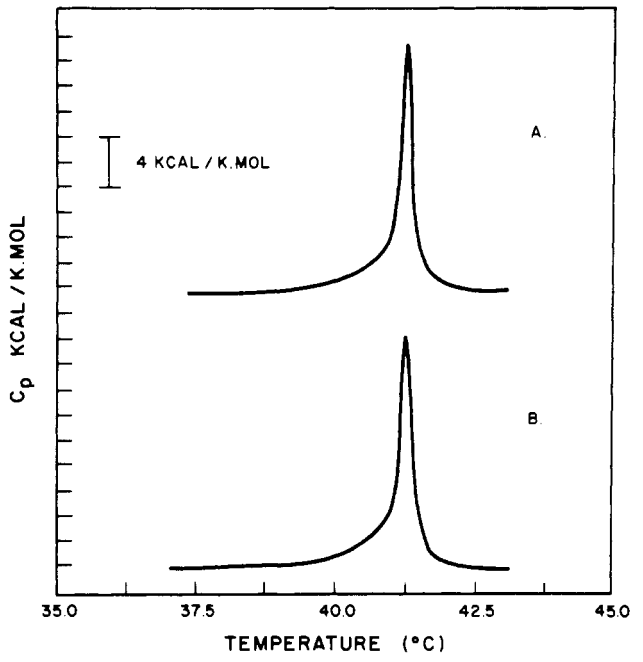

FIGURE 1: Excess heat capacity function vs. temperature for (A) large unilamellar DPPC vesicles and (B) large unilamellar DPPC vesicles with 5×10^{-4} M [D-Ala^2]methionine-enkephalinamide.

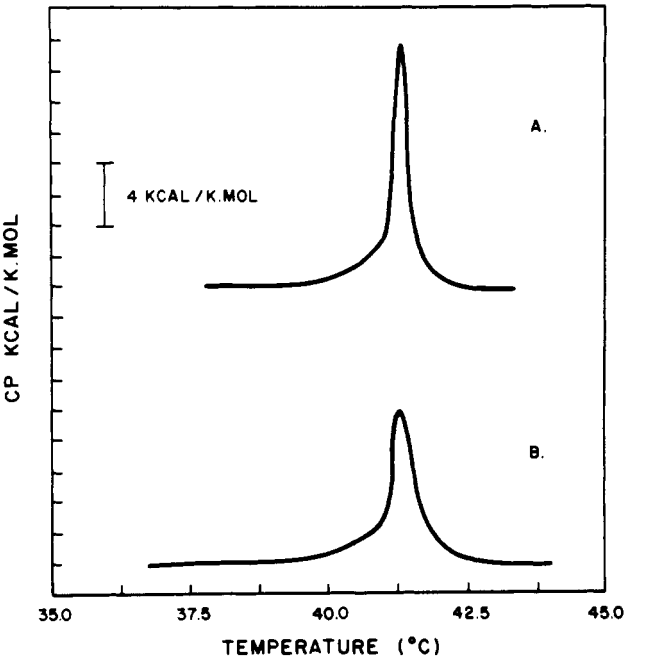

FIGURE 2: Excess heat capacity function vs. temperature for (A) large unilamellar DPPC vesicles containing 10% ganglioside G_{M1} and (B) the same with 2.2×10^{-4} M [D-Ala^2]methionine-enkephalinamide.

High-Sensitivity Scanning Calorimetry. All calorimetric experiments were performed with a Microcal MC1 differential scanning calorimeter. The sensitivity and precision of the basic calorimetric unit have been improved by the use of two separate Keithley amplifiers connected to the heat capacity and temperature outputs of the instrument and interfaced to an IBM PC microcomputer system for automatic data collection and processing. The calorimetric data were digitized by a Data Translation DT-805 A/D converter board and stored in floppy disks at 0.05 °C intervals for subsequent analysis. In this way it is possible to perform experiments using very dilute biological materials. With pure lipid dispersions, concentrations smaller than 0.5 mg/mL can be used with a total sample volume of 0.7 mL. For these studies the concentration of lipid in the calorimetric cell was about 4 mg/mL. All the calorimetric scans were performed at a scanning rate of 15 °C/h.

Steady-State Fluorescence Anisotropy. The thermotropic behavior of the membrane preparations was also studied by steady-state fluorescence depolarization using a Perkin-Elmer LS-5 spectrofluorometer equipped with a thermostated cell holder and 3M 105-Ml glass polarizers in the excitation and emission beams. The sample temperature was controlled by a Precision RDL 20 refrigerated bath circulator and the temperature monitored within 0.1 °C by a Keithley digital thermometer. 1,6-Diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, Junction City, OR) was dissolved in acetonitrile and added to suspensions of SESM or SPM at a ratio of 1 probe molecule per 500 lipid molecules. The total lipid concentration was 0.1 M. Equilibration of the probe was assured by incubation at 45 °C for 1 h. The samples were excited at 360 nm, and the emission intensity was measured at 430 nm parallel and perpendicular to the plane of excitation. Anisotropy was calculated as described by Barenholz et al. (1976).

RESULTS

Thermotropic Behavior of DPPC–Ganglioside FUV's and Enkephalin. The excess heat capacity vs. temperature function for large unilamellar DPPC vesicles is shown in Figure 1A. The gel–liquid-crystalline phase transition of these vesicles is centered at 41.3 °C and is characterized by an enthalpy change (ΔH) of 8.4 kcal/mol of lipid. The addition of up to 5×10^{-4} M [D-Ala^2]methionine-enkephalinamide (Figure 1B) does not have any appreciable effect on the thermodynamic parameters associated with this transition. This is consistent with the studies of Jarrell et al. (1980) which report little or no binding of enkephalinamide to phosphatidylcholine and Abood & co-workers (1977) which indicate that phosphatidylcholine has no effect on opiate binding to neural membrane preparations.

Figure 2 illustrates the effects of enkephalinamide on the thermotropic behavior of DPPC large unilamellar vesicles containing 10 mol % ganglioside G_{M1} asymmetrically localized on the outer membrane surface. The presence of ganglioside G_{M1} broadens the calorimetric transition profile of DPPC without any detectable effect on the enthalpy change for the transition (Figure 2A). The presence of 2×10^{-4} M enkephalinamide (Figure 2B), however, causes an additional broadening of the transition profile, suggesting that at this enkephalin concentration some interaction of the opiate peptide with ganglioside G_{M1} is taking place. The interaction, even though stronger than the one with DPPC alone, is still relatively weak and cannot be detected calorimetrically at enkephalin concentrations lower than 10^{-4} M.

Figure 3 shows the enkephalinamide effect on the thermotropic behavior of DPPC large unilamellar vesicles containing 10 mol % ganglioside G_{d1a} asymmetrically localized on the outer membrane surface. In this case, the presence of enkephalinamide has a very dramatic effect on the thermotropic behavior of the vesicles. At enkephalinamide concentrations as low as 1×10^{-5} M (Figure 3B) the heat capacity function becomes strongly asymmetric toward the high-temperature end of the transition, suggesting that the overall curve is the superposition of two distinct peaks. At enkephalinamide concentrations of 2×10^{-5} M and higher (Figure 3C,D), the second high-temperature peak centered at about 42 °C becomes clearly visible in the calorimetric scan, indicating that the enkephalin is inducing a phase separation process within the membrane. The appearance of two distinct peaks in the calorimetric profile suggests that the membrane surface is no longer compositionally homogeneous and that different regions

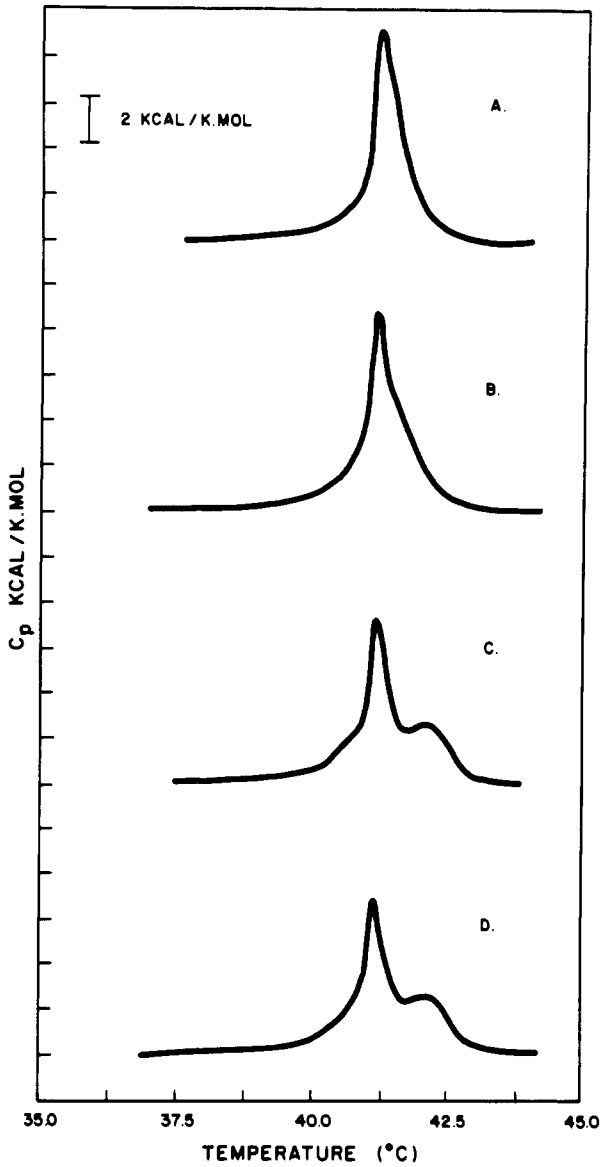

FIGURE 3: Excess heat capacity function vs. temperature for (A) large unilamellar DPPC vesicles containing 10% ganglioside G_{dla}, and the same DPPC–G_{dla} vesicles with (B) 1.1×10^{-5} M [D-Ala^2]methionine-enkephalinamide, (C) 2.2×10^{-5} M [D-Ala^2]methionine-enkephalinamide, and (D) 3.3×10^{-5} M [D-Ala^2]methionine-enkephalinamide.

of the membrane possess a different molecular composition. In the present case, the area under the second high-temperature peak can be as large as 40% of the total area, suggesting that the phase-separated domains are not pure ganglioside domains, since ganglioside comprises only 10% of the total lipid, but mixed phosphatidylcholine–ganglioside domains. Further addition of enkephalinamide did not induce additional changes in the heat capacity function of the DPPC–G_{dla} vesicles.

Figure 4 illustrates the enkephalinamide effect on the thermotropic behavior of large unilamellar DPPC vesicles containing 10 mol % ganglioside G_{tlb} asymmetrically localized on the outer membrane surface. As reported before (Myers et al., 1984), ganglioside G_{tlb} has an intrinsic tendency to phase separate from DPPC judging from the existence of a second peak in the calorimetric profile even in the absence of divalent cations or enkephalin. In the presence of enkephalinamide concentrations of 10^{-4} M or larger (Figure 4B,C), the phase separation process is accentuated and the high-temperature peak is shifted to higher temperatures. It must be noted that

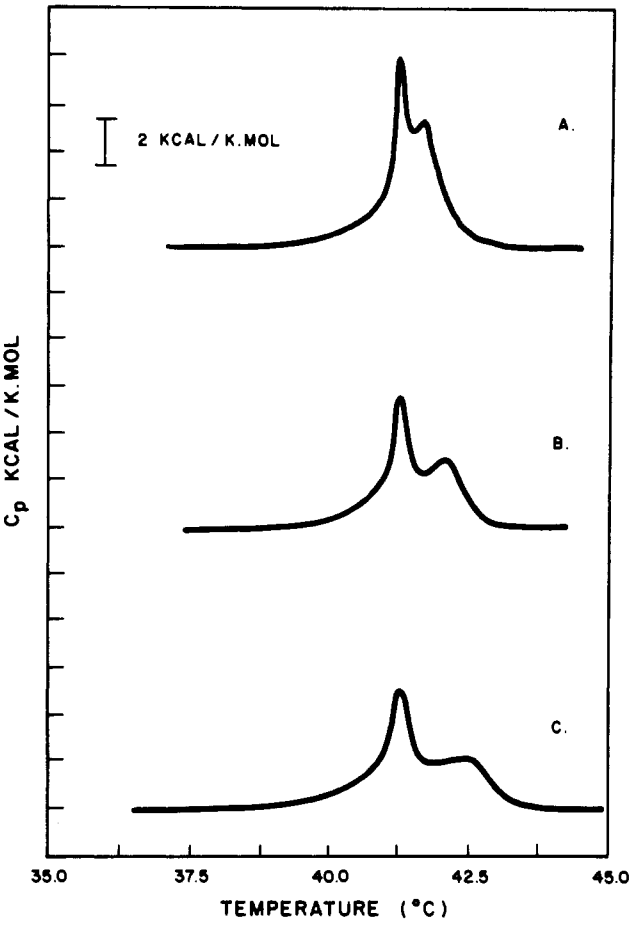

FIGURE 4: Excess heat capacity function vs. temperature for (A) large unilamellar DPPC vesicles containing 10% ganglioside G_{tlb} and the same with (B) 1.1×10^{-4} M [D-Ala^2]methionine-enkephalinamide and (C) 2.2×10^{-4} M [D-Ala^2]methionine-enkephalinamide.

in this, as well as in all the other cases under study in this paper, the enthalpy change for the overall lipid transition remained unchanged within the experimental error, suggesting that the interaction of the enkephalin with the ganglioside-containing lipid bilayer does not induce a significant perturbation of the hydrophobic core of the bilayer. In this respect, it should be mentioned that the bilayer perturbation induced by intrinsic membrane proteins and even small hydrophobic peptides located within the hydrophobic core of the bilayer, like the antibiotic gramicidin (Chapman et al., 1977; Freire et al., 1983), is characterized by a decrease in the enthalpy change for the phospholipid phase transition. On the other hand, membrane proteins that are primarily localized on the bilayer surface do not generally affect the enthalpy change of the lipid transition (Wiener et al., 1983). The absence of such an enthalpic effect for the case of enkephalin suggests that the primary site of interaction is at the head-group level. This conclusion is strengthened by the observation that different gangliosides respond differently to the enkephalinamide. Recently, Behnam & Deber (1984) have presented a model, based upon nuclear magnetic resonance data, for the association of methionine- and leucine-enkephalin to a membrane environment and also concluded that the enkephalin is primarily bound to the membrane surface.

Intact and DMPC Selectively Enriched Synaptic Plasma Membranes. Differential scanning calorimetric experiments of synaptic plasma membranes did not show any evidence of a lipid phase transition. This observation was confirmed by DPH steady state anisotropy measurements as shown in Figure 5. The anisotropy values decreased monotonically with tem-

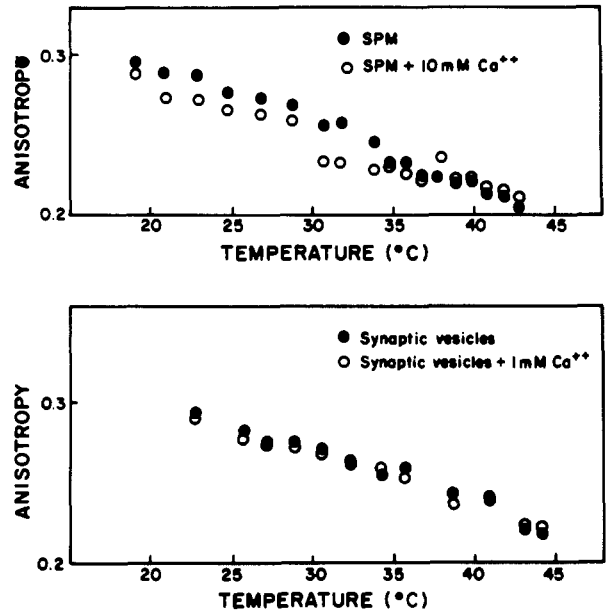

FIGURE 5: (Upper panel) Fluorescence anisotropy of DPH incorporated into SPM's with (○) and without (●) 10 mM Ca^{2+}. (Lower panel) Fluorescence anisotropy of DPH incorporated into synaptic vesicles with (○) and without (●) 1 mM Ca^{2+}.

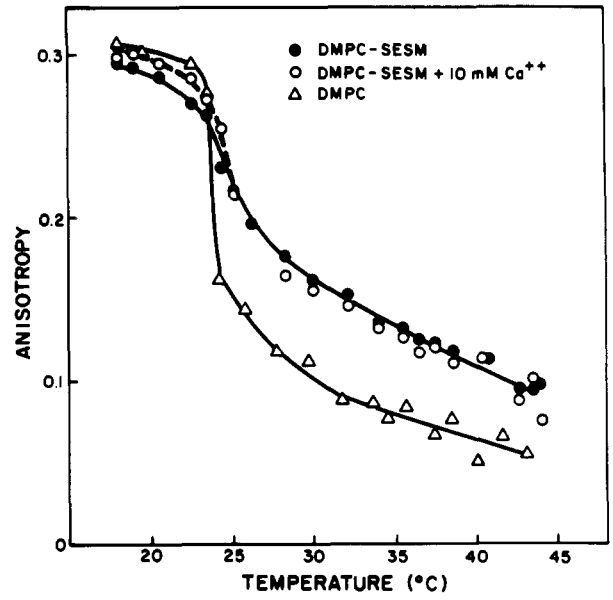

FIGURE 6: Fluorescence anisotropy of DPH incorporated into DMPC FUV's (△), DMPC–SESM's (●), and DMPC–SESM's plus 10 mM Ca^{2+} (○).

perature from a value of approximately 0.3 at 20 °C to a value of about 0.2 at 43 °C. The anisotropy values were insensitive to the presence of up to 10^{-2} M Ca^{2+} concentration, suggesting that Ca^{2+} does not induce a significant change in the overall fluidity of the synaptic plasma membrane. The same was true for synaptic vesicles as shown in the lower panel of Figure 5.

In order to assess the effects of lipid composition and membrane physical state on the properties of synaptic plasma membranes, these membranes were enriched with DMPC and/or gangliosides as described above. The enrichment procedure resulted in a rather homogeneous population of unilamellar vesicles with a diameter ranging between 0.5 and 0.8 μm as indicated by negative-stain electron microscopy. The temperature dependence of the steady-state fluorescence anisotropy of DPH incorporated into DMPC–SESM containing 80 mol % DMPC is shown in Figure 6. For comparison, the anisotropy values for pure DMPC vesicles are also shown in the figure. Below 23 °C the DPH steady-state anisotropy of DMPC–SESM's is about 0.3, very close to the value obtained for pure DMPC in the gel phase. At approximately 24 °C, the phase transition temperature of DMPC, the anisotropy of the DMPC–SESM's decreases rapidly even though the decrease is not as sharp as for pure DMPC. Above the lipid phase transition temperature (24 °C), the anisotropy values for DMPC–SESM's are always larger than those for pure DMPC, indicating that DMPC–SESM's are less fluid than pure DMPC vesicles above the lipid phase transition temperature but not as rigid as intact synaptic plasma membranes.

Figure 7 shows the calorimetric profile of DMPC–SESM's before and after the incorporation of 10 mol % of ganglioside G_{d1a}. In the absence of ganglioside G_{d1a} the heat capacity function of DMPC–SESM's is characterized by a broad peak centered at 24 °C as expected for a membrane preparation of heterogeneous molecular composition containing a significant fraction of DMPC. The calorimetric scan of pure DMPC (not shown) gives a very sharp peak with a half-height width of 0.4 °C and a peak maximum of 15 kcal/(K·mol). In contrast, the peak maximum for the DMPC–SESM's is less than 4 kcal/(K·mol), and the half-height width is 2 °C. The absence of any fine structure in the heat capacity function of the DMPC–SESM's indicates that the DMPC is readily mixed with the endogenous synaptosomal lipid. The incorporation of ganglioside G_{d1a} into the DMPC–SESM's induces a further broadening of the transition peak as in the case of the pure phospholipid. In the absence of ganglioside G_{d1a} the enkephalinamide does not have an observable effect on the phase transition profile of DMPC–SESM's. However, DMPC–SESM's containing 10 mol % ganglioside G_{d1a} exhibit a distinct broadening and the appearance of a second broader peak at a higher temperature in the presence of enkephalinamide. As in the case of the phospholipid vesicles, the appearance of this second peak is indicative of a phase separation process within the bilayer matrix. Thus, judging from these results, the enkephalin molecule appears to be able to induce phase separation processes in both natural and liposomal membranes containing ganglioside G_{d1a}.

Discussion

Gangliosides, sialic acid containing glycosphingolipids, constitute about 10 mol % of the total plasma membrane lipid of the neural synaptic membrane and about 20 mol % of the lipid on the outer surface of these membranes (Ledeen, 1978; Tettamanti et al., 1979). If we consider that the ganglioside head-group cross-sectional area is much larger than that of other lipid constituents of the membrane, it follows that a significant fraction of the plasma membrane lipidic surface is occupied by gangliosides. This large figure should underline the potential biochemical impact of interactions between endogenous or exogenous ligands with cell surface gangliosides.

The calorimetric experiments presented in this paper indicate that an active opioid peptide, [D-Ala^2]methionine-enkephalinamide, is capable of inducing phase separation of gangliosides, particularly ganglioside G_{d1a}, in both liposomal and synaptic plasma membranes at concentrations considerably lower than those necessary for noticeable effects on other lipids. In fact, several experiments designed to assess the in vivo effect of enkephalins are performed at concentrations high enough to cause ganglioside phase separation (Dawson et al., 1979; McLawhorn et al., 1981). Because of the drastic change in ganglioside distribution due to phase separation and the subsequent alteration of the entire outer surface of the synaptic membrane, such enkephalinamide–ganglioside interactions

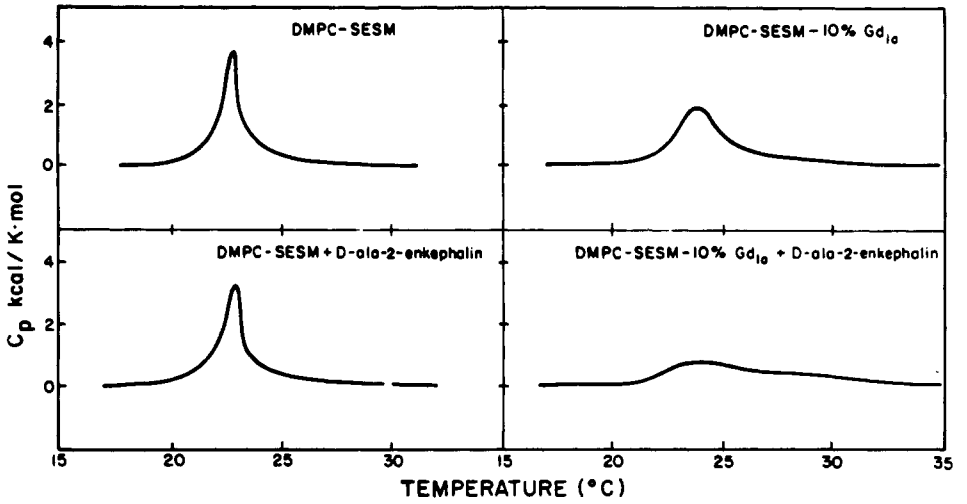

FIGURE 7: Excess heat capacity function vs. temperature for (A) DMPC–SESM, (B) DMPC–SESM's plus [D-Ala^2]methionine-enkephalinamide, (C) DMPC–G_{d1a}–SESM's, and (D) DMPC–G_{d1a}–SESM's plus [D-Ala^2]methionine–enkephalinamide.

may have important biological implications. For example, Dawson et al. (1979) have previously shown that enkephalins may inhibit ganglioside synthesis in mouse neuroblastoma cells. Earlier work in this laboratory (Myers et al., 1984) indicates that membrane-bound ganglioside aggregates are less suitable substrates for soluble sialidase than gangliosides dispersed throughout the membrane. Tettamanti and co-workers (1979, 1980) have postulated that changes in the lateral distribution of gangliosides might be important for the functioning of presynaptic nerve membranes in three specific manners: (1) aggregation of these potential receptors might facilitate ligand binding, (2) ganglioside-free areas might permit easier collision with apolar ligands, and (3) the formation of polar channels might be facilitated by the presence of ganglioside-free phospholipid domains (Tettamanti et al., 1980).

Changes in the distribution of molecules within the membrane have been associated with a variety of processes such as clustering of receptor sites, patching and cap formation, exocytosis, and enzymatic activities to name only a few (Papahadjopoulos et al., 1976; Beisson et al., 1976; Fishman & Brady, 1976). These changes in molecular organization are capable of eliciting rather dramatic changes in the physical and functional properties of the membrane by creating microdomain environments with properties different from those of the rest of the membrane, by bringing together and assembling functionally active molecular aggregates or receptors, and/or by modulating diffusion rates and therefore reaction rates of membrane-associated processes.

The enkephalinamide-dependent ganglioside phase separation described in this paper should result in the formation of ganglioside-rich domains. Judging from the area under the calorimetric phase-separated peaks, these ganglioside-rich domains may contain up to one ganglioside molecule for every four lipid molecules. The exact molecular structure of these domains as well as their sidedness (whether they involve phospholipid molecules from the inner face of the bilayer) remains to be elucidated. The results presented in this paper indicate that these domains must have a different thermotropic behavior from the rest of the membrane, that above the phase transition temperature they have a lower fluidity than the bulk lipid, and that because of the expected ganglioside clustering, these domains must have a rather large sialic acid density. These modified physical parameters are likely to affect functional properties of the synaptic plasma membrane. For example, recently we have shown that the insertion of the hydrophobic active subunit of cholera toxin into the membrane is facilitated by ganglioside G_{M1} phase separation (Goins & Freire, 1985). A redistribution of ganglioside molecules within the membrane surface may, in fact, affect the functioning of the opiate receptor even in the absence of a direct involvement of glycolipid in the biochemical reactions occurring at the receptor level. Ganglioside clustering might bring ligand molecules closer to the receptor, essentially acting as a membrane surface carrier, thus facilitating receptor–ligand interactions. On the other hand, the activity of the opiate receptor may be dependent on aggregation parameters as is the case for other receptors and be affected by lateral phase separation processes. A mechanism of this type could explain apparently contradictory literature reports concerning the susceptibility of the opiate receptor to neuraminidase treatment.

Registry No. DPPC, 2644-64-6; DMPC, 13699-48-4; ganglioside G_{M1}, 37758-47-7; ganglioside G_{d1a}, 12707-58-3; ganglioside G_{t1b}, 59247-13-1; (D-Ala^2,Met^5)-enkephalin amide, 61090-95-7.

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Spontaneous Transfer of Gangliotetraosylceramide between Phospholipid Vesicles†

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ABSTRACT: The transfer kinetics of the neutral glycosphingolipid gangliotetraosylceramide (asialo-GM_1) were investigated by monitoring tritiated asialo-GM_1 movement from donor to acceptor vesicles. Two different methods were employed to separate donor and acceptor vesicles at desired time intervals. In one method, a negative charge was imparted to dipalmitoylphosphatidylcholine donor vesicles by including 10 mol % dipalmitoylphosphatidic acid. Donors were separated from neutral dipalmitoylphosphatidylcholine acceptor vesicles by ion-exchange chromatography. In the other method, small, unilamellar donor vesicles (20-nm diameter) and large, unilamellar acceptor vesicles (70-nm diameter) were coincubated at 45 °C and then separated at desired time intervals by molecular sieve chromatography. The majority of asialo-GM_1 transfer to acceptor vesicles occurred as a slow first-order process with a half-time of about 24 days assuming that the relative concentration of asialo-GM_1 in the phospholipid matrix was identical in each half of the donor bilayer and that no glycolipid flip-flop occurred. Asialo-GM_1 net transfer was calculated relative to that of [^{14}C]cholesteryl oleate, which served as a nontransferable marker in the donor vesicles. A nearly identical transfer half-time was obtained when the phospholipid matrix was changed from dipalmitoylphosphatidylcholine to palmitoyloleoylphosphatidylcholine. Varying the acceptor vesicle concentration did not significantly alter the asialo-GM_1 transfer half-time. This result is consistent with a transfer mechanism involving diffusion of glycolipid through the aqueous phase rather than movement of glycolipid following formation of collisional complexes between donor and acceptor vesicles. When viewed within the context of other recent studies involving neutral glycosphingolipids, these findings provide additional evidence for the existence of microscopic, glycosphingolipid-enriched domains within the phospholipid bilayer.

Spontaneous, nonprotein-mediated transfer of phospholipids and phospholipid derivatives between model membranes is a well established phenomenon (Martin & McDonald, 1976, Papahadjopoulos et al., 1976; Duckwitz-Peterlein et al., 1977; Nichols & Pagano, 1981, 1982; Roseman & Thompson, 1980; McLean and Phillips, 1981; De Cuyper et al., 1983; Schroit & Madsen, 1983). Nonprotein-mediated, intervesicular transfer of cholesterol and other lipid derivatives is also well documented (Backer & Dawidowicz, 1981; McLean & Phillips, 1981, 1982; Kao et al., 1977; Doody et al., 1980). However, very little information exists on the spontaneous interbilayer transfer of glycosphingolipids. Insight into the behavior and structural organization of glycosphingolipids in membranes is important because these molecules have been implicated in cell–cell identification and recognition processes (Horowitz, 1978; Hakomori, 1981), and they can also serve as cellular attachment sites for certain bacterial toxins (Fishman, 1982) and viruses (Holmgren et al., 1980).

In previous studies from this laboratory (Correa-Freire et al., 1982) the spontaneous interbilayer transfer of the simple neutral glycosphingolipid glucosylceramide was examined and

†Supported by U.S. Public Health Service Grants GM-23573 and GM-14628.
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0006-2960/85/0424-4082$01.50/0