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Comparison of the Calcium Release Channel of Cardiac and Skeletal Muscle Sarcoplasmic Reticulum by Target Inactivation Analysis†

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Received July 20, 1988; Revised Manuscript Received September 22, 1988

ABSTRACT: The calcium release channel of sarcoplasmic reticulum which triggers muscle contraction in excitation–contraction coupling has recently been isolated. The channel has been found to be morphologically identical with the feet structures of the junctional face membrane of terminal cisternae and consists of an oligomer of a unique high molecular weight polypeptide. In this study, we compare the target size of the calcium release channel from heart and skeletal muscle using target inactivation analysis. The target molecular weights of the calcium release channel estimated by measuring ryanodine binding after irradiation are similar for heart (139 000) and skeletal muscle (143 000) and are smaller than the monomeric unit (estimated to be about 360 000). The target size, estimated by measuring polypeptide remaining after irradiation, was essentially the same for heart and skeletal muscle, 1 061 000 and 1 070 000, respectively, indicating an oligomeric association of protomers. Thus, the calcium release channel of both cardiac and skeletal muscle reacts uniquely with regard to target inactivation analysis in that (1) the size by ryanodine binding is smaller than the monomeric unit and (2) a single hit leads to destruction of more than one polypeptide, by measuring polypeptide remaining. Our target inactivation analysis studies indicate that heart and skeletal muscle receptors are structurally very similar.

In excitation–contraction coupling, excitation at the sarcolemma leads to an elevated intrafiber Ca^{2+} concentration and thereby to muscle contraction. In vertebrate skeletal muscle, essentially all of the calcium for contraction is stored intracellularly within the sarcoplasmic reticulum (SR)[1] (Fleischer & Tonomura, 1985). Calcium is released from SR as a consequence of depolarization of the transverse tubule. This process is referred to as "depolarization induced calcium release". In heart, there are two pools of calcium: (1) extracellular calcium first enters the fiber through voltage-gated sarcolemmal calcium channels, and (2) the elevated Ca^{2+} then triggers calcium release from the SR compartment. This two-step process is referred to as "calcium induced calcium release" (Fabiato, 1983; Endo, 1977). A basic question to be resolved is whether the calcium release machinery in SR in heart is different from that in skeletal muscle.

The calcium release channels of SR from heart and skeletal muscle have recently been isolated and identified in molecular terms (Inui et al., 1987a,b; Lai et al., 1988a,b; Hymel et al., 1988a,b; Fleischer & Inui, 1988; Imagawa et al., 1987). The breakthrough was based on two key advances: (a) the isolation of a terminal cisternae fraction of SR containing well-defined feet structures which served as a test system (Saito et al., 1984) and (b) the finding that ryanodine is a specific ligand for the calcium release channel of SR (Fleischer et al., 1985). The isolated ryanodine receptor from heart and skeletal muscle consists of an oligomer of a single high molecular weight polypeptide (Inui et al., 1987b; Lai et al., 1988a,b; Imagawa et al., 1987). The receptor has been identified morphologically as the foot structure (Inui et al., 1987a,b) which spans the triad or dyad junction (Franzini-Armstrong & Nunci, 1983) between terminal cisternae and transverse tubules or plasmalemma. The identity of the ryanodine receptor as the channel was achieved by reconstituting it into bilayers. Channel gating behavior was obtained, and the response to specific ligands reflected the permeability changes characteristic of terminal cisternae of SR (Hymel et al., 1988a,b; Lai et al., 1988a,b).

In this study, we compare structural characteristics of the calcium release channel from heart and skeletal muscle by target inactivation analysis.

Materials and Methods

All chemicals were reagent grade or the best available and were prepared in deionized water. Protein was measured according to the method of Lowry et al. (1951) using bovine plasma albumin as the standard. Cardiac microsomes were

† This work was supported in part by grants from the National Institutes of Health (DK 14632 and HL 32711), by the Muscular Dystrophy Association of America, and by a Biomedical Research Support grant from the National Institutes of Health administered by Vanderbilt University.

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[1] Abbreviations: SDS–PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; SR, sarcoplasmic reticulum; HMW polypeptide, high molecular weight polypeptide.

0006-2960/89/0428-1319$01.50/0

prepared from canine heart as described by Inui et al. (1988) with the omission of DTT in the preparation procedure. Triads were prepared from rabbit skeletal muscle according to Mitchell et al. (1983). Ryanodine was obtained from the Penick Corp. (Lyndhurst, NJ).

Irradiation Procedure. Cardiac microsomes were diluted to 1 mg of protein/mL in 0.65 M KCl, 0.29 M sucrose, and 10 mM imidazole, pH 6.8. The skeletal muscle triads were diluted to 1 mg of protein/mL in 0.3 M sucrose and 5 mM Hepes, pH 7.4. The suspension was then plated out in 1- or 1.2-mL aliquots in open aluminum trays and frozen immediately using liquid nitrogen as previously described (McIntyre et al., 1983). The freezing time was approximately 10–15 s per tray. The samples were shipped to Buffalo in a portable liquid nitrogen refrigerator where they were irradiated under a nitrogen atmosphere at a temperature of −40 to −50 °C in a Van de Graaff generator. The generator produced a 0.3- or 0.65-mA beam of 1.5-MeV electrons. The irradiation dose for each sample was controlled by varying the milliamperes of the beam and/or the number of passes through the electron beam. The total dosage was varied between 0 and 7.2 Mrad. Sham-irradiated controls were placed in the irradiation chamber under identical conditions, but with the electron beam off. These samples showed no change in activity when compared with samples which were retained in the laboratory. The radiation dose was measured at the sample irradiation temperature using the transmittance change of blue cellophane calibrated against the Fricke dosimeter. The samples were maintained at liquid nitrogen temperature until ready for analysis. The samples were rapidly thawed (McIntyre et al., 1983) and 50–160-μL aliquots were refrozen in 1 mM DTT for densitometry. Ryanodine binding assay was performed on the remaining sample immediately after thawing.

Ryanodine Binding. Tritiated ryanodine, radiolabeled as previously described (Fleischer et al., 1985), was used for ligand binding studies (cardiac microsomes) or diluted with carrier to approximately 5000 cpm/pmol (skeletal muscle triads) prior to use for binding. Scatchard analysis of ryanodine binding to the cardiac microsomes and skeletal muscle triads was performed, and the dissociation constant (K_D) and maximum number of binding sites (B_{max}) for the ryanodine receptor were determined. Total binding was subsequently measured in the irradiated samples and controls by using a saturating concentration of ryanodine calculated for each tissue based on the K_D. For measurement of total ryanodine binding (Inui et al., 1987a; Fleischer et al., 1985), the assay in 1 mL contained binding buffer (1 M KCl, 10 mM Hepes, and 1 mM DTT for cardiac microsomes only, pH 7.4), 0.1 mg of protein and [^{3}H]ryanodine. For Scatchard analysis, the [^{3}H]ryanodine concentration was varied between 1 nM and 1.44 μM for cardiac microsomes and between 30 and 270 nM for skeletal muscle triads. The saturating concentrations of [^{3}H]ryanodine selected for use in the target inactivation experiments were 30 or 50 nM (cardiac) or 270 nM (skeletal muscle triads).[2] Nonspecific binding was measured by including a 100-fold excess of unlabeled ryanodine in the binding medium. In general, nonspecific binding was 10% or less of the total binding. Specific binding was determined for each radiation point by subtracting the nonspecific from the total binding. Each total binding point was determined in triplicate and each nonspecific binding point in duplicate. The samples, after mixing, were incubated at 37 °C (cardiac) or room temperature (skeletal muscle triads) for 30 min. Following incubation, samples were filtered in an Amicon filtration apparatus at a negative pressure equivalent to 25 mmHg using 0.22-μM filters (GSWP, Millipore Corp., Bedford, MA) which had been presoaked for 30 min in binding buffer. The filtered samples were then washed twice with 2-mL aliquots of binding buffer and 4 times with 2-mL aliquots of 10% ethanol in water. The washed filters were collected and placed in 12 mL of ACS scintillation fluid (Amersham Corp., Arlington Heights, IL). Radioactivity was measured to 0.5% of σ accuracy in a Searle Analytic 81 scintillation counter using quench correction. Counting efficiency was approximately 55%.

For the skeletal muscle receptor, irradiation was found to decrease the number of binding sites (B_{max}) and within experimental error did not alter the K_d (McGrew et al., 1987).

Densitometry To Determine Polypeptide Remaining. Aliquots of cardiac microsomes (45- or 140-μL aliquots of 1 mg/mL protein) were sedimented in a Beckman airfuge (A-100 rotor) at 29 psi at room temperature for 30 min. Skeletal muscle triads (1 mg of protein/mL) were not concentrated. The pellets (cardiac) or aliquots (skeletal muscle) from each irradiated sample and sham control were solubilized in Laemmli solubilization media at a final concentration of 3 or 5 mg of protein/mL (cardiac) or 0.75 mg of protein/mL (skeletal muscle). The solubilized cardiac or skeletal muscle protein (10 μL) for each irradiation point was placed in a multiwell stacking gel and separated by SDS–PAGE according to Laemmli (1970). A different gel was used for each experiment. The amount of high molecular weight protein (molecular weight of about 340 000 for cardiac and 360 000 for skeletal muscle) was quantitated by densitometry after staining with Coomassie blue as previously described (McIntyre et al., 1983; Hymel et al., 1984). The gels were scanned at 633 nm using an LKB Ultrascan XL densitometer. The band referable to the high molecular weight protein was identified, and the base line for the peak was drawn manually from trough to trough. The area of the peak was then determined by computer integration using LKB 2400 Gel Scan XL software. The target size of the calcium pump protein, previously found to be a dimer in the membrane (Hymel et al., 1984), served as an internal control of irradiation dose.

Data Presentation and Calculation. The data were analyzed with a single-target, single-hit model of radiation inactivation (Kempner & Fleischer, 1989; Setlow & Pollard, 1953). Plots of the logarithm of the percentage of surviving activity (ryanodine binding or polypeptide remaining) were linear over 1–1.5 orders of magnitude with correlation coefficients generally ≥ -0.94 (in two cases ~ -0.83–0.85). Apparent molecular weights were calculated by using the formula: molecular weight (M_r) = $(6.4 \times 10^{11})/D_{37}$ (rad), where D_{37} is the dose of absorbed radiation required to reduce the activity to 37% of the original (Kepner & Macey, 1968).

Results

Ryanodine binding for the high-affinity site was performed on nonirradiated control cardiac microsomes and skeletal muscle triads. Scatchard analysis of ryanodine binding data gave a dissociation constant (K_D) of approximately 7.1 ± 1.7 nM (n = 2) for the cardiac microsomes and 39.1 ± 21.8 nM (n = 3) for skeletal muscle triads using the conditions described.[2]

Ryanodine binding to the high-affinity site was then measured for the irradiated samples using near-saturating concentrations of ryanodine: 30 or 50 nM for cardiac microsomes

[2] The high-affinity binding constant (K_{D1}) for ryanodine binding is very dependent on the conditions used. Careful binding studies carried out under identical conditions (37 °C for 1 h) indicate that the high-affinity binding of ryanodine to the receptor is about the same ($K_{D1} \sim$ 6 nM) for both heart and skeletal muscle (McGrew et al., 1988).

Table I: Target Molecular Weight Measuring Ryanodine Binding and High Molecular Weight Polypeptide

characteristic studied	membrane fraction	target mol wt ($\times 10^{-3}$)
ryanodine binding[a]	cardiac microsomes	139 ± 1 (2)
	skeletal muscle triads	143 (1)
	skeletal muscle terminal cisternae[b]	138 ± 21 (10)
high mol wt polypeptide[c]	cardiac microsomes	1063 ± 78 (3)
	skeletal muscle triads	1069 ± 243 (2)
Ca pump protein polypeptide remaining (internal control)	skeletal muscle triads	277 ± 12 (2)

[a] Ryanodine binding in cardiac microsomes and skeletal muscle triads was measured as a function of radiation dosage. Near-saturating concentrations of [^{3}H]ryanodine were used: 30 or 50 nM [^{3}H]ryanodine for cardiac microsomes and 270 nM [^{3}H]ryanodine for skeletal muscle triads (see Figure 1). The data are expressed as the mean molecular weight ± standard deviation, with the number of experiments given in parentheses. [b] Data from McGrew et al. (1987). [c] Cardiac (or skeletal muscle) fractions from each irradiated sample and sham-irradiated controls were solubilized, placed on a multiwell stacking gel, and separated by SDS–PAGE using the conditions of Laemmli (1970). A different gel was used for each experiment. Correlation coefficients for the inactivation curves varied between −0.83 and −0.99 (see Figure 2). Data are tabulated as the mean molecular weight and standard deviation, with the number of experiments given in parentheses.

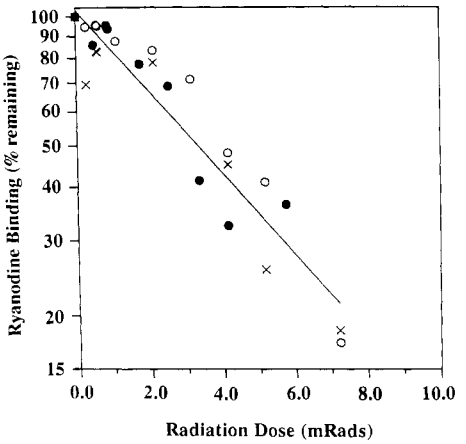

FIGURE 1: Semilog plot of ryanodine binding as a function of irradiation dose. The sarcoplasmic reticulum fractions were irradiated in the frozen state at different doses, and ryanodine binding was carried out as described under Materials and Methods. The 100% value for ryanodine binding, obtained from the "sham irradiated" control (electron beam off), was the same as for the sample prior to dilution and preparation for irradiation. For different membrane preparations, the maximum ryanodine binding (B_{max}) ranged from 5 to 10 pmol/mg of protein. Data are shown for binding of 30 nM (●) and 50 nM (○) ryanodine in cardiac microsomes and 270 nM (×) ryanodine in skeletal muscle triads. The linear regression line for all three experiments is essentially the same.[2] The slope and D_{37} of the inactivation profile were determined by linear regression (correlation coefficients ≥0.94), and the target molecular weights, calculated by using the established relationship (Kepner & Macey, 1968) $M_r = (6.4 \times 10^{11})/D_{37}$, were 138 000, 140 000, and 143 000 for 30 and 50 nM (cardiac) and 270 nM (skeletal muscle) ryanodine, respectively.

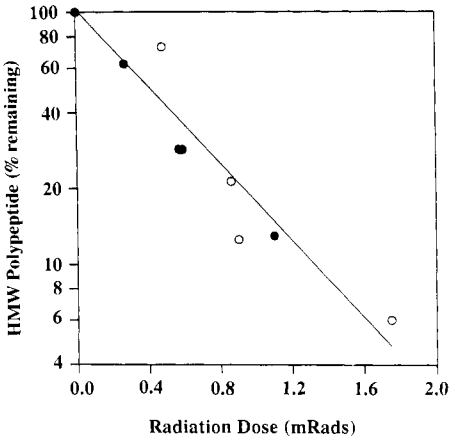

FIGURE 2: Semilog plot of high molecular weight polypeptide remaining as a function of irradiation dose. The cardiac microsomes (sedimented) or skeletal muscle triads (not sedimented) from each irradiation point and sham-irradiated control were solubilized in Laemmli solubilization buffer to a final protein concentration of 3 or 5 mg/mL for cardiac microsomes and 0.75 mg/mL for skeletal muscle triads. A 10-μL aliquot of the solubilized sample was placed on a multilevel stacking gel and separated by SDS–PAGE according to Laemmli (1970). A separate gel was used for each experiment. The amount of high molecular weight polypeptide (HMW) (molecular weight of about 360 000 for skeletal muscle and 340 000 for heart) was quantitated by densitometry after staining with Coomassie blue as previously described (McIntyre et al., 1983; Hymel et al., 1984). The gels were scanned at 633 nm using an LKB Ultrascan XL densitometer. The band referable to the high molecular weight polypeptide was identified, and the area was determined by computer integration using LKB 2400 Gel Scan XL software. Data are shown for cardiac microsomes (○) and skeletal muscle triads (●). The linear regression line for both experiments is essentially the same. The slope and the D_{37} of the inactivation profile were determined by linear regression (correlation coefficient 0.95–0.99), and the target molecular weights calculated from the D_{37} as in Figure 1 were 1.1×10^6 (cardiac) or 1.2×10^6 (skeletal muscle triads).

and 270 nM [^{3}H]ryanodine for skeltal muscle triads. Semilog plots of inactivation of ryanodine binding as a function of irradiation dose were linear (Figure 1). The target size for the cardiac microsomes (139 000) was experimentally the same as the target molecular weight for the skeletal muscle triads (143 000) obtained in this study. These values are essentially the same as that previously reported, 138 000, for skeletal muscle terminal cisternae (McGrew et al., 1987) (Table I).

The amount of high molecular weight protein remaining after irradiation was quantitated by densitometry on SDS–PAGE gels. Semilog plots of the percentage of high molecular weight protein remaining as a function of irradiation dose were linear for both cardiac and skeletal muscle tissue (Figure 2). The target molecular weight was essentially the same for cardiac microsomes (1 063 000 ± 78 000) and skeletal muscle triads (1 069 000 ± 243 000) (Table I). The calcium pump protein in the skeletal muscle triads served as an internal standard.

Discussion

In this study, we compared the calcium release channel (ryanodine receptor) from heart and skeletal muscle sarcoplasmic reticulum fractions using target inactivation analysis. The target size based on ryanodine binding is essentially the same for both. The value obtained here for skeletal muscle triads is the same as that previously determined (138 000 ± 21 000) for skeletal muscle terminal cisternae (McGrew et al., 1987). The ryanodine binding domain therefore is experimentally the same in size in heart and skeletal muscle. It is substantially smaller than the size of the high molecular weight polypeptide estimated to be ~360 000 for skeletal muscle (Inui et al., 1987a) and ~340 000 for heart (Inui et al., 1987b) and more recently substantially higher, ~450 000 (Imagawa et al., 1987).

The target molecular weight can be greater than the size of the subunit polypeptide when the energy from a single hit is transferred from one polypeptide to another in a complex. An oligomeric association is indicated when this occurs

(McIntyre et al., 1983; Hymel et al., 1984; Kempner & Fleischer, 1989). The findings in this study suggest such energy transfer occurs, indicating that the high molecular weight polypeptide in the foot structure of the terminal cisternae of sarcoplasmic reticulum is associated into an oligomeric structural unit.

The target sizes of the high molecular weight protein molecule on the basis of polypeptide remaining for cardiac muscle (1 063 000 ± 78 000) and skeletal muscle triads are experimentally the same (1 069 000 ± 243 000). Several conclusions about the structure of the calcium release channel are warranted from the target inactivation analysis obtained in this study: (1) the size of the ryanodine binding domain is considerably smaller than the protomer; (2) the feet structures consist of structural units of several protomers of the high molecular weight polypeptide. This conclusion is indicated by the observation that one hit leads to the destruction of more than one polypeptide and (3) the feet structures in heart and skeletal muscle are similar. The latter conclusion is especially significant considering this combination of traits (observations 1 and 2) is rare in target inactivation analysis studies (Kempner & Fleischer, 1989). Inui et al. (1987a) found by gel filtration HPLC a molecular weight of approximately 1.1×10^6 for the high molecular weight polypeptide, similar to that observed by target inactivation measuring polypeptide remaining in this study [see also Lai et al. (1988)].

The molecular weight of the foot structure, calculated from its dimensions and the density of protein (1.37 g/cm^3), was estimated to be $4.4–4.8 \times 10^6$ (Inui et al., 1987a). This is an upper limit, assuming that all of the space is filled with protein. The observation in crystals is that the space occupied by protein can vary widely, approximately 20–70% (Matthews, 1968). We have recently obtained an image-enhanced view of the calcium release channel (Saito et al., 1988) which reveals spaces devoid of mass. The foot structure approximates a square rectangle with 4-fold symmetry. Fourfold symmetry could readily be explained by a tetramer consisting of protomers, dimers, or trimers. Nonetheless, we cannot at present assign subunit structure on this basis since (a) the symmetry is only approximate in molecular terms and (b) the target size could be referable to an oligomer consisting of only a portion of the entire foot structure. Further, there is as yet uncertainty regarding the molecular weight of the protomer. The molecular weight has been obtained by an empirical method (mobility on SDS–PAGE), and even so, the reported estimate for protomer molecular weight varies substantially (Inui et al., 1987a; Lai et al., 1988a; Imagawa et al., 1987).

Classical target theory presumes that one hit destroys a single polypeptide. However, recent studies reveal interesting nuances [see review by Kempner and Fleischer (1989)]. An oligomeric structure is indicated when one hit leads to the destruction of more than one polypeptide. The target size can be smaller than the protomer when the measured characteristic is localized to specific polypeptide(s) not complexed in a way to allow energy transfer [e.g., see Goll et al. (1984)]. In several studies, the target size was found to be less than the polypeptide size. In these cases, rather than random fragmentation with a "hit", the polypeptide appears to fracture in such a way that a minimum protein domain for the measured characteristic is retained. For example, this has been reported for nitrate reductase (Solomonson & McCreery, 1986), for binding of inhibitor to the anion channel of erythrocytes (Verkman et al., 1986), and for the ryanodine binding domain from skeletal muscle (McGrew et al., 1987). In this study, we now report that the high molecular weight polypeptide in heart contains such a ryanodine binding domain comparable to that of skeletal muscle. The target molecular weight for ryanodine binding reflects the size of the smallest domain which retains activity (smaller than the protomer), whereas measurements of polypeptide remaining appear to reflect oligomeric association of the HMW polypeptide. In this regard, the structural similarity of the receptors from heart and skeletal muscle is especially noteworthy.

The sarcoplasmic reticulum calcium release channels in heart and skeletal muscle are similar despite apparent important differences in the nature of the excitation–contraction coupling and reported differences in conductance and sensitivity to ligands (Smith et al., 1986; Rousseau et al., 1986; Hymel et al., 1988b) and different immunological cross-reactivity (M Inui and S. Fleischer, unpublished studies). The channel from both heart and skeletal muscle consists of an oligomer of a unique high molecular weight polypeptide which is activated by specific ligands such as Ca^{2+}, ATP, and ryanodine and inhibited by ruthenium red and Mg^{2+} in both tissues. We now report that the target size of the calcium release channel of sarcoplasmic reticulum is structurally similar in heart and skeletal muscle as studied by target inactivation analysis.

Acknowledgments

We are pleased to acknowledge the helpful advice of Dr. J. Oliver McIntyre of this laboratory and Dr. Charles Berenski for carrying out the irradiation.

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Reversed Hexagonal Phase Formation in Lecithin–Alkane–Water Systems with Different Acyl Chain Unsaturation and Alkane Length†

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Received April 12, 1988; Revised Manuscript Received July 29, 1988

ABSTRACT: Investigations of lipid–alkane systems are important for an understanding of the interactions between lipids and hydrophobic/amphiphilic peptides or other hydrophobic biological molecules. A study of the formation of nonlamellar phases in several phosphatidylcholine (PC)–alkane–^2H_2O systems has been performed. The PC molecules chosen in this work are dipalmitoyl-PC (DPPC), 1-palmitoyl-2-oleoyl-PC (POPC), dioleoyl-PC (DOPC), and dilinoleoyl-PC (DLiPC), lipids that in excess water form just a lamellar liquid–crystalline phase up to at least 90 °C. The addition of *n*-alkanes (C_8–C_{20}) to these PC–^2H_2O systems induces the formation of reversed hexagonal (H_{II}) and isotropic phases. The water and dodecane concentrations required to form these phases depend on the degree of acyl chain unsaturation of the PC molecules and increase in the order DLiPC ≈ DOPC < POPC < DPPC. The most likely explanation to this result is that the diameter of the lipid–water cylinders in the H_{II} phase grows gradually larger with increased acyl chain saturation and more water and dodecane are consequently needed to fill the water cylinders and the void volumes between the cylinders, respectively. The ability of the alkanes to promote the formation of an H_{II} phase is strongly chain length dependent. Although the number of alkane carbon atoms added per DOPC molecule in the DOPC–*n*-alkane–^2H_2O mixtures was kept constant, this ability decreased on going from octane to eicosane. The thermal history of a DPPC–*n*-dodecane–^2H_2O sample was important for its phase behavior. A large fraction of the sample formed an isotropic phase at 65 °C when heated at 10 °C/24 h, while an H_{II} phase dominated at this temperature when the sample was heated at 10 °C/h. The results obtained are consistent with the theories presented by Gruner [Gruner, S. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3655–3669] and Siegel [Siegel, D. P. (1986) *Chem. Phys. Lipids 42*, 279–301].

Studies of phase transitions between lamellar and nonlamellar phases in biological membrane lipid–water systems are important for several reasons: (1) Such transitions are induced by a number of biologically relevant factors (Rilfors et al., 1984). (2) Most biological membranes contain at least one lipid species (Ansell et al., 1973; Goldfine, 1982) which does not form a lamellar phase under physiological conditions. Moreover, the balance between lipids forming a lamellar phase and lipids forming a nonlamellar phase is metabolically regulated in some bacterial membranes (Wieslander et al., 1980; Lindblom et al., 1986; Goldfine et al., 1987). (3) Many processes are associated with biological membranes during which the lipid bilayer structure most probably is transiently disrupted (e.g., fusion and exo- and endocytosis) (Lucy, 1970; Rilfors et al., 1984; Cullis et al., 1985; Bentz & Ellens, 1988). (4) The activity of some membrane-bound enzymes or protein assemblies is greatly enhanced in the presence of lipids forming nonbilayer structures (Jensen & Schutzbach, 1984; Navarro et al., 1984; Cheng & Hui, 1986; Siefermann-Harms et al., 1987; Pick et al., 1987).

Some investigations have been performed with phosphatidylethanolamine (PE)[1]–*n*-alkane–H_2O, phosphatidylcholine (PC)–*n*-alkane–H_2O, and PE–PC–*n*-alkane–H_2O systems to elucidate the principles underlying the transition from a la-

† This work was supported by the Swedish Natural Science Research Council and the Foundations of Carl Trygger and Magnus Bergvall.

[1] Abbreviations: L_α, lamellar liquid-crystalline phase; H_{II}, reversed hexagonal phase; DO, dioleoyl; PO, 1-palmitoyl-2-oleoyl; DP, dipalmitoyl; DLi, dilinoleoyl; PC, phosphatidylcholine; PE, phosphatidylethanolamine; T_{LH}, temperature interval for the transition from an L_α to an H_{II} phase.

0006-2960/89/0428-1323$01.50/0