6. SUMMARY

The basic structure of all the biological membranes, including the plasma membrane and the internal membranes of eukaryotic cells, possesses a common construct, which is comprised of macromolecular aggregates of amphipathic lipids and proteins held together by virtue of the hydrophobic force. The amphipathic lipid components are arranged as a continuous bilayer, 40-50 Å in thickness. The three classes of lipids in the cell membranes, phospholipids (which comprise the majority), neutral lipids (mainly cholesterol) and glycolipids (largely glycosphingolipids), usually constitute almost half the plasma membrane mass.

Phospholipid molecules within the membrane have got a characteristic feature in which the polar head-group lies parallel to the plane of the bilayer and the both phospholipid acyl chains extend into the membrane bilayer and form its core. The first two carbon atoms of the *sn*-2 acyl chain lie parallel to the surface. therefore, for a phospholipid with two identical fatty acyl groups, the *sn*-1 chain extends further into the bilayer than does the *sn*-2 chain.

In model membranes, a lipid phase transition is observed, in which the phospholipid acyl chains change from an ordered (gel) to a more ordered (liquid crystalline) state. In the gel state, acyl chain orientation is generally tilted to the plane of the membrane, but above the liquid crystalline state, the acyl chains lie perpendicular to the surface. A number of studies have been performed to understand determine the

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structure and dynamics of membrane bilayer phospholipids to understand their functional and structural roles in biomembranes. These molecules in crystals, aggregated form or solution have preferred conformation which seems to be determined primarily by the glycerol backbone. Further, the preferred conformation of the glycerol backbone has been shown to remain unaffected by the length of the acyl chains in phosphatidylcholines. The role of the acyl chain stacking in determining the preferred conformation of the glycerol backbone in phosphatidylcholines, have been analyzed by using modified phospholipid analogs wherein the glycerol moiety has been replaced by the butanetriol residue such that the two acyl chains having 1,2 and 1,3 arrangement. The interface structure of the phosphatidylcholine bilayers is not affected by introducing one methylene residue between C2 and C3 carbons, but it gets altered if a similar chemical change is introduced between the carbons that carry the two acyl chains (DPBPC). High resolution ^{1}H and ^{13}C-NMR studies on the racemic form of DPBPC have shown that the conformational preference around the butanetriol C2-C3 bond in this phospholipid is almost similar to that observed for the glycerol C1-C2 bond in DPPC (Arora and Gupta, 1997a). Based on this observation, it has been suggested that structurally, the C2 methylene residue in the butanetriol backbone of the phospholipid analog represents the proximal beginning of the primary acyl chain (Arora and Gupta, 1997a). Since the preferred conformation of phosphatidylcholines in micelles or bilayers is such that *sn*-2 ester group aligns at the micelle (or bilayer) interface while the *sn*-1 ester group remains almost perpendicular to the *sn*-2 ester region (Seelig ans Seelig, 1980; Pascher, 1992), introduction of an additional

methylene residue between the glycerol C1 and C2 carbon atoms in DPPC should lead to an altered phospholipid structure in the interface region due to further pushing of the *sn*-1 ester group in the hydrophobic interior of the micelles. To investigate the specificity of the glycerol backbone or the interface region of the phospholipids in various phospholipid-protein interactions, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine analogs hving 1,3,4-butanetriol residue, instead of glycerol moiety, were synthesized, and studied their interactions with *Naja naja* phospholipase A_2, aminophospholipid translocases and human apolipoprotein A-II.

Phospholipases A_2 catalyzes the hydrolysis of the fatty acyl group at the *sn-2 position of membrane phospholipids in a* Ca^{2+} dependent manner. The binding of phospholipase A_2 with phosphatidylcholines involves interactions of the enzyme active site calcium with the phospholipid phosphate oxygen and the *sn*-2 carbonyl oxygen followed by the hydrophobic interactions between the phospholipid fatty acyl chains and the enzyme catalytic site (Arni and Ward, 1996; Dennis, 1983; Scott et. al., 1990; White et. al., 1990). Also, the increase in the hydrophobicity of the *sn*-1 acyl residue increases the affinity between the phospholipid molecule and the enzyme (Yu et. al., 1990). However, the role of the *sn*-1 ester moiety (-CO-O-) of the acyl chain in the phospholipase A_2-glycerophospholipid interactions is not yet shown.

To find out whether the *sn*-1 ester moiety plays any role in the phospholipase A_2-glycerophospholipid interactions, the hydrolysis of the secondary ester group in

both phosphatidylcholines and their butanetriol-containing analogs by *Naja naja* phospholipase A_2 was carried out by employing the phospholipids that contained NBD-labeled secondary acyl chain. NBD-labeled phosphatidylcholine analogs (fig. 18, 19) viz. C_6-NBD-PC, C_6-NBD-bPC, C_{12}-NBD-PC and C_{12}-NBD-bPC were synthesized, purified and characterized. Time and concentration-dependent hydrolysis of these phosphatidylcholine analogs by *Naja naja* phospholipase A_2 showed that under identical conditions the hydrolysis of glycerophospholipid analogs (C_6-NBD-PC and C_{12}-NBD-PC) was higher than that of the corresponding butanetriol-containing phospatidylcholine analogs (C_6-NBD-bPC and C_{12}-NBD-bPC) (fig. 22). Similar results were obtained even at saturated concentration of phospholipase A_2 (fig. 23).

The further analysis of the problem was done by studying the phospholipid concentration-dependent hydrolysis of C_{12}-NBD-PC and C_{12}-NBD-bPC (Fig. 24). The dissociation constant, K_s , of C_{12}-NBD-bPC was almost 7 times higher than that of C_{12}-NBD-PC, indicating the lesser binding of C_{12}-NBD-bPC to phospholipase A_2 than that of C_{12}-NBD-PC. Also, the value of V_{max}, in case of C_{12}-NBD-PC was almost 6 times higher as compared to that of C_{12}-NBD-bPC. This proved that C_{12}-NBD-PC is a much better substrate for phospholipase A_2 than C_{12}-NBD-bPC. It was then confirmed by measuring the change in protein tryptophan fluorescence with varying concentrations was measured. The tryptophan fluorescence was measured after adding varying concentrations of mixed micelles of DPPC or DPBPC with octylglucoside to phospholipase A_2 (fig. 26).Nearly 2.6 times higher

concentration of DPBPC was required to reach the half maximal binding as compared to DPPC, suggesting that the affinity of diacyl glycerophospholipids (DPPC) is higher than that of butanetriol-containing phospholipids (DPBPC).

In order to ascertain whether butanetriol-containing phosphatidylcholine analogs could competitively inhibit the phospholipase A_2-catalyzed hydrolysis of phosphatidylcholines, the effects of DPPC and DPBPC on the C_{12}-NBD-PC hydrolysis were analyzed (fig. 27). The hydrolysis of C_{12}-NBD-PC was not much affected by mixing increasing quantities of DPPC, but it was appreciably decreased when it was mixed with increasing quantities of DPBPC. The results were interpreted to suggest that the C1 eater moiety plays a crucial role in the phospholipase A_2-phospholipid interactions.

The ability of phospholipids to move across the two leaflets of the membrane bilayer is important in establishment and maintenance of the phospholipid asymmetry in biological membranes. The out-to-in translocation of aminophospholipids is ATP dependent and protein mediated (Daleke & Huestis, 1985; Seigneuret & Devaux, 1984; Tilley et al., 1986). It has been reported that aminophospholipid translocase recognizes not only the amino group on the lipid polar moiety but also the glycerol and the ester bonds (Morrot et al., 1989). To investigate the role of glycerol backbone in aminophospholipids–translocase interactions, fluorescently labeled butanetriol-containing phospholipid analogs viz. C_6-NBD-bPC, C_6-NBD-bPE, C_6-NBD-bPS, C_{12}-NBD-bPC, C_{12}-NBD-bPE and C_{12}-NBD-bPS, and their corresponding glycerophospholipid analogs, viz. C_6-NBD-PC, C_6-NBD-PE, C_6-NBD-PS, C_{12}-NBD-

PC, C_{12}-NBD-PE and C_{12}-NBD-PS (fig. 18, 19), were synthesized and their out-to-in translocation in the human erythrocyte membrane was studied. The out-to-in movements of phospholipids was monitored by incorporating the NBD-labeled phospholipids in the human erythrocytes and then 'back exchanging' the outer surface-incorporated phospholipids using bovine serum albumin. No significant difference was observed in the extents of out-to-in movements of glycerol and butanetriol-containing phospholipid analogs of PC, PE and PS (fig. 28-30). Almost similar results were obtained in out-to-in translocation of the two types of phospholipids even when human erythrocytes were depleted of ATP (fig. 31). This study demonstrates that replacement of the glycerol residue in PE and PS by the 1,3,4-butanetriol moiety does not effect the ATP-dependent out-to-in translocation of these phospholipids in the human erythrocyte membrane. Further, it suggests that this translocation besides requiring the specific phospholipid polar head-group, may also require specific phospholipid backbone conformation.

HDL is a heterogeneous macromolecule containing equal proportions of protein and lipid and small amount of carbohydrate. The phospholipid binding site or sites of apoA-II are localized in the carboxyl-terminal portion of the molecule (Chen et. al., 1979; Mao et. al., 1981). Extensive studies of the structural and lipid binding properties of human apoA-II and several of its derivative fragments have been carried out and on the basis of the space filling models the amino acid sequences that associates with the phospholipids have been suggested (Benetollo et. al., 1996; Mao et. al., 1977; Segrest et. al., 1974). To further analyze the specificity of apoA-II–phopholipid interaction, the

binding of this protein with phosphatidylcholine analogs, DPPC and DPBPC, have been studied. The interactions of apoA-II with both PC (DPPC) and modified PC (DPBPC) were monitored by microcalorimetric analysis and protein tyrosine fluorescence measurements. It was found out that the binding of DPPC with apoA-II is biphasic (fig. 32, 33). The tyrosine fluorescence remained unchanged upon addition of DPBPC, palmitic acid and 1,2-diacyl glycerol (fig. 34A). Also, the binding of DPPC with apoA-II was not affected by the presence of palmitic acid, 1,2-diacyl glycerol and DPBPC (fig. 34B). Moreover, the role of the ionic interactions was also confirmed by measuring the binding of DPPC in the presence of NaCl with respect to the increase in tyrosine fluorescence (fig. 35). On the basis of these studies, it was concluded that the replacement of the glycerol backbone in DPPC by the 1,3,4-butanetriol residue leads to an altered phospholipid interaction with human apoA-II and that apoA-II contains a unique binding site for phosphatidylcholines.