The molecular structure of phospholipids and the regulation of cell function


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The effects of phospholipid structure on the function of a calcium pump

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Introduction

All biological membranes contain a complex mixture of lipids. To what extent is this complexity required for the proper functioning of the membrane? One role of the lipids is to provide a suitable support for the proteins in the membrane; the activity of an intrinsic membrane protein, embedded in the lipid bilayer, might then be expected to be sensitive to at least some features of lipid structure. We have been exploring this possibility for the Ca2+-ATPase purified from skeletal muscle sarcoplasmic reticulum (SR). The Ca2+-ATPase is particularly suitable for such studies because it is both an enzyme and a transport protein; it is therefore possible to study the effects of lipid structure on both the scalar process of ATP hydrolysis and the vectorial process of Ca2+ transport. The purified ATPase can be mixed with phospholipid in a detergent, such as cholate, followed by dilution of the mixture into buffer (so that the detergent concentration drops below its critical micellar concentration, or cmc), to give membrane fragments in which the ATPase is surrounded by the phospholipid of choice; because the membrane is present as fragments, the rate of ATP hydrolysis can be measured in this system, independently of any accumulation of Ca2+. The ATPase can also be reconstituted into sealed phospholipid vesicles, allowing the Ca2+ transport process to be studied. Sealed vesicles can be produced by mixing the ATPase and lipid in cholate as detergent, followed by slow detergent removal, either

Abbreviations used: BrPC, dibromostearoylphosphatidyleroline; dibromocholesterol, 5,6-dibromocholestan-3β-ol; di(C14:1)PC, dimyristoylphosphatidylcholine; di(C16:0)PC, dioleoylphosphatidylcholine; di(C18:2)PC, dielueroylphosphatidylcholine; di(C18:3)PA, dioleoylphosphatidic acid; SR, sarcoplasmic reticulum.
by dialysis or on a column of Sephadex; these methods give relatively small vesicles, about 60 nm in diameter [1]. An alternative procedure in which the ATPase is dissolved in the detergent octa(ethylene glycol) dodecyl monoether and then mixed with giant liposomes that have been treated with a small concentration of octyl β-D-glucopyranoside produces larger vesicles showing higher levels of uptake [2]; diameters of these vesicles are typically about 90 nm.

**Effects of phospholipid structure on ATPase activity**

The major species of phospholipid found in the SR membrane is the bilayer-favouring, zwitterionic phosphatidylcholine. However, SR membranes also contain phosphatidylethanolamines, which favour a non-bilayer structure, and a variety of anionic phospholipids including phosphatidylserine and the phosphatidylinositols. Studies in which the ATPase is reconstituted into membrane fragments have defined the effects of phospholipid structure on ATPase activity. ATPase activity is highest in bilayers of dioleoylphosphatidylcholine [di(C₁₄:₁)PC] and activities are low in bilayers with shorter (C₁₂:₀) or longer (C₁₈:₀) fatty acyl chain length, and thus bilayer thickness, is important for the proper functioning of the ATPase [3-5]. Activities in bilayers of phosphatidylethanolamine are the same as in phosphatidylcholine so that the exact structure of a zwitterionic phospholipid headgroup appears not to be important [6]. However, activities are low in bilayers of phosphatidylserine or phosphatidic acid so that a negatively charged headgroup supports low activity [7]. Activities in gel phase lipid are very low, and activities in phosphatidylethanolamines under conditions where the phosphatidylethanolamine is in the hexagonal HII phase are also low [6,8,9]. Thus, a liquid crystalline bilayer of the appropriate thickness is required for high activity; these experiments provide no evidence that the exact phospholipid composition of the native SR membrane is in any way special, at least as far as ATPase activity is concerned.

We have also shown that there is no one common mechanism that explains the effects of all phospholipids on the function of the ATPase; the activity observed in a bilayer of any one particular phospholipid depends on the unique conformational state of the ATPase in that particular bilayer. The observed changes in activity for the ATPase can be interpreted in terms of the reaction scheme for the ATPase shown in Figure 1. The scheme proposes that two Ca²⁺ ions bind in a cooperative fashion to the E1 conformation of the ATPase, from the cytoplasmic side of the membrane [10-12]. Following binding of MgATP to the ATPase, the ATPase is phosphorylated and undergoes a change in conformation to a state in which the two Ca²⁺ binding sites are of low affinity and inward facing (E₂PC₄). Ca²⁺ is lost from this phosphorylated intermediate to the lumen of the SR. Dephosphorylation of E₂P then allows recycling to E₁ [13,14]. All of these steps can be affected by phospholipid structure. In short- (C₁₂:₀) or long- (C₁₄:₀) chain phosphatidylcholines, the stoichiometry of Ca²⁺ binding changes from the usual two Ca²⁺ ions bound per ATPase molecule to one Ca³⁺ ion bound [3,15]. In dimyristoylphosphatidylcholine [di(C₁₄:₀)PC] the rate of phosphorylation of the ATPase by ATP decreases, the rate of dephosphorylation of E₂P decreases and the E₁/E₂ equilibrium shifts towards E₁ [3,16-18]. In gel phase lipid the rate of phosphorylation becomes very low [9] and in hexagonal HII phase lipid, although the rate of phosphorylation is normal, the rate of dephosphorylation becomes low [6]. The effects of chain length on the stoichiometry of Ca²⁺ binding and on the rate of dephosphorylation are distinct; for example, in dierucylphosphatidylcholine [di(C₂₀:₁)PC] the stoichiometry of Ca³⁺ binding is normal, but the rate of dephosphorylation is decreased [19] and similarly in gel or hexagonal phase lipid, Ca²⁺ binding is normal, despite effects on the rates of phosphorylation or dephosphorylation.

An effect of bilayer thickness on Ca²⁺ binding is, perhaps, not surprising since the Ca²⁺ binding sites on the ATPase are located between transmembrane α-helices embedded in the lipid bilayer (Figure 1). Since Ca²⁺ ions bind to the ATPase at binding sites made up by helices M4, M5, M6, and M8 [20], transport of Ca²⁺ across the membrane must involve changes in the packing of these helices. Changes in the stoichiometry of Ca²⁺ binding to the ATPase as a result of changes in the thickness of the lipid bilayer must also reflect changes in the packing of the helices making up the Ca²⁺ binding sites. What is surprising is that Ca²⁺ binding is unaffected by lipid head-group structure [7] or by a lipid phase transition from the liquid crystalline into the gel or hexagonal HII phases, whereas phos-
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Phosphorylation or dephosphorylation are affected by these changes [6,9].

**Effects of phospholipid structure and cholesterol on transmembrane α-helices**

Phosphorylation and dephosphorylation of the Ca\(^{2+}\)-ATPase occur on the cytoplasmic part of the ATPase, at a site located a considerable distance above the bilayer surface [21,22]. Since the transmembrane α-helices of the Ca\(^{2+}\)-ATPase are the parts embedded in the lipid bilayer, effects of changes in the lipid bilayer must be transmitted to the phosphorylation and nucleotide binding domains via changes in the packing of the transmembrane α-helices. Some of these changes, at least, can be explored using simple model transmembrane α-helices, incorporated into bilayers of defined composition. We have synthesized a series of peptides of the type acetyl (Ac)-Lys-Gly-Leu-Trp-Leu-Lys-Aa-amide (P<sub>n+10</sub>) containing a central Trp residue to act as a fluorescence reporter group and a pair of Lys residues at each end to anchor the peptide across the lipid bilayer [23]. The peptides have been incorporated into bilayers of phosphatidylincholines with chain lengths between C\(_{14}\) and C\(_{24}\) by mixing peptide and excess phospholipid in organic solvent, removing the solvent, and hydrating the mixture to give a bilayer containing the peptide. The peptide P\(_{22} (m = 10, n = 12)\) incorporates into all bilayers but P\(_{16} (m = 7, n = 9)\), although it incorporates into bilayers of chain length C\(_{20}\) or less, only partly incorporates into bilayers where the chain length is C\(_{22}\) and does not incorporate at all into bilayers when the fatty acyl chain length is C\(_{24}\). This difference

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**Figure 1**

The mechanism of the Ca\(^{2+}\)-ATPase

The scalar hydrolysis of one ATP molecule is linked to the vectorial transport of two Ca\(^{2+}\) ions across the membrane. The nucleotide binding domain to which ATP binds, and the phosphorylation domain containing the Asp-351 residue phosphorylated by the ATPase, are located in the cytoplasmic region of the ATPase, whereas the two Ca\(^{2+}\) binding sites are located in the transmembrane region. In the reaction scheme, the E1 conformation of the ATPase has two Ca\(^{2+}\) binding sites of high affinity exposed to the cytoplasm. Following the binding of Ca\(^{2+}\) and ATP, the ATPase becomes phosphorylated and undergoes a conformational change to E2PCa\(_{2}\), containing two low-affinity binding sites for Ca\(^{2+}\), exposed to the lumen of the SR. Following the loss of Ca\(^{2+}\) from E2PCa\(_{2}\), the ATPase can dephosphorylate and recycle to E1. In an alternative, 'leak' pathway, E2PCa\(_{2}\) can dephosphorylate, losing Ca\(^{2+}\) to the cytoplasmic side of the membrane.
between the properties of the long and short peptides follows because a too-long peptide can be matched to a too-thin bilayer both by stretching or compressing the lipid and by tilting the peptide. However, a too-thin peptide can only be matched to a too-thick bilayer by compression of the lipid, which becomes energetically unfavourable when the difference between the bilayer thickness and the peptide length (the hydrophobic mismatch) exceeds about 10 Å [23].

In the region of hydrophobic mismatch where the peptide does incorporate into the bilayer, there will be an energetic cost associated with stretching or compressing the lipid fatty acyl chains, which will be reflected in values of relative lipid binding constants. These can be determined by studying tryptophan fluorescence intensities for the peptide in bilayers containing mixtures of normal phospholipid and a phosphatidylycholine in which some of the C–H bonds in the fatty acyl chains have been replaced by C–Br bonds; brominated phospholipids can be made readily by bromination of a lipid such as di(C18:1)PC-containing unsaturated fatty acyl chains [24]. Quenching of tryptophan fluorescence is static and short range; the extent of quenching is therefore dependent on the proportion of the lipid ‘sites’ around the peptide that are occupied by brominated lipid. Equilibrium in the system can be considered as a series of displacement reactions at each of these ‘sites’ of the following type:

$\text{Peptide}\cdot\text{BrPC} + \text{di(C18:1)PC} \rightleftharpoons \text{Peptide}\cdot\text{di(C18:1)PC} + \text{BrPC}$

where BrPC is the brominated phospholipid di-bromostearoylphosphatidylcholine. Fluorescence quenching in mixtures of BrPC and non-brominated lipid can be fitted to the equation:

$$F/F_0 = F_{\text{min}} + (F_0 - F_{\text{min}}) (1 - f_{\text{br}})^n$$

where $F_0$ and $F_{\text{min}}$ are the fluorescence intensities for the peptide in lipid and in BrPC respectively, $F$ is the fluorescence intensity in the phospholipid mixture when the mole fraction of BrPC is $x_{\text{Br}}$, and the fraction of sites at the lipid–peptide interface occupied by BrPC is $f_{\text{br}}$, and $n$ represents the number of lipid sites making contact with a tryptophan residue [24]. The fraction of sites occupied by BrPC is related to $x_{\text{Br}}$ by:

$$f_{\text{br}} = x_{\text{Br}}/(x_{\text{Br}} + K[1 - x_{\text{Br}}])$$

where $K$ is the relative binding constant of BrPC with respect to the non-brominated phospholipid [25]. Fluorescence quenching of the peptides in mixtures of BrPC and di(C18:1)PC fits to these equations with a value of $n$ of about 2.5, suggesting that about 2.5 phospholipid molecules, or five fatty acyl chains, can make contact with a tryptophan residue; this would be consistent with an approximately hexagonal packing arrangement in the bilayer. Strongest binding of lipid to peptide P16 is observed for di(C18:1)PC whereas for peptide P22, strongest binding is observed with di(C20:1)PC; in both cases the strongest binding lipid gives a bilayer thickness that matches the hydrophobic length of the peptide. However, effects of a too-thin bilayer are relatively small, and level out at 2–2.5-fold reduction in binding constant at a mismatch of about 6 Å [23].

Addition of cholesterol is known to increase the thickness of a lipid bilayer in the liquid crystalline phase [26], and addition of cholesterol can have a major effect on the incorporation of the peptides into phospholipid bilayers. Effects of cholesterol on incorporation of P16 into bilayers of di(C14:1)PC or di(C18:1)PC are relatively small but for P16 in di(C18:1)PC the effect of cholesterol is very much more marked, with a very low level of incorporation at a 1:1 molar ratio of cholesterol to phospholipid [23]. Thus, although di(C16:1)PC is the optimal phospholipid for incorporation of P16 in the absence of cholesterol, in the presence of cholesterol the bilayer becomes too thick so that P16 is unable to incorporate at all. An effective increase in chain length is also suggested by fluorescence quenching experiments which show that, in the presence of cholesterol, the relative binding constant of di(C16:1)PC to P16 increases from 0.4 to about 1, as expected if the presence of cholesterol increases the effective chain length of the C18 chain so that it more nearly matches the hydrophobic length of the peptide [23].

We can also use these fluorescence quenching methods to study interactions between the peptide and cholesterol in the membrane (S. Ma, R. J. Webb and A. G. Lee, unpublished work). Figure 2(B) shows that the tryptophan fluorescence emission of the peptide P22 is quenched by 5,6-dibromocholesterol-3β-ol (dibromocholesterol) in bilayers of di(C18:1)PC so that dibromocholesterol must be able to interact with the peptide. Conversely, cholesterol can displace BrPC from around the peptide (as shown by an increase in fluorescence intensity; Figure 2A), again showing that cholesterol can interact with the peptide. The experimental quenching curves
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for P22 in BrPC/cholesterol mixtures can be fitted to equations (1) and (2) with \( n = 2.3 \) and a relative binding constant \([\text{peptide.cholesterol}]/[\text{peptide}]\) of 0.7. This same relative binding constant also fits the data for P22 in di(C18,1)PC/brominated cholesterol mixtures, assuming a value of \( n \) for brominated cholesterol of 5.3, suggesting a hexagonal packing arrangement for cholesterol around the peptide, as suggested above for the phospholipid fatty acyl chains. The observation of a relative binding constant only slightly less than 1 shows that the interaction between peptide and cholesterol is only slightly less favourable than that between peptide and phospholipid. In other studies, we have shown that cholesterol binds relatively weakly at the lipid–protein interface of the ATPase [25,27]; comparison with the peptide studies reported here suggests that weak binding of cholesterol to the ATPase is the result of interactions in the lipid headgroup region, rather than interactions between the sterol ring and the hydrophobic transmembrane \( \alpha \)-helices.

These studies therefore suggest that the activity of the Ca\(^{2+}\)-ATPase is sensitive to lipid bilayer thickness because of tilting of the transmembrane \( \alpha \)-helices to match the thickness of the bilayer. Changes in the packing of helices M4, M5, M6 and M8 could lead to changes in the stoichiometry of Ca\(^{2+}\) binding. Changes in the packing of the other helices in response to changes in bilayer phase or lipid headgroup structure could transmit changes to the nucleotide binding and phosphorylation domains, leaving Ca\(^{2+}\) binding unchanged.

**Effects of lipid structure on accumulation of Ca\(^{2+}\)**

The ATPase can be reconstituted into sealed vesicles, allowing measurements of ATP-driven accumulation of Ca\(^{2+}\). Surprisingly, the level of accumulation of Ca\(^{2+}\) into vesicles of di(C18,1)PC, the phospholipid supporting highest ATPase activity, is low (Figure 3). The level of accumulation is increased significantly in the presence of 10 mol% dioleoylphosphatidic acid [di(C18,1)PA] or PtdIns, levels of anionic lipid higher than 10 mol% having no additional effect. Effects of

**Figure 2**

Fluorescence intensities for peptide P22 in mixtures containing BrPC and cholesterol (A) or dibromocholesterol and di(C18,1)PC (B), as a function of the mole fraction of BrPC (A) or dibromocholesterol (B).

P22 was incorporated into mixtures of phosphatidylycholine and cholesterol at a molar ratio of P22/phosphatidylycholine of 1:100. Fluorescence intensities are expressed as a fraction of that in a mixture of di(C18,1)PC and cholesterol. The solid line in (A) shows the best fit to the data with \( n \) fixed at 2.3, with a relative binding constant \( K \) of 0.7. The dotted lines show that range of \( K \) values (0.5–0.8) that bracket the experimental data. The solid line in (B) shows the best fit to the data with \( K \) fixed at 0.7, with an \( n \) value of 5.3. The dotted lines show the range of \( n \) values (4.2–7.2) that bracket the experimental data.

**Figure 3**

Accumulation of Ca\(^{2+}\) by the Ca\(^{2+}\)-ATPase reconstituted into sealed vesicles

The solid lines show the experimental data for di(C18,1)PC (a), di(C18,1)PC/10% di(C18,1)PA and di(C18,1)PC/10% PtdIns (b), and di(C18,1)PC/10% PtdIns4P (c). The dotted lines show simulations of the experimental data with the parameters described in the text. The upper broken line (d) shows a simulation with zero leak rate. The lipid/ATPase ratio was 40:1 (w/w), and the medium was 10 mM PIPES, pH 7.1, 100 mM K\(_2\)SO\(_4\), 5 mM Mg\(^{2+}\), 120 \( \mu \)M Ca\(^{2+}\), 8 \( \mu \)M antipyrpylazo III, 25°C. The sample contained 1.6 mg lipid/ml, and uptake was initiated by addition of 0.8 mM ATP.
PtdIns4P are more marked than those of PtdIns (Figure 3). At low concentrations, anionic phospholipids such as di(C18,1)PA or PtdIns have no effect on ATPase activity, and at higher concentrations they actually reduce activity [7], so that the effects on accumulation of Ca\(^{2+}\) are unexpected. As described elsewhere [28] these effects of anionic lipid are seen in the presence of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (to equilibrate H\(^{+}\) across the membrane), and any effects of valinomycin and K\(^{+}\) (to collapse membrane potentials) are very small. However, the presence of phosphate trapped within the vesicles does lead to increased levels of accumulation of Ca\(^{2+}\), suggesting that there is a significant rate of Ca\(^{2+}\) leak from the vesicles; effects of anionic phospholipids on the levels of accumulation of Ca\(^{2+}\) are therefore likely to follow from effects on the rate of leak.

Simulations have been performed according to the model shown in Figure 1. The pattern of accumulation of Ca\(^{2+}\) cannot be reproduced if it is assumed that Ca\(^{2+}\) leak follows a simple, passive pathway - in a simple leak model, Ca\(^{2+}\) accumulation increases almost linearly to a maximal level, determined by the balance between the rate of uptake and the rate of leak and this is very unlike the pattern of Ca\(^{2+}\) accumulation observed experimentally (Figure 3). However, the pattern of Ca\(^{2+}\) accumulation can be reproduced if it is assumed that the Ca\(_{2}\)E2P intermediate, as well as releasing Ca\(^{2+}\) to the lumen of the vesicles giving E2P, can follow an alternative pathway, releasing Ca\(^{2+}\) to the external medium and forming E2. The experimental data have been reproduced well assuming rates for this step of 400 s\(^{-1}\) in di(C18,1)PC, 150 s\(^{-1}\) in di(C18,1)PC/10% di(C18,1)PA or di(C18,1)PC/10% PtdIns, and 60 s\(^{-1}\) in di(C18,1)PC/10% PtdIns4P, with an affinity of E2P for Ca\(^{2+}\) of 3 mM. The observation that the observed time course of accumulation of Ca\(^{2+}\) can only be reproduced through a leak pathway involving a phosphorylated intermediate of the ATPase shows that leak cannot be attributed to the presence of denatured ATPase or to ATPase with the incorrect orientation in the vesicles (since ATPase molecules oriented with their ATP binding sites facing the lumen will not be able to react with ATP in sealed vesicles). Thus the leak pathway is an intrinsic property of the ATPase, and the observation that the extent of leak is reduced at the level of anionic phospholipid actually found in the native SR membrane suggests that this could be the role for the anionic phospholipids in the native membrane.

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Why is docosahexaenoic acid essential for nervous system function?

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Introduction

It has long been known that the nervous system accumulates very high concentrations of highly unsaturated phospholipids including species containing docosahexaenoic acid (C22:6,n-3; DHA) and, to a lesser extent, arachidonate (C20:4,n-6; AA) [1,2]. DHA is found primarily in the brain on the sn-2 position of the aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) [3,4] in neural membranes with little in myelin [2]. However, the retina is rich in phospholipid species with both fatty acids containing 4–6 double bonds and di-DHA species occurring in significant amounts [5]. DHA is tenaciously retained once accumulated and can survive a prolonged fat-free diet, and the levels may actually increase when expressed as a percentage of the total phospholipid during starvation or sperm ejaculation, situations where other lipid is metabolized, presumably for energy [6]. Many studies have shown that the period of early development is a time when the nervous system must be provided with adequate sources of dietary n-3 fatty acids in order that 'normal'

Accretion may take place as the neural membranes are formed.

Evidence of a more direct nature has accumulated more recently, suggesting suboptimal nervous system development and function where long-chain polyunsaturates such as DHA are not provided [6-9]. For example, Birch et al. in a double-blind, randomized trial found that a fish oil-supplemented formula led to greater visual acuity in premature infants relative to those fed vegetable oil-based formulae [10]. Carlson et al. found an improved score in Bayley’s Mental Developmental Index in premature infants at 12 months of age when they were fed formula containing marine oil (principally DHA) from birth only until 2 months past term [11]. Werkman and Carlson also observed a shorter look-duration to novel and familiar stimuli in supplemented infants; this was associated with more rapid information processing [12]. Animal studies have also supported these findings as a variety of behavioural features are altered by a low α-linolenic acid (C18:3,n-3; LNA) diet relative to one containing primarily linoleic acid (C18:2,n-6) as the source of essential fatty acid [9,13,14].

The remarkable observation in these studies of nervous system function is that there is a reciprocal replacement of DHA with docosapentaenoic acid (C22:5,n-6; DPA,n-6) [15-17] since there is n-6 fatty acid deficiency. Since the n-6 replacement for DHA is also a C22 molecule and is highly unsaturated with five double bonds, the contribution of the behavioural effects to the loss of a single double bond would seem to follow. This is a rather remarkable hypothesis that may be unparalleled in modern neuroscience! An alternative hypothesis is that DPA,n-6 can substitute for DHA but is provided in insufficient

Abbreviations used: DHA, docosahexaenoic acid, C22:6,n-3; AA, arachidonic acid, C20:4,n-6; PE, phosphatidylethanolamine; PS, phosphatidylserine; LNA, α-linolenic acid, C18:3,n-3; DPA,n-6, docosapentaenoic acid, C22:5,n-6; EPA, eicosapentaenoic acid, C20:5,n-3; di-C16:0 PC, 1,2-myristoyl-sn-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; di-C22:6,n-3 PC, 1,2-docosahexaenoyl-sn-glycero-3-phosphocholine; di-C20:4,n-6 PC, 1,2-arachidonoyl-sn-glycero-3-phosphocholine; di-C16:0 PC, 1,2-stearoyl-sn-glycero-3-phosphocholine; MI, metarhodopsin II; Keq, metarhodopsin II–metarhodospin I equilibrium constant; PC, phosphatidylcholine; ROS, retinal rod outer segment.

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