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Effect of cholesterol on the dipole potential of lipid membranes

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Abstract The membrane dipole potential, ψ_d, is an electrical potential difference with a value typically in the range 150 – 350 mV (positive in the membrane interior) which is located in the lipid headgroup region of the membrane, between the linkage of the hydrocarbon chains to the phospholipid glycerol backbone and the adjacent aqueous solution. At its physiological level in animal plasma membranes (up to 50 mol%), cholesterol makes a significant contribution to ψ_d of approximately 65 mV; the rest arising from other lipid components of the membrane, in particular phospholipids. Via its effect on ψ_d, cholesterol may modulate the activity of membrane proteins. This could occur through preferential stabilization of protein conformational states. Based on its effect on ψ_d, cholesterol would be expected to favour protein conformations associated with a small local hydrophobic membrane thickness. Via its membrane condensing effect, which also produces an increase in ψ_d, cholesterol could further modulate interactions of polybasic cytoplasmic extensions of membrane proteins, in particular P-type ATPases, with anionic lipid headgroups on the membrane surface, thus leading to enhanced conformational stabilization effects and changes to ion pumping activity.

Keywords Lipid headgroup; Oxysterols; Lipid packing; Hydrophobic thickness; Ion channels; Ion pumps

1 Introduction

The plasma cell membrane and organellar membranes of all animal cells contain significant amounts of cholesterol, with between 5 and 50 mol% of the total membrane lipid composed of cholesterol [1]. The largest percentages are found in the plasma membrane, between 10 – 30 mol% up to 50 mol% [2]. The membranes of intracellular organelles, in contrast, contain significantly lower percentages, with the endoplasmic reticulum, Golgi apparatus and mitochondrial membranes, all with <10 mol% cholesterol [2]. It is highly likely that these variations in cholesterol composition between different animal membranes, which occur even within a single cell, are related to different functions of the plasma membrane versus organellar membranes and those of the proteins that they contain. In the subsequent volume of this series modulation of protein function by direct interactions between cholesterol and a variety of proteins are discussed. However, cholesterol is known to cause changes in a number of physical properties of lipid membranes, which could, by a variety of mechanisms, indirectly modify protein function. In this chapter we concentrated predominantly on the effect of cholesterol on the membrane dipole potential and how cholesterol-induced changes in dipole potential might modulate membrane protein function.

2 Membrane dipole potential

The membrane dipole potential, ψ_d, is an electrical potential difference which drops across the lipid headgroup region of a lipid bilayer (see Fig. 1), i.e., approximately from the position of the glycerol backbone of phospholipids and the nearest neighbouring aqueous solution (cytoplasm, extracellular fluid or organellar lumen, depending on the membrane concerned). It arises because of the anisotropic structure of a lipid membrane, with the polar lipid headgroups pointing towards the aqueous phase and the hydrocarbon chains pointing towards the centre of the membrane. Because the lipids are anisotropically arranged, then any dipolar groups associated with them (including hydrating water dipoles) must also be anisotropically arranged.

This necessarily gives rise to an electrical potential difference, ψ_d, across the headgroup region of the membrane. The same is true for any other self-assembled molecular colloidal system, e.g. micelles and microemulsions [3, 4]. In the case of lipid membranes, although its origin is not entirely resolved, it appears likely that the major contribution to the magnitude of ψ_d is oriented water dipoles, which hydrogen-bond to the carbonyl oxygen in the ester linkage between the glycerol backbone and the *sn*-2 hydrocarbon chain of ester phospholipids [5-9].

The dipole potential is much less widely known and investigated than the transmembrane electrical potential, $\Delta\psi$, or the surface potential, ψ_s. One reason for this is that $\Delta\psi$ and ψ_s can be relatively easily directly measured or controlled by electrophysiological or electrophoretic techniques. In contrast, the measurement of ψ_d, an electrical potential difference located totally within the membrane and which drops over a distance of not more than 0.5 nm [10], relies mostly on indirect observations, e.g. the effect it has on the transport rates of ions across the membrane or the electronic polarization that it causes to membrane-bound probes [11-13]. However, just because it is difficult to measure doesn't mean that it is unimportant. Depending on the lipid concerned, the magnitude of ψ_d has been estimated to be in the range 100-400 mV, positive in the membrane interior, which would be expected to produce local electric field strengths of 10^8-10^9 V m^{-1} [12], i.e., at least an order of magnitude greater than the field strengths produced by the transmembrane potential, $\Delta\psi$, which is known to be capable of regulating the opening and closing of voltage-sensitive ion channels [14]. Thus, it seems reasonable to expect that ψ_d could play a significant role in modulating the activity of membrane proteins.

The existence of the dipole potential was discovered in 1969 by two Russian scientists, Liberman and Topaly [15]. In studies using the hydrophobic ions tetraphenylborate (TPB^-) and tetraphenylphosphonium (TPP^+) as models to investigate the carrier-mediated mechanism of ion transport, they discovered that TPB^- produced a bilayer phospholipid membrane conductance approximately 10^5 times greater than TPP^+, although the two ions have virtually identical radii and very similar chemical structures. They reasoned that there must, therefore, be a greater energy barrier for the transport of cations across the membrane than anions, i.e.,

dipoles associated with the lipid headgroups and their hydrating water molecules must be arranged so that the positive ends of the dipoles point on average more towards the centre of the membrane, whereas the negative ends point more towards the aqueous phase. Based on the relative magnitudes of the membrane conductances towards these two ions it is possible to estimate a value of ψ_d [6,16,17]. Depending on the lipid concerned, these purely experimental values vary between around 100 and 230 mV. However, as many authors have pointed out [6,16,17], the calculation of these values relies on an assumption that there is no difference in the free energies of hydration of TPB^- and TPP^+, and this assumption is unlikely to be completely true. Indeed, theoretical calculations indicate that TPB^- is more strongly hydrated than TPP^+ [18,19]. Using conductance ratios again, but correcting for the difference in hydration energies, yields values of ψ_d which are at least 60 mV more positive, i.e., in the range 150 – 350 mV [19]. This is in reasonable agreement with values of ψ_d estimated from the change in electrical surface potential produced on spreading a lipid monolayer above an aqueous subphase in a Langmuir trough [20,21].

Before discussing the effect of cholesterol on ψ_d, it is useful to review the factors which have been found to influence the magnitude of ψ_d, because this will help later to explain the origin of changes caused by cholesterol. If the dipole moments of all the molecules within the lipid headgroup region of the membrane were precisely known, then, in principle, ψ_d could be theoretically calculated from the Helmholtz equation for a parallel-plate capacitor:

$$\psi_d = \frac{\mu_\perp}{A\varepsilon_0\varepsilon} \qquad (1)$$

where $\mu_\perp$ is the average component of the lipid molecular dipole moment (including membrane-associated water dipoles) perpendicular to the plane of the membrane, *A* is surface area of the membrane, ε_0 is the permittivity of free space, and ε is the local dielectric constant. From this equation it can be seen that ψ_d is directly proportional to the dipole packing density, ($\mu_\perp$/A). This theoretical prediction has been borne out in experimental results. Thus, an increase in the degree of saturation of phospholipid hydrocarbon chains causes a decrease in ψ_d [22]. *Cis* double bonds produce a larger

drop in ψ_d than *trans* double bonds because a *cis* double bond produces a kink in the hydrocarbon chain, and, thus, causes a greater decrease in lipid packing density than a *trans* double bond. Similarly, the introduction of a heteroatom in the hydrocarbon chain reduces ψ_d [8]. Peterson et al [8] found that the replacement of a CH_2 carbon atom of a hydrocarbon chain by sulfur causes a drop in ψ_d, but that the magnitude of the drop depends on the position of substitution. If the substitution is near the end of the chain, close to the terminal CH_3 group, there is no significant effect on ψ_d, but if the substitution is at the other end of the hydrocarbon chain, close to the headgroup, there is a significant drop in ψ_d. This result seems perfectly logical, since one would expect that a disruption to lipid packing near the headgroup would be magnified along the chain, as the hydrocarbon tails continue to spread towards the centre of the bilayer. A further result strongly supporting the role of lipid packing as an important determinant of the magnitude of ψ_d was obtained by Warshaviak et al [23], who showed that membrane expansion due to the exposure of lipid vesicles to osmotic stress causes a drop in ψ_d.

A further important factor in determining the magnitude of ψ_d is the chemical nature of the linkage between phospholipid hydrocarbon tails and the headgroup. As discussed already, a lipid with an ester linkage, i.e. including a carbonyl group, produces a significantly greater ψ_d than the corresponding lipid with an ether linkage [6,22]. Because the carbonyl bond is itself a dipole, the cause for the difference in ψ_d between ester and ether lipids is most likely not simply due to changes in lipid packing, but rather a change in the component of the average dipole moment perpendicular to the membrane, $\mu_\perp$ (see Eq. 1). Experiments comparing a single-chain ester phospholipid to its corresponding double-chain derivative [9] have shown that the single-chain lipid has a much lower $\mu_\perp$. The likely cause of this difference is the orientation of the carbonyl group relative to the membrane surface. In a single-chain lipid the carbonyl bond is expected to be oriented on average more parallel to the membrane surface, similar to the carbonyl of an *sn*-1 hydrocarbon chain [5,6]. In double-chain ester phospholipids, on the other hand, the carbonyl bond of the extra chain, i.e. the *sn*-2 chain, is expected to be oriented more perpendicular to the membrane, thus contributing to the membrane inside-positive polarity of ψ_d [5,6].

However, the orientation of the *sn*-2 chain's carbonyl is likely to have a further effect on ψ_d in addition to the contribution of its dipole moment. The oxygen atom of the carbonyl can act as a hydrogen bond acceptor and thus cause an alignment of hydrating water molecules, with the positive hydrogen-end of the water dipole directed towards the membrane interior. This would further increase the positive magnitude of ψ_d.

The chemical nature of the phospholipid headgroup is another important factor which can alter the magnitude of ψ_d. In the case of the phosphatidylcholine (PC) headgroup, the $P^- – N^+$ dipole between the phosphate group and the nitrogen of the choline is thought to lie approximately parallel to the membrane surface and, therefore, not be a major contributor to ψ_d. Nevertheless, NMR studies have shown that the angle it makes to the membrane surface can change with the surface charge of the membrane, which could lead to a modulation in ψ_d [24]. However, substitution of the PC headgroup by the negatively charged phosphatidylserine (PS) has not been found to cause major changes in ψ_d [9,10]. Presumably the negative charge on the carboxylate residue of the PS group is far enough out into the adjacent aqueous solution that it is effectively screened by physiological levels of salt. A similar situation seems to exist in the case of the negatively charged phosphatidylglycerol (PG) headgroup, which also shows very little effect on ψ_d relative to PC [9]. The hydroxyl dipoles of the PG headgroup are probably sufficiently far out into the aqueous phase that the electric fields they produce are screened by the surrounding water dipoles with a dielectric constant of 80. In contrast, the zwitterionic phosphatidylethanolamine (PE) headgroup causes a significant increase in ψ_d. A possible reason for this is a difference in hydration of the PE versus the PC headgroup. Rand and Parsegian [25] have shown that as the degree of methylation of the nitrogen of the headgroup increases from 0 in PE to 3 in PC, there is an increase in number of hydrating water molecules. If the additional water molecules of PC polarize themselves so as to oppose the existing ψ_d, then this could account for the higher ψ_d of PE relative to PC. Finally, the small negatively charged headgroup of phosphatidic acid (PA) causes a significant increase in ψ_d [9]. This result stresses the importance of the precise location of charges relative to the membrane surface. The

increase in ψ_d caused by PA implies that its negative phosphate group is located close to the membrane surface at the level of the negative pole of the dipole potential, so that the electrical potential gradient is magnified and ψ_d increases (see Fig. 2).

The change in ψ_d caused by PA can be compared to the effects of ion binding to the membrane from the adjacent aqueous phase. Anions with relatively low hydration energies, such as perchlorate, have been found to bind to PC membranes and cause a significant drop in ψ_d [26]. The drop could be accounted for by binding within the membrane at the level of the positive pole of the dipole potential, thus yielding a partial neutralization and hence a ψ_d decrease. Binding of divalent or trivalent cations has also been found to produce a drop in ψ_d [26]. It might seem paradoxical that both anions and cations cause a change in ψ_d in the same direction. However, divalent and trivalent cations have very high hydration energies relative to monovalent anions and are, thus, unlikely to bind very deeply within membranes. Their hydrophilic nature makes it much more likely that they bind to a negatively charged polar site at the membrane surface, i.e., to the phosphate residue of PC. Binding at this position would cause a partial neutralization of the negative pole of the dipole potential, thus yielding the observed decrease in ψ_d. In principle then, because ψ_d arises from the alignment of dipoles, the membrane binding of anions and cations could produce either an increase or a decrease in ψ_d depending on where in the membrane they bind.

Before turning to the effect of cholesterol, there is one further factor worth mentioning. From the Helmholtz equation (see Eq. 1) it can be seen that there is an inverse relationship between ψ_d and the local dielectric constant, ε, of the medium. Thus, any decrease in lipid packing density which allows further mobile water dipoles (as opposed to oriented water) to penetrate into the lipid headgroup region will result in an increase in ε. The polarisation of these water molecules around existing oriented dipoles will lead to a further decrease in ψ_d, thus magnifying the drop caused by the decrease in dipole packing density alone. Similarly, any increase in lipid packing density could cause the exclusion of some mobile water from the headgroup region,

thus causing a drop in ε and a further increase in ψ_d above that caused by the increase in $\mu_\perp$/A alone.

3 Influence of cholesterol on the dipole potential

The fact that cholesterol is able to significantly alter the magnitude of the dipole potential of a lipid bilayer was first recognized by Szabo [27], who found that the addition of cholesterol to the membrane caused an increase in membrane conductance towards TPB^- of up to 30 fold and a decrease in conductance towards TPP^+ of up to 100 fold. Based on the conductance changes Szabo [27] estimated cholesterol-induced increases in ψ_d of up to approximately 100 mV. However, he did not speculate on the molecular origin of the effect. Subsequent studies using a variety of cholesterol derivatives have shown that sterol-induced changes in ψ_d are very sensitive to the sterol's molecular structure [28-30] (see Fig. 3). 6-Ketocholestanol, for example, causes an increase in ψ_d which is even greater than that produced by cholesterol [29,31]. For this reason 6-ketocholestanol has sometimes been used as a ψ_d-enhancing molecule in the testing of dipole potential probes [10,32]. Other cholesterol derivatives, however, have been found to decrease ψ_d. Thus, the incorporation of 5-cholesten-3β-ol-7-one (hereafter referred to by its more common name 7-ketocholesterol) into phosphatidylcholine vesicles causes a significant drop in ψ_d [29]. It has been found [29] that the magnitude of the effect of a range of cholesterol derivatives on ψ_d correlates with the component of the derivatives' dipole moments perpendicular to the membrane, $\mu_\perp$, assuming as a first approximation that each adopts the same orientation within the membrane as cholesterol itself [33,34]. Such a correlation would be expected based on the Helmholtz equation (Eq. 1). However, the magnitude of the changes in ψ_d are far too large to be accounted for by the dipole moments of the cholesterol derivatives alone. Starke-Peterkovic et al [29] estimated that the derivative's dipole moments could only account for approximately 20% of the observed ψ_d changes.

A further important effect to consider is the influence that cholesterol and its derivatives have on lipid packing, i.e, on the value of $\mu_\perp$/A in Eq. 1. It has long been known that cholesterol has a condensing effect on lipid membranes [35-38]. However, the observed cholesterol-induced increase in ψ_d is still significantly greater than what one would expect from the combined effect of cholesterol's dipole moment plus the increase in lipid packing density of the phospholipid [29]. Therefore, in addition to these two effects, it seems that there must be some cholesterol-induced structural reorganization of the lipid headgroup region. A possibility could be that cholesterol causes an increase in the local dielectric constant, ε, perhaps either by increasing the proportion of oriented immobilized water molecules within the membrane or by decreasing the penetration of mobile orientationally polarizable water molecules into the membrane. A decrease in ε, by either of these mechanisms, would be expected to further enhance cholesterol's effect on ψ_d (see Eq. 1).

The effect of 7-ketocholesterol on ψ_d is particularly interesting and also physiologically relevant, because it is a major oxidation product leading from the cholesterol biosynthetic pathway. It is produced either directly from cholesterol or from its precursor in the pathway 7-dehydrocholesterol [39]. 7-Ketocholesterol has been found to accumulate in atherosclerotic plaques and to cause apoptosis in vascular cells [40,41]. As explained above, 7-ketocholesterol has been found to cause a significant reduction in ψ_d [29], i.e., the complete opposite of the effect of cholesterol. Measurements of lipid monolayers using a Langmuir trough have shown, however, that, although significantly less effective than cholesterol, 7-ketocholesterol also causes a lipid condensation effect, i.e., an increase in lipid packing density [42-44]. In the absence of any reorganization of the lipid interface, one would expect the increase in phospholipid packing density caused by addition of 7-ketocholesterol to cause a smaller increase in ψ_d than observed on addition of cholesterol, but not to completely reverse the direction of the ψ_d change. To explain this effect one can think of ψ_d consisting of two components, one due to the phospholipids (plus associated water dipoles) within the membrane, $\mu_\perp^{PL}$, and one due to 7-ketocholesterol molecules (plus associated water dipoles), $\mu_\perp^{KC}$. The resultant ψ_d would then be given by an expanded form of the Helmholtz equation:

$$\psi_d = \frac{\left|\mu_\perp^{\mathrm{PL}}\right|}{A\varepsilon_0\varepsilon} - \frac{\left|\mu_\perp^{\mathrm{KC}}\right|}{A\varepsilon_0\varepsilon} \qquad (2)$$

In the absence of any change in ε, the only way that addition of 7-ketocholesterol to the membrane could lead to a decrease in ψ_d would be if the modulus $|\mu_\perp{}^{\mathrm{KC}}|$ is greater than $|\mu_\perp{}^{\mathrm{PL}}|$. The subtraction in Eq. 2 signifies the opposite polarity of the dipole moments due to phospholipid and 7-ketocholesterol. Thus, the compression-induced drop in the component of ψ_d caused by 7-ketocholesterol must overcompensate for the increase expected from the phospholipid component of ψ_d. It is possible that this massive difference in the effects of cholesterol and its common oxidative product 7-ketocholesterol on ψ_d may to some extent be involved in 7-ketocholesterol's cytotoxic effects. It has even been proposed that a cause of neurodegenerative diseases, such as Parkinson's and Alzheimer's disease, could be an overproduction of oxysterols [45].

Another interesting effect of cholesterol and some of its oxidized derivatives on ψ_d is that the concentration profile is biphasic [29]. Thus, up to a level of approximately 40 mol%, cholesterol steadily increases ψ_d, but if one incorporates more cholesterol into the membrane, ψ_d starts to drop again. It is interesting that 40 mol% corresponds to the level of cholesterol often found physiologically in the plasma membrane of animals [1,2]. Thus, it appears that for some reason the cholesterol content of the plasma membrane is optimized to maximize the value of ψ_d. This could perhaps be to minimize the cation permeability of the lipid component of the membrane, as suggested by Szabo [27], or it could be due to an effect of ψ_d on the activity of transmembrane proteins, which will be discussed in the following section. The precise reason why the effect of cholesterol on ψ_d goes through a maximum at 40 mol% is not entirely clear. However, it is not completely surprising. Optimal concentrations are often observed in three-dimensional solvent mixtures, e.g. boiling point maxima or minima at a particular mixture composition. These are attributed to differences in the strengths of the intermolecular forces between the components of the mixture. Cholesterol is thought to interact more strongly with the hydrocarbon chains of phospholipids than with itself [46-48]. Therefore, as the cholesterol

composition increases, the overall net strength of intermolecular forces in the membrane and its stability would be expected to initially increase. However, if the cholesterol composition increases too far and the membrane becomes too cholesterol-rich, the lower strength of the forces between two cholesterol molecules relative to a cholesterol molecule and a phospholipid would be expected to cause a drop in the strength of intermolecular forces. The fact that ψ_d goes through a maximum at a particular cholesterol concentration is an indication of the key role that intermolecular forces play in determining its magnitude.

Another interesting observation is that the effect of cholesterol on the magnitude of ψ_d varies depending on the phospholipid composition of the membrane. Removal of cholesterol via treatment with methyl-β-cyclodextrin from lipid vesicles formed from lipids extracted from a variety of animal tissues has shown [29] that cholesterol makes a contribution of approximately 65 mV to the total ψ_d. However, in experiments with synthetic phospholipid vesicles, it has been found [29] that cholesterol produces a far greater increase in ψ_d when the hydrocarbon chains of the phospholipid are saturated than when they are unsaturated. This would seem to be further evidence supporting an important role of intermolecular forces in determining cholesterol's effect on ψ_d, in this case a differential interaction between cholesterol and saturated versus unsaturated hydrocarbon chains.

4 Physical basis of modulation of membrane protein function

The activity of many membrane proteins is critically dependent on the composition of their surrounding lipid environment, and there are many possible mechanisms by which lipid sensitivity could come about. Here the discussion is limited purely to the mechanisms by which ψ_d could modulate membrane protein activity. Because ψ_d produces an electric field within the headgroup region of the membrane, ion-transporting membrane proteins, e.g. ion channels and ion pumps, first come to mind as possible candidates that could potentially be sensitive to the value of ψ_d in their surrounding membrane. Therefore, we will first concentrate on this class of proteins.

In spite of the large electric field strength that ψ_d produces of $10^8 - 10^9$ Vm^{-1}, it seems that, apart from small pore-forming peptides such as gramicidin and syringomycin [49,50], ψ_d has little effect on binding or conduction of ions through membrane proteins. Theoretical calculations indicate that the reason for this is that most ion channels or pumps are so large that the electric field strength originating from ψ_d is effectively screened from the ion binding sites by the intervening protein mass [51,52]. However, this only means that ion conduction rates are not affected, not that ion channel or ion pump activity are totally insensitive to ψ_d. As a prime example, let us consider the voltage-gated Na^+ channels of neurons.

From cell-attached patch clamp measurements of Na^+ channels of neuroblastoma cells, Zhang et al [53] found that, although the single-channel conductance was constant regardless of where on the cell it was measured, the kinetics of activation, i.e. the gating of the channel, varied across the surface of the cell. In previous measurements [54] the same group had already found that the magnitude of ψ_d also varies spatially across the cell surface. Comparison [53] of the spatial variations of ψ_d with those of channel activation showed that they matched. This suggests, therefore, that Na^+ channel gating kinetics are dependent on the local value of ψ_d in the membrane surrounding the protein. Because the plasma membrane contains such a high proportion of cholesterol, and cholesterol is known to modulate ψ_d, Bedlack et al [54] suggested that the local variations in ψ_d arise because of local variations in the cholesterol composition across the surface of the membrane.

The measurements of Zhang et al [53] on ion channels are consistent with results on ion pumps. In particular, it has been found [52,55] that the kinetics of ion occlusion reactions are dependent on the local value of ψ_d, even though, based on electrophysiological studies [56-61], ion occlusion reactions do not involve significant movement of the transported ions, i.e., they are non-electrogenic reactions. Both the ion occlusion reactions of ion pumps and the gating reactions of ion channels are protein conformational changes. Therefore, the question is, how could a conformational change of a membrane protein be sensitive to ψ_d. A possible mechanism which has recently been presented [52,62] will now be described.

Any conformational change of a membrane protein, whether it be a channel, pump, secondary transporter or a receptor is likely to lead to some change in the protein's hydrophobic thickness. In order to avoid any exposure of protein hydrophobic regions to the surrounding aqueous medium, the membrane can undergo local deformations around the protein so that the hydrophobic thickness of the protein matches that of the membrane [63-66]. Any distortion to the membrane must be associated with a change in the density of lipid packing and hence in the value of ψ_d (see Eq. 1). Any membrane distortion will also involve a change in energy, which must be considered as a component of the total energetics describing the conformational change a membrane protein undergoes. Here we consider the energy changes which arise due to changes in ψ_d alone.

Because the dipoles which give rise to ψ_d are aligned more or less parallel to one another, the energy of interaction is repulsive, or destabilizing. For an infinite planar lattice of parallel dipoles the energy of interaction, *E*, is given by:

$$E = \frac{1}{4\pi\varepsilon_0\varepsilon}\frac{\mu^2}{r^3}M \qquad (3)$$

where *r* is the distance between two neighbouring dipoles of dipole moment, μ, and *M* is termed a Madelung constant and represents the factor by which the energy changes on going from a pair of dipoles to an infinite lattice. The value of *M* depends on the geometrical arrangement of the lattice. For a hexagonally close-packed array of dipoles, *M* has been estimated to have a value of 10.2. For such a lattice, if one considers each net dipole to be associated with a single lipid within the membrane, *r* is related to the cross-sectional area, *A*, occupied by a lipid in the membrane by:

$$r = \sqrt{A/\cos 30} \qquad (4)$$

Substituting this expression for *r* into Eq. 3 and including the value of *M* of 10.2, yields the following repulsive energy per mole of lipid:

$$E = \frac{8.22N_A}{4\pi\varepsilon_0\varepsilon}\frac{\mu^2}{A^{3/2}} \qquad (5)$$

In order to use this equation to estimate *E* one first needs to know values of μ, *A* and ε. Based on a typical ψ_d value of 300 mV, an *A* of 0.65 nm^2 [22], and an ε of 75 (i.e., a polarity slightly less than water) [52,67] one can estimate the value of μ to be 1.3×10^{-28} Cm or 39 D. Inserting these values into Eq. 5 yields a repulsive energy of interaction of 19 kJ mol^{-1}. This is comparable to the attractive energy of interaction on hydrogen bond formation. If one considers that any membrane protein is surrounded by many annular lipids and membrane distortions due to protein conformational changes are likely to extend much further out from the protein than the annular lipids, then it is clear that the repulsive energy of interaction associated with ψ_d could play a substantial role in determining the relative stabilities of protein conformations, e.g. between the open and closed state of channels or the occluded and deoccluded states of pumps. For example, the Na^+,K^+-ATPase and the sarcoplasmic reticulum Ca^{2+}-ATPase possess approximately 35 and 20 annular lipids, respectively [68]. The repulsive energy of interaction due solely to the 35 annular lipids of the Na^+,K^+-ATPase amounts to 665 kJ mol^{-1}. Even if a membrane distortion caused a relatively small perturbation of 10% to this repulsive energy, this would be of the same order of magnitude as the energy released by ATP hydrolysis of approximately – 60 kJ mol^{-1}. Thus, even relatively small changes in ψ_d could be expected to result in significant changes in ion pump kinetics.

Now let us consider at a more molecular level the effects of ψ_d on the conformational equilibrium of a membrane protein. Figure 4 shows an exaggerated picture of a membrane protein conformational change in which a large change in protein hydrophobic thickness occurs. In the protein conformation on the left the protein has a large hydrophobic thickness. In this situation the membrane must increase its own thickness in order to cover the protein's hydrophobic domains. The only way the membrane can do this is by extending the hydrocarbon chains of the surrounding lipids. This necessarily causes an increase in lipid packing and thus an increase in the local ψ_d around the protein. Conversely, in the protein conformation on the right the protein has a small hydrophobic thickness and the membrane must, therefore, thin so that its own hydrophobic thickness matches that of the protein. The only way this can occur is if the hydrocarbon tails of the lipids spread. This naturally

causes a local decrease in the lipid packing density and a decrease in ψ_d. Thus, protein conformations with a large hydrophobic thickness are expected to be associated with a large local ψ_d and conformations with a small hydrophobic thickness are expected to be associated with a small local ψ_d. Now if a molecule is added to the membrane that changes ψ_d the distribution of the protein between the two states would be expected to change. This can be explained qualitatively using Le Châtelier's principle, i.e., any equilibrium shifts in order to decrease the magnitude of a perturbation. Thus, if one adds cholesterol to a membrane, and it is known that cholesterol increases ψ_d, then the equilibrium must shift to decrease ψ_d again. This necessitates a shift to the protein conformation on the right, with a lower ψ_d. Based purely on the energetics of dipole-dipole interactions at the membrane interface, increasing cholesterol concentrations, up to the physiological level, would, therefore, be expected to favour protein conformational states with a small hydrophobic thickness. In contrast the addition of substances to the membrane which decrease ψ_d would be expected to favour protein conformational states with a large hydrophobic thickness. The theoretical framework described here for the effect of ψ_d on membrane protein conformational transitions arose out of the author's work on the lipid sensitivity of the Na^+,K^+-ATPase. Recently it has also been applied by others [69] to explain the effects of activators of the hERG (human Ether-a-go-go Related Gene) K^+ channel, which is involved in coordinating beating of the heart. Interference in the activity of the hERG channel can potentially lead to sudden death via the condition known as long QT syndrome. For this reason the Food and Drug Administration of the USA requires that all new drugs be tested for any effect on the hERG channel. If the hERG channel is in fact sensitive to ψ_d, it would seem advisable that any new drugs, whatever their planned therapeutic purpose, be initially screened for their effect on ψ_d. It is hard to imagine a more severe side effect of a drug than sudden death.

5 Membrane-binding terminal extensions of membrane proteins

The calculations presented in the previous section indicate that any membrane bending required to accommodate changes in protein hydrophobic thickness would be

expected to be associated with very significant changes in energy. However, rather than the membrane distorting to encompass a protein's changing hydrophobic thickness, another possibility is that the protein could change its orientation relative to the membrane. Thus, if a membrane protein can tilt in the membrane sufficiently so that any expansions to its hydrophobic domains are moved into the membrane, the membrane's hydrophobic thickness could remain unchanged and still fully cover the hydrophobic domains of the protein. Indeed evidence for such a mechanism has recently been found from X-ray crystallographic studies on the sarcoplasmic reticulum Ca^{2+}-ATPase [70]. This paper presented the first structures of a P-type ATPase in which the surrounding lipid bilayer could be resolved. The results revealed that between different protein conformational states, the protein tilts relative to the membrane plane by angles up to 18.4 degrees (see Fig. 5). Such a mechanism would minimize effects of ψ_d on the protein's function via the mechanism described in the previous section, i.e., preferential stabilization of conformational states with small protein hydrophobic thicknesses with increasing ψ_d.

However, if membrane proteins do rock backwards and forwards in the membrane in order to avoid membrane bending, other mechanisms need to be considered to explain the effect of their membrane environment on protein activity. It is now clear that many membrane proteins contain polybasic sequences, i.e., clusters of lysine and arginine residues, which can interact with the headgroups of negatively charged phospholipids such as phosphatidylserine on the cytoplasmic face of the plasma membrane [71]. Such an interaction has been clearly shown from experiments with model membrane systems and synthetic peptides and polyamino acids (e.g. polylysine) [72-74]. Prime examples are the Na^+,K^+-ATPase and its closest relative amongst the P-type ATPase family, the gastric H^+,K^+-ATPase, which is responsible for the acidification of the stomach, necessary for activation of the digestive enzyme pepsin. Both of these proteins possess lysine-rich N-termini on the proteins' cytoplasmic face [75]. The N-terminus of the H^+,K^+-ATPase from the Chinese carnivorous fish *Siniperca chuatsi* even has seven consecutive lysine residues. Based on both equilibrium and kinetic experiments it appears that the N-terminus interacts electrostatically with negatively charged phospholipids in the surrounding membrane

and that this interaction stabilizes the enzymes' E2 conformational state [75-77], which is the state to which K^+ ions preferentially bind. Now that it is known from the x-ray studies on the Ca^{2+}-ATPase [70] that these enzymes rock backwards and forwards in the membrane as the enzyme cycles between E1 and E2 conformations, this provides a structural basis to understand the conformational preference of the N-terminus-membrane interaction. It seems possible then that this membrane interaction could continually be switching on and off as the proteins pump ions across the membrane. What the purpose of this might be is still unclear. A possibility is that the interaction may provide a membrane anchoring, thus locking in E2-like conformations and helping to drive the pumping cycle forward by inhibiting back reactions. The interaction could also be involved in the occlusion process of the transported ions or in regulation of the proteins' activities. However, whatever the functional purpose, if a membrane interaction exists, this would seem to be an obvious locus to seek an explanation for the strong sensitivity of the activity of the Na^+,K^+-ATPase on the cholesterol content of the membrane [68,78-81].

As mentioned earlier, cholesterol is known to cause an increase in lipid packing density [35-38]. Any increase in the cholesterol content of the inner cytoplasmic leaflet of the plasma membrane, which is known to contain a high level of negatively charged lipids such as phosphatidylserine, would be expected to increase the negative surface charge density of the membrane, thus promoting interaction with the positively charged N-terminus of the Na^+,K^+- or H^+,K^+-ATPase and stabilizing the E2 conformation. Any stabilization of one conformation over another will alter the kinetics of the entire ion pumping cycle and could lead to either pump stimulation or inhibition. What role the ψ_d may have in this mechanism of membrane protein modulation awaits further investigation. In principle this depends on the degree of penetration of the N-terminus into the membrane. If the interaction is purely at this surface, ψ_d may have minimal effect, but if the penetration extends to the level of the phospholipid glycerol backbone, where the gradient of ψ_d is expected to be at its greatest, then ψ_d may play a significant role.

6 Conclusions

It is clear that the cholesterol molecule has a significant effect on the magnitude of ψ_d. That much at least is certain. The exact origin of cholesterol's effect on ψ_d is less clear, but several possibilities have been here discussed. How and by how much cholesterol affects the function of membrane proteins via its influence on ψ_d is also in need of further investigation. Whatever the mechanism, any effects on ion-transporting membrane proteins are likely to be on the kinetics of protein conformational changes (i.e., gating or occlusion reactions), not ion conduction.

The effects of lipids, including cholesterol, on membrane proteins are normally classified as being either general membrane-mediated (i.e., due to the physical properties of the membrane) or specific (i.e., due to a direct interaction between the protein and a specific lipid). Effects of ψ_d on membrane proteins would normally be classed in the first category. However, it is worthwhile pointing out that such classifications, although sometimes useful, tend to impose a limitation to the imagination. In actual fact there is no reason why a mechanism by which cholesterol modulates membrane protein function couldn't combine both general and specific aspects. The final mechanism presented in the previous section of this chapter is a case in point. It is proposed that a specific section of a protein, its N-terminus interacts with a negatively charged membrane surface. There is no suggestion at this stage that cholesterol interacts directly with the N-terminus, but it modulates a general physical property of the membrane, namely lipid packing, and alters surface charge density (and, as described in section 6.2, also ψ_d), thus influencing the strength of the N-terminus interaction with the membrane. This mechanism is also in need of further investigation and how widely applicable it is remains to be seen. The final message, however, is that a complete understanding of the role that cholesterol plays in membrane function requires an open mind willing to consider the possibility of complex mechanisms which are not able to be pigeon-holed as either specific or general.

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Figure Captions

Fig. 1 Electrical potential, ψ, profile across a lipid bilayer. The transmembrane potential, $\Delta\psi$, is due to the difference in anion and cation concentrations between the two bulk aqueous phases. The surface potential, ψ_s, arises from charged residues at

the membrane-solution interface. The dipole potential, ψ_d, results from the anisotropic arrangement of dipoles associated with the lipid headgroups and their solvating water molecules. It is defined at the potential drop from interior of the membrane (at the level of the linkage between lipid glycerol backbone and the hydrocarbon chains) to the adjacent aqueous solution. Reproduced from [82] with permission from Springer Nature.

Fig. 2 Mechanism for the increase in ψ_d by the incorporation of phosphatidic acid (PA) into a phosphatidylcholine (PC) membrane. The solid lines represent the profile of the electrical potential, ψ, for PC alone. The dotted lines represent the profile of ψ for a PC membrane incorporating PA. The electrical potential is defined to be zero in the aqueous solution far from the membrane surface. ψ_d^{PC} and ψ_d^{PA} represent the dipole potentials of a pure PC membrane and one in which PA has been incorporated, respectively. PC, because it as a zwitterionic headgroup, is assumed to produce no surface potential, whereas PA with its negatively charged phosphate group produces a negative surface potential. Reproduced from [9] with permission from Springer Nature.

Fig. 3 Chemical structures of cholesterol and some of its oxidized derivatives. (a) Cholesterol, (b) 6-ketocholestanol, and (c) 5-cholesten-3β-ol-7-one (7-ketocholesterol).

Fig. 4 Conformational transition between two membrane protein conformational states with large (left) and small (right) hydrophobic thicknesses. Adapted from [61] with permission from Elsevier.

Fig. 5 Changes in orientation of the sarcoplasmic reticulum Ca^{2+}-ATPase molecule during its ion pumping reaction cycle. The horizontal solid lines show each side of the membrane. The inclined dotted lines show the previously thought position of the membrane, in alignment with the protein's M7–M10 transmembrane helices. The angle values associated with the arrows between different protein conformational

states indicate the rotation of the protein relative to the membrane surface required for it to proceed to the next conformational state. Reproduced from [69] with permission from Springer Nature.

Figure 1

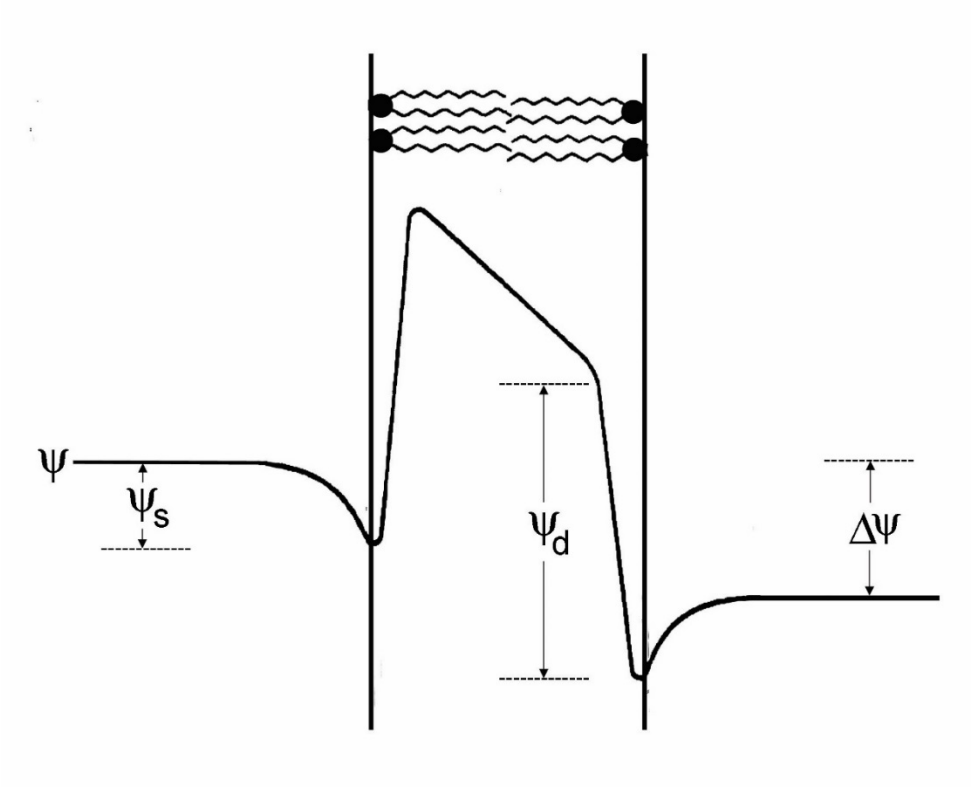


Figure 2

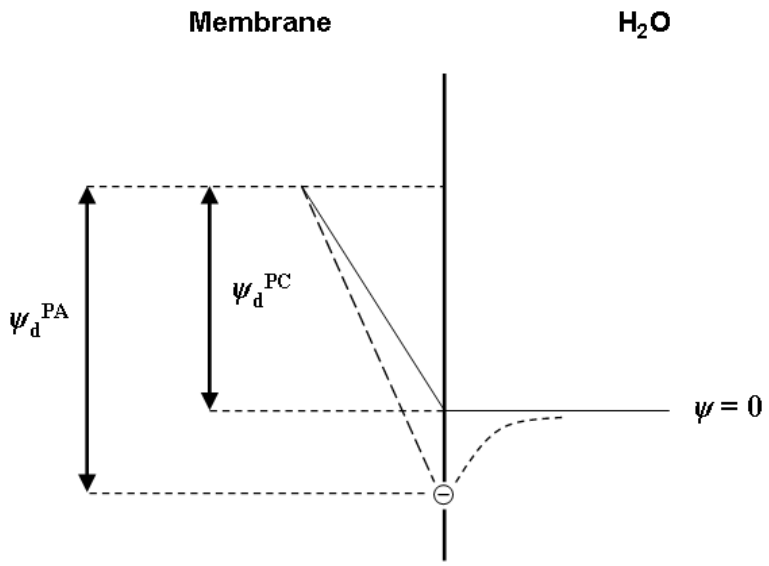


Figure 3

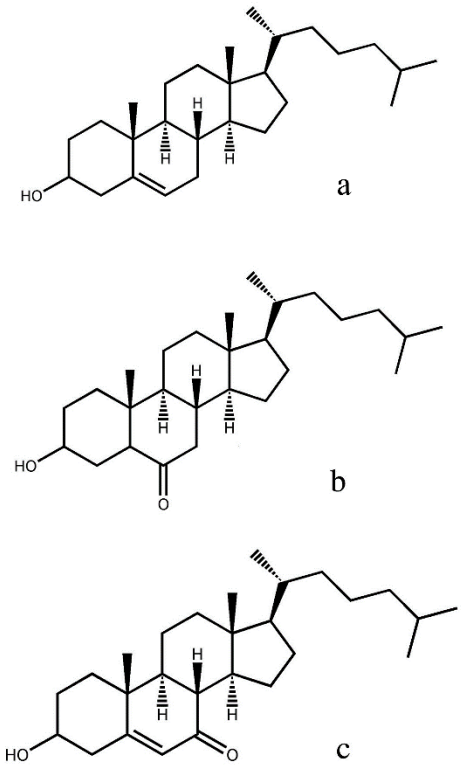


Figure 4

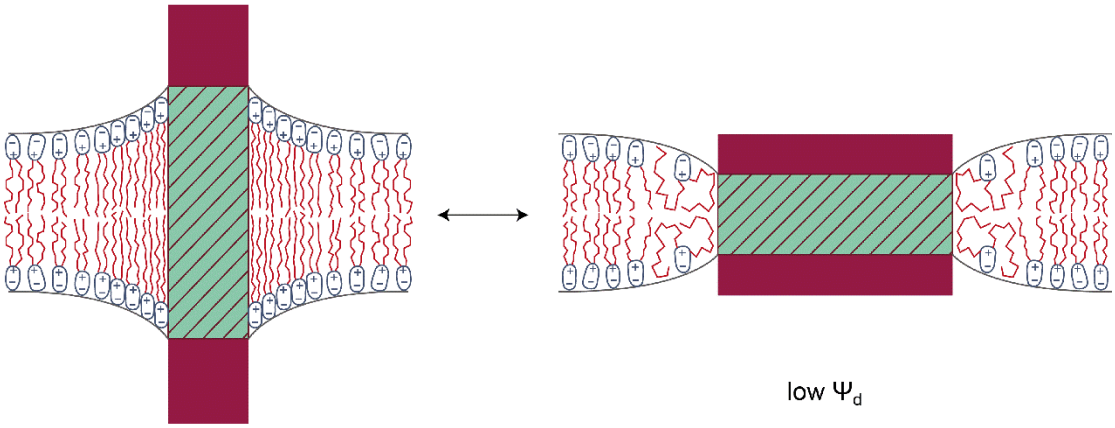
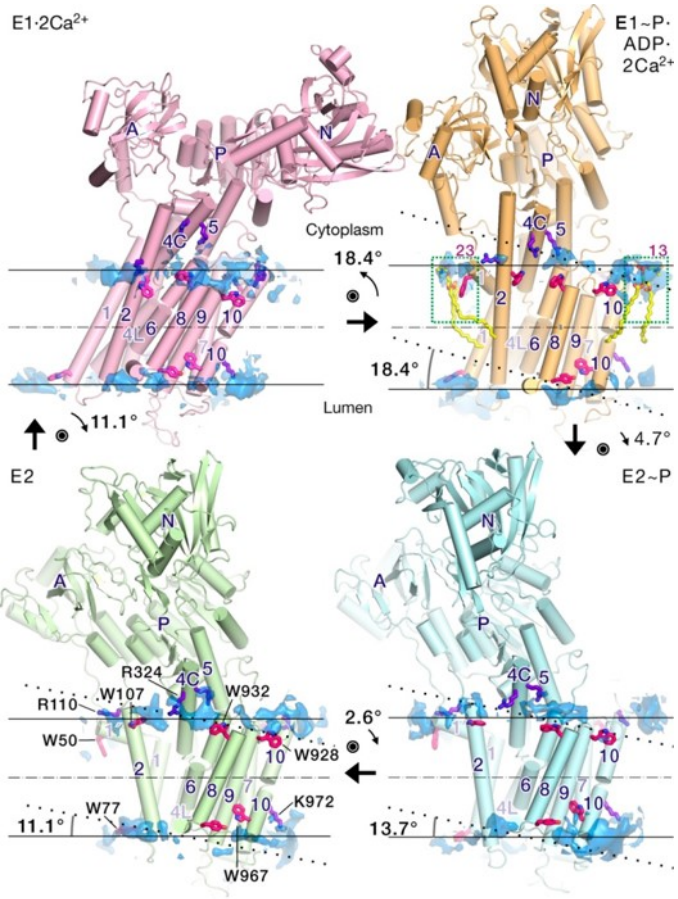


Figure 5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