A METHOD FOR ESTIMATING THE CHOLESTEROL AFFINITY OF INTEGRAL MEMBRANE PROTEINS FROM EXPERIMENTAL DATA

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Short title: Determination of the cholesterol affinity of integral membrane proteins

ABSTRACT: The cholesterol affinities of many integral plasma membrane proteins have been estimated by molecular computation. However, these values lack experimental confirmation. We therefore developed a simple mathematical model to extract sterol affinity constants and stoichiometries from published isotherms for the dependence of the activity of such proteins on membrane cholesterol concentration. The binding curves for these proteins are sigmoidal with strongly-lagged thresholds that we attribute to competition for the cholesterol by bilayer phospholipids. Using the model, we matched computed to experimental isotherms using sterol association constants and stoichiometries for the phospholipids taken from the literature. Three oligomeric transporters were found to bind cholesterol without cooperativity with dimensionless association constants of 35 for Kir3.4* and 100 for both Kir2 and a GAT transporter. (The corresponding $\triangle G^o$ values are -8.8, -11.4 and -11.4 KJ/mol, respectively.) These association constants are significantly lower than those for the phospholipids, which range from ~100 to 6,000. The BK channel, the nicotinic acetylcholine receptor and the M192I mutant of Kir3.4* appear to bind multiple cholesterol molecules cooperatively (n = 2 or 4) with subunit affinities of 563, 950 and 700, respectively. The model predicts that the three less avid transporters are

approximately half-saturated in their native plasma membranes; hence, sensitive to variations in cholesterol in vivo. The more avid proteins would be nearly saturated in vivo. The method can be applied to any integral protein or other ligand in any bilayer for which there are reasonable estimates of the sterol affinities and stoichiometries of the phospholipids.

INTRODUCTION

Cholesterol binds to many plasma membrane proteins and affects their disposition and activity in diverse ways. These associations have been characterized by various techniques, including X-ray, cryo-EM, NMR, molecular computation and functional analysis.[1-10] Two cardinal features of the interaction of the proteins and sterols are the strength of their association and their binding stoichiometry. We have found only one experimental determination in the literature of the affinity of cholesterol for an integral membrane protein in situ.[11] However, that study is not secure.[12] In the absence of experimental values, binding constants for numerous proteins have been obtained by molecular dynamics, docking and other computational methods.[4, 13-21]

Here, we describe an indirect method to characterize the binding of cholesterol to integral proteins and other membrane-embedded ligands based on the sterol dependence of their activity. Basically, a simple mathematical model was used to generate cholesterol dependence curves that simulate published activity isotherms for such proteins. The model posits the formation of complexes between cholesterol and membrane phospholipids which, perforce, compete with the protein for the sterol.[22-24] Using published values for the sterol affinity and stoichiometry of the membrane phospholipids, the model delivers estimates of the sterol affinity and stoichiometry for the proteins.

At one extreme, very avid proteins outcompete the phospholipids for the cholesterol and so bind it with nearly hyperbolic isotherms. At the other extreme, very weak proteins bind cholesterol poorly until the phospholipids become saturated with sterol at their stoichiometric

equivalence point and the uncomplexed excess sterol becomes increasingly available to the protein.[25] Between these extremes, proteins have sigmoidal sterol dependence curves with shapes and thresholds determined by the attributes both of the ligands and the phospholipids (see Results). We assume that two forms of cholesterol are available to the protein: (a) that extracted from membrane phospholipid complexes by strong competition; and (b) that readily available beyond the stoichiometric equivalence point of the complexes.[25]

METHODS

The model. This model simulates the binding of sterol molecules to a ligand within a phospholipid bilayer so as to estimate the affinity and stoichiometry of the reaction. It resembles an earlier model that treats the binding of water-soluble ligands such as cytolysins to membrane sterols.[25] In contrast, the present version places the ligand in the same membrane compartment as the sterol and the phospholipid. We assume that a ligand (L) associates with cholesterol (C) in competition with the membrane phospholipids (P). The important parameters are therefore the sterol association constants and stoichiometries for the phospholipids and the ligand. The compartment size is $\alpha = (C_T + P_T + L_T)$ where the subscript T denotes total. The chemical activities of the reactants are ideal and expressed as their concentrations in mole fractions. These are C_f/α, P_f/α and L_f/α, where f denotes the free form of the reactant. CP_r denotes lipid complexes and C_nL denotes cholesterol-ligand (namely, protein) complexes.

We postulate an association equilibrium for the sterol and phospholipid:

$$C_f + r\,P_f \leftrightarrow CP_r \qquad (1)$$

The dimensionless association constant for complexation is then

$$K_P = [(CP_r)/\alpha] \,/\, \{[(C_f)/\alpha] \times [(P_f)/\alpha]^r\} = (CP_r) \times \alpha^r \,/\, [(C_f) \times (P_f)^r] \qquad (2)$$

Many integral membrane proteins such as the transporters considered here are oligomeric.[26, 27] For simplicity, we allow the ligand (L) to be an oligomer composed of identical subunits that bind n sterol molecules in a fully cooperative all-or-none fashion; a concerted rather than sequential reaction.[28] Hence,

$$n\ C_f + L_f \leftrightarrow C_nL \qquad (3)$$

Designating the dimensionless association constant for the formation of an n-mer of L as K_{Ln}, we have

$$K_{Ln} = [(C_nL)/\alpha] / \{[(C_f)/\alpha]^n \times (L_f)/\alpha\} = (C_nL) \times \alpha^n / [(C_f)^n \times (L_f)] \qquad (4)$$

Application of the model. Eqs.1-4 were used to generate isotherms that simulate the binding of an integral membrane protein to cholesterol in competition with the phospholipids. Simulations are numerical solutions computed in MATLAB. The computer code is presented in the Supporting Information. Reactant quantities are in moles. The abundance of the phospholipid (P_T) was set to unity and the mole fraction of the sterol (C_T/α) was varied across its physiologic range, from zero to 50 mol%. The abundance of the protein (L_T) was set at 1×10^{-3} for biological membranes and 1×10^{-5} for planar bilayers. (Variation in this small fraction of protein did not affect the results significantly nor should competition by extraneous proteins matter because of the large excess of the sterol.) Cholesterol/protein binding ratios of n = 1 to 5 were examined.

Accurate values for the sterol affinity and stoichiometry of the phospholipid mixtures (i.e., K_P and CP_r in Eq. 2) were not available. We therefore used plausible estimates based on a published data set.[29] We assumed that the stoichiometry of binding of phospholipid to cholesterol, r, was one or two.[24, 25, 29] The program allows membranes to contain two types of phospholipids with different cholesterol affinities and stoichiometries in different proportions. The membrane sterol concentration was expressed as mol% cholesterol; *i.e.*, 100 x moles

cholesterol / (moles cholesterol + moles phospholipid). Free energies of sterol binding were calculated as $\Delta G^o = -RT \ln K_{L1}$ at 298 ^oK.

We assigned various values to the cholesterol affinity and stoichiometry of the phospholipids and protein and matched computed isotherms to experimental data by eye. The experimental data in Figures 1-6 were kept in their published form, scaled on the left-hand ordinates. Simulations were scaled from zero to one on the right-hand ordinates. In addition to K_{Ln} and n, the simulations yielded cholesterol-dependence curves for the fractional saturation of the protein (C_nL/L_T), the fraction of each phospholipid associated with the sterol ($r \times CP_r/P_T$) and the fraction of uncomplexed sterol (C_f/C_T).

A satisfactory match of simulation to data was found in each case. However, estimates of K_{Ln} depended on K_P; consequently, the experimental data could be matched by various pairs of values for K_P and K_L. Thus, the delivered association constants are not uniquely determined and are therefore not definitive.

It is shown in Section S3.a of Supporting Information that, at half-saturation of the protein,

$$(C_f)_{1/2} = \alpha / (K_{Ln})^{1/n} = \alpha / K_{L1} \qquad (5)$$

Eq. (5) implies that the half-saturation of a protein is independent of the assumed sterol binding stoichiometry, n, and, furthermore, that the cholesterol-dependent isotherms of a protein will all intersect at $(C_f)_{1/2}$ (not shown).

In addition, note that the calculated isotherms in Figures 3-6 all intersect close to the same *total* cholesterol concentration (C_T) for all values of n. As shown in Section S3.b of the Supporting Information, this intersection point is given by:

$$(C_T)_{1/2} \approx [\alpha / K_{L1}] \times \{1 + [K_P \times (P_f)^r / \alpha^r]\} \qquad (6)$$

Thus, the concentration of cholesterol at the half-saturation point of a protein with affinity K_{Ln} is independent of n.

RESULTS

Many integral plasma membrane proteins associate with cholesterol (see Introduction). However, sterol-dependence curves suitable for our analysis of these interactions are few.[30] Six such isotherms were tested here.

GAT. These oligomeric transporters facilitate the recovery of secreted γ- aminobutyric acid (GABA) across presynaptic plasma membranes symported by gradients of NaCl.[31] The data in Figure 1 show the cholesterol dependence of the transport activity of a purified rat brain GAT reconstituted in liposomes.[32] The overall sterol affinity of the phospholipids was not known, so we tested various K_P values for this polyunsaturated mixture.[29]

Assuming a phospholipid affinity for the sterol of $K_P = 200$, a protein affinity of $K_{L1} = 100$ and a protein stoichiometry of n = 1 gave a very good match to the data (curve 3 in Figure 1). Because the protein competes moderately well with the phospholipids, the isotherm is gently sigmoidal and ascends well before the appearance of free cholesterol at its presumed stoichiometric equivalence point with the phospholipids, 42 mol% (curve 6). This shows the ability of the protein to extract cholesterol from the phospholipid complexes.

The other curves in Figure 1 explore the model. Curve 1 shows that, in the presence of phospholipids with low sterol affinity, a protein such as this binds cholesterol strongly with a nearly hyperbolic isotherm. Curves 2 and 4 show the effect of doubling and halving the sterol affinity of the best-matched phospholipids (curve 3). The spread among curves 2, 3 and 4 gives an indication of the sensitivity of sterol binding to K_P. Curve 5 illustrates how the cholesterol binding curve of a protein is shifted to the right when the bilayer phospholipids are very avid.

Curve 6 shows that free cholesterol increases sharply beyond saturation of the phospholipid mixture at 42 mol%.

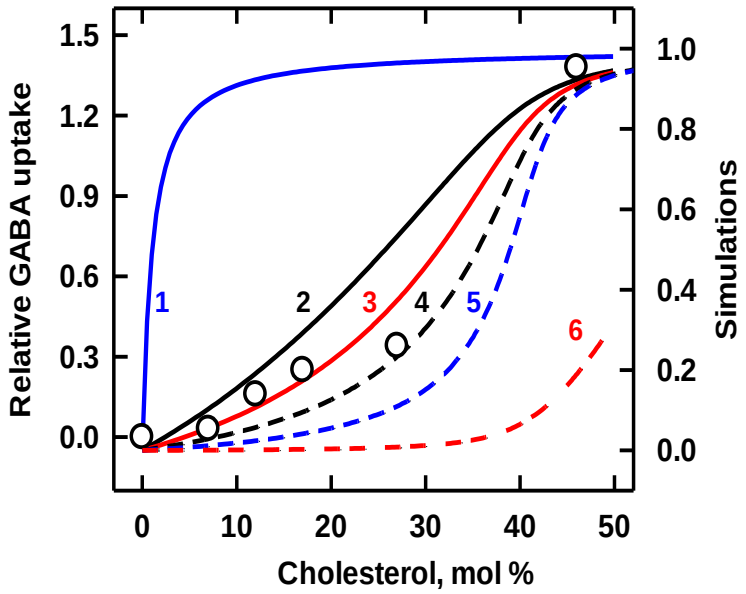

Figure 1. Simulation of the binding of cholesterol to a GAT transporter. Experimental data (symbols) depict the uptake of γ-aminobutyric acid (GABA) by liposomes containing the transporter, asolectin, brain phospholipids and varied cholesterol.[32] Curves 1-5 assigned K_{L1} = 100 and n = 1 to the protein. Lacking experimental values for the phospholipid mixture, we assumed that the bilayer contained equal proportions of two phospholipid species with stoichiometries of C:P = 1:1 and 1:2, with the K_P association constants shown in Table 1.[29] Curve 6 shows free cholesterol in moles/mol phospholipid in the absence of protein.

Table 1 Values used for GAT in Figure 1

Curve	K_P	Code
1	1×10^{-6}	Blue line
2	100	Black line
3	200	Red line
4	400	Black dash
5	1,000	Blue dash
6	Free cholesterol	Red dash

We also matched the experimental data to simulations that assumed that the protein has two or four identical sterol binding sites; that is, n = 2 and 4. The affinity of the monomer was again taken to be K_{L1} = 100. The curves had lagged thresholds and very acute ascents and so did not match the experimental data well (not shown). We infer that the binding of cholesterol to the GAT is not cooperative. Nevertheless, multiple cholesterol molecules might associate with the oligomer at non-interacting sites.

KIR3.4*. This recombinant homotetramer was derived from a subunit of the G protein-activated potassium channel, GIRK.[8] In the study shown in Figure 2, the protein was reconstituted into planar bilayers and single channel activities were recorded as a function of membrane cholesterol concentration.[33] Curve 2 provides a good match to the data using K_{L1} = 35 and n = 1. Doubling and halving the sterol affinity of the simulated protein (curves 1 and 3, respectively) shifted the binding isotherm moderately. Curve 4 assumed n = 2; it paralleled the data at low cholesterol concentrations but rose acutely and overshot the plateau. Hence, we preferred the match for n = 1. For illustrative purposes, curve 5 shows a protein with a weak affinity that nevertheless extracts cholesterol from the phospholipids. Curve 6 shows the concentration of free cholesterol in the absence of protein; it rises acutely as the total cholesterol exceeds the complexation capacity of the phospholipids at their stoichiometric equivalence point, 42 mol%.

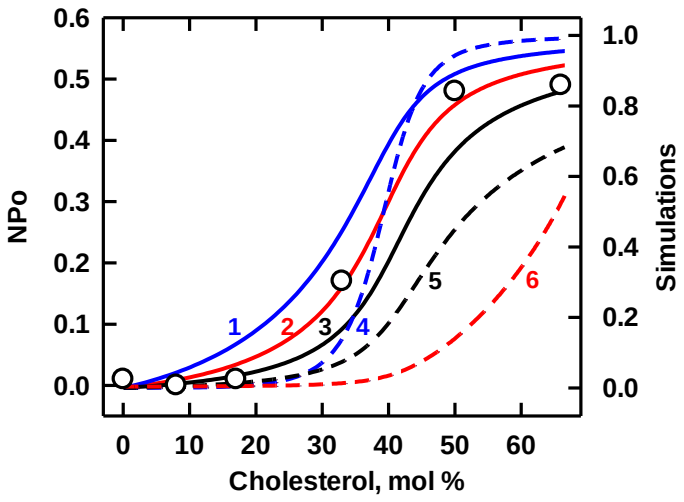

Figure 2. Simulation of the binding of cholesterol to Kir3.4*. Experimental data (open circles) represent the open probability (NPo) of single channels in a planar bilayer composed of 1:1 mixtures of 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) and brain phosphatidylethanolamine (POPE).[33] The former phospholipid was assigned a sterol stoichiometry of C:P = 1:2 and an affinity of K_P = 210; the latter species was assigned a C:P = 1:1 and a K_P = 130 (the value published for 1,2-dimyristoyl-phosphatidylethanolamine, DMPE).[29] The protein values in Table 2 were used in the calculation of the isotherms. Curve 6 shows the concentration of free cholesterol in moles/mol phospholipid in the absence of protein. As predicted by the model, curves 2 and 4 intersect at their half-saturation values (see Methods).

Table 2 Values used for Kir3.4* in Figure 2

Curve	n	K_{Ln}	Code
1	1	70	Blue line
2	1	35	Red line
3	1	17.5	Black line
4	2	1,225	Blue dash
5	1	5	Black dash
6	Free cholesterol		Red dash

Kir2. As seen in Figure 3, the activity of this tetrameric inward rectifier potassium

channel is inhibited by cholesterol.[34, 35] The simulation of these data was slightly better for n = 1(curve 1) than for n = 4 (curve 2), both by eye and from the value of R^2

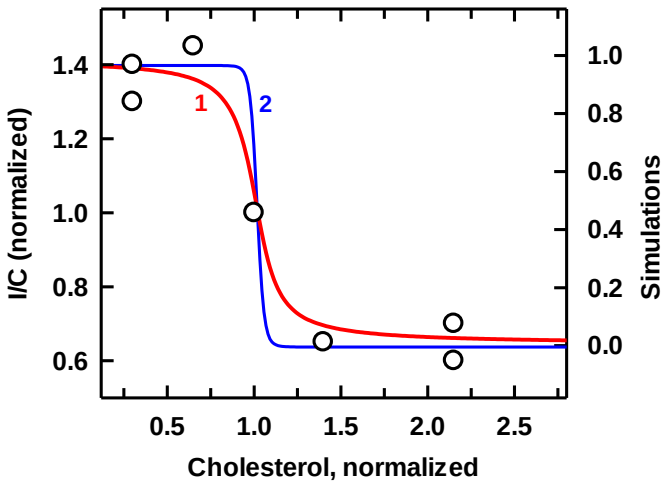

Figure 3. Simulation of the binding of cholesterol to Kir2 channels. Experimental data (open circles) give the peak current density (normalized I/C) obtained by patch clamping cultured bovine aortic endothelial cells that had been treated with methyl-β-cyclodextrin plus varied cholesterol to adjust the plasma membrane sterol concentration.[34] We took the unperturbed plasma membrane cholesterol concentration, normalized to 1.0, to be 41 mol% (mole fraction = 0.41 or C/P = 0.69).[24] The plasma membrane was assumed to contain a mixture of equal proportions of two phospholipids with stoichiometries of C:P = 1:1 and 1:2 and affinities of 5,000 and 2,500, respectively.[24] The calculations used the values in Table 3. As predicted by the model, the curves intersected at their half-saturation values (see Methods). [Much of the excess sterol loaded into the cells presumably moved to intracellular organelles.[36] However, this should not have affected the derived K_{Ln}.]

Table 3. Values used in Kir2 in Figure 3

Curve	n	K_{Ln}	R^2	Code
1	1	100	0.968	Red line
2	4	1×10^8	0.952	Blue line

(Table 3). Curves for n = 2 and 3 fell between these two extremes (not shown). It appears that the protein does not extract appreciable cholesterol from the avid phospholipid complexes but depends on the free cholesterol emerging at the stoichiometric equivalence point of the plasma membrane. This undermines the estimation of the stoichiometry, n, which depends on the shape of the isotherm.

BK. This voltage-gated potassium channel is activated by cytoplasmic calcium ions with high positive cooperativity.[37, 38] However, as shown in Figure 4, it is inhibited by cholesterol.[38, 39] The simulation for n = 4 (curve 2; R^2=0.73) was better matched to the data than that for n = 1 (curve 1; R^2 = 0.58). Rejecting the stray point at ~20 weight % further favored the match for n = 4 compared to n = 1 (R^2 = 0.90 vs. 0.66). The sterol affinity of a putative subunit was K_{L1} = 563 (Table 4). Thus, BK was much more avid for cholesterol than the three transporters analyzed above.

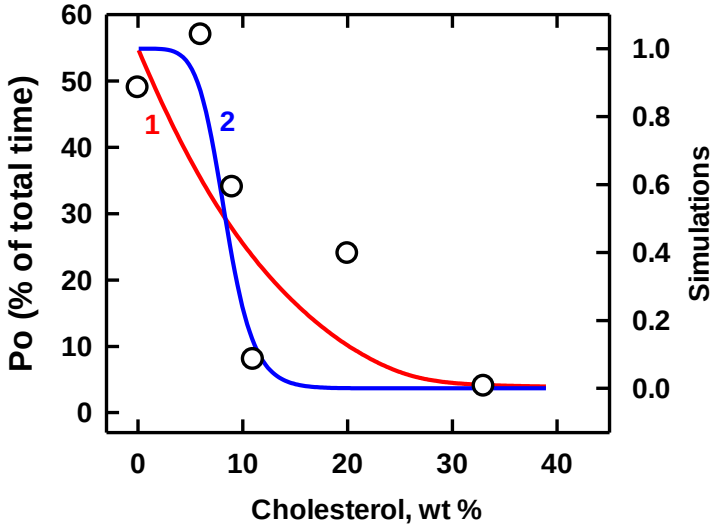

Figure 4. Simulation of the binding of cholesterol to BK channels. (These were presumably tetramers of the alpha subunit.[40]) Experimental data (open circles) represent the open probability (P_o) of single channels in a planar bilayer composed of a 1:1 mixture of 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) plus 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) in the presence of 0.1 mM $CaCl_2$.[39] We assumed that the two phospholipids had stoichiometries of C:P = 1:2 and 1:1 and affinities of K_P = 210 and 130, respectively.[29] To convert the experimental cholesterol concentrations from the reported percent of total lipid weight to mol%, we assumed molecular weights of 387 and 740 for C and P, respectively. The midpoint of the curve, 8.3 weight %, corresponds to 12.7 mol% cholesterol. The values for BK listed in Table 4 were used in the calculations. As predicted by the model, the curves intersect at their half-saturation values (see Methods).

Table 4. Values used for BK in Figure 4

Curve	n	K_L	R^2	Code
1	1	563	0.66	Red line
2	4	1×10^{11}	0.90	Blue line

M192I variant of Kir3.4*. This mutant recombinant homotetrameric potassium channel was recently characterized.[41] Our analysis showed that the cholesterol dependence of the conductance was altered by the mutation in three ways. First, while cholesterol activated the wild type (Figure 2), it inhibited the variant (Figure 5). Second, whereas the open probability of the unmodified channel in Figure 2 appeared to have first-order dependence on cholesterol concentration, the data for the mutant were better matched by assuming n = 4 rather than n = 1. Third, the mutant protein was far more avid for cholesterol than was the wild type (i.e., K_{L1} = 700 versus 35, respectively). Figure 1 shows that the sterol affinity of the phospholipid strongly influences the binding isotherm of a protein. Because the Kir3.4* and the M192I variant were analyzed in the same bilayer environment, the striking difference in their isotherms can be attributed to the alteration in the protein.

Nicotinic acetylcholine receptor (AChR). This pentameric cation channel has complex allosteric behavior and specific binding sites for cholesterol.[42, 43] The data in Figure 6 show the sterol-dependence of a conformational switch in the receptor from a desensitized to a resting state.[44] Simulation with n = 1 (curve 1) gives a poor match to the data, and the threshold for n = 5 (curve 3) is too sharp. On the other hand, assuming modest cooperativity (i.e., n = 2, curve 2) provides an excellent match. Calculations using n = 3 or n = 4 give intermediate simulations while varying the affinity of the phospholipids moves these cooperative curves to lower and higher thresholds (not shown). We infer that both the cooperativity of the protein and competition by the phospholipid contributed to the position and contour of curve 2, the best-matched simulation.

The sterol affinity of the protein subunit, K_{L1} = 950, is an order of magnitude greater than that of the proteins shown in Figures 1-3. The corresponding free energy change is $\triangle G^o$ = -17.2 KJ/mole. Molecular dynamics simulation suggested an energy of interaction of cholesterol with

subunits of the receptor of -25 to -50 KJ/mole.[45] However, those determinations were performed in vacuo rather than in a bilayer.

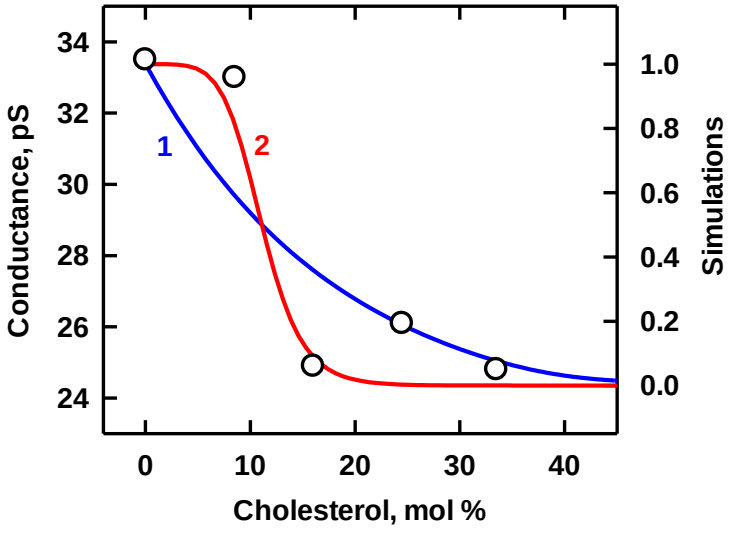

Figure 5. Simulation of the binding of cholesterol to Kir3.4* M182I channels. Experimental data (open circles) represent the conductance of single channels in a planar bilayer composed of a 1:1 mixture of 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) and 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE).[41] We assumed that the two phospholipids had C:P stoichiometries of 1:2 and 1:1 and affinities of K_p = 210 and 130, respectively.[29] The computed curves used the protein values listed in Table 5. As predicted by the model, the curves intersect at their half-saturation values (see Methods).

Table 5. Values used for Kir3.4* M182I in Figure 5

Curve	n	K_L	R^2	Code
1	1	7×10^2	0.77	Blue line
2	4	2.4×10^{11}	0.93	Red line

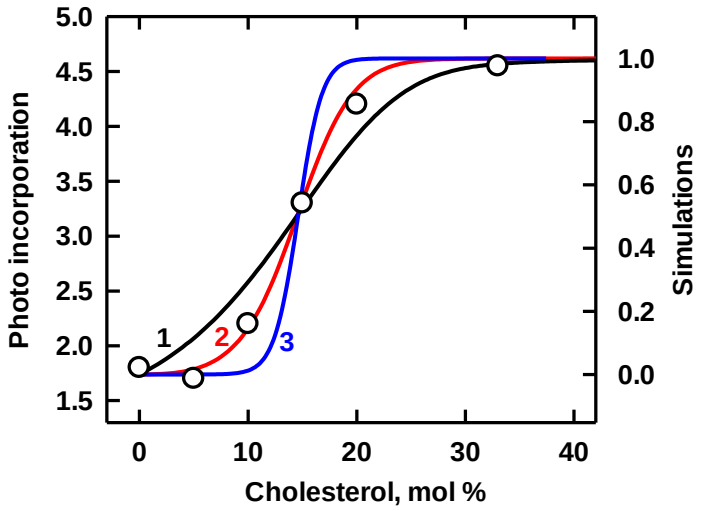

Figure 6. Simulation of the binding of cholesterol to nicotinic acetylcholine receptors. Experimental data (open circles) represent the labeling of the protein by a conformationally-sensitive radioactive diazirine probe in vesicles composed of 3:1 dioleoylphosphatidylcholine:dioleoylphosphatidic acid (DOPC:DOPA).[44] We assumed sterol affinities of K_P = 930 and 200, respectively, for the two phospholipids and a C:P stoichiometry of 1:2 for both.[29] The calculations used the protein values listed in Table 6. As predicted by the model, the curves intersect at their half-saturation values (see Methods).

Table 6. Values used in Figure 6

Curve	n	K_L	Code
1	1	9.5×10^2	Black
2	2	9×10^5	Red
3	5	7.7×10^{14}	Blue

Simulated isotherms for five transporters in cell membranes. The model posits that the value for K_{Ln} does not vary with the phospholipid environment of a protein. This allowed us to apply the K_{Ln} values obtained above to model five of those proteins in a plasma membrane bilayer (Figure 7). (We did not include the M192I mutant of Kir3.4* from Figure 5 because its

biological relevance is uncertain.) The midpoints of the simulations for the three low-affinity transporters (curves 3, 4 and 5 in Figure 7) fell near the resting cholesterol concentration assumed for plasma membrane, ~41 mol%.[24] In contrast, the two high-affinity transport proteins with n = 4 (curve 2) and n = 2 (curve 1) had thresholds to the left of the physiological rest point, predicting their saturation with the sterol in the plasma membrane.

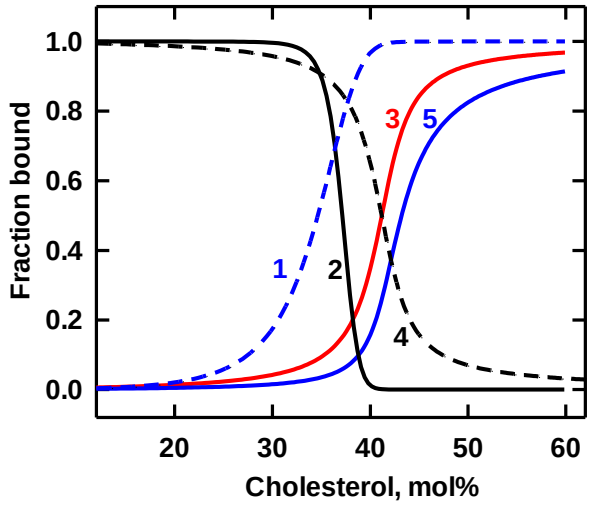

Figure 7. Simulation of the association of cholesterol with five transporters in a hypothetical plasma membrane. Using the values obtained above for K_{Ln} and n, we simulated sterol binding curves for the proteins in their native environment, the plasma membrane. The bilayer was represented by a 1:1 mixture of two phospholipids with stoichiometries of C:P = 1:1 and 1:2 and sterol association constants of 5,000 and 2,500, respectively.[24] The values for the affinities of the proteins are given in Table 7.

Table 7. Values used in Figure 7

Curve	Protein	n	K_{Ln}	Code
1	AChR	2	9×10^5	Blue dash
2	BK	4	1×10^{11}	Black line
3	GAT	1	100	Red line
4	Kir2	1	100	Black dash
5	Kir 3.4*	1	35	Blue line

In Figure 8, we simulated the association of the sterol with five of the integral membrane proteins in a hypothetical intracellular compartment. We assumed that the cytoplasmic endomembranes, taken as a whole, have a very low cholesterol content (namely, ≤ 10 mol%) and phospholipids of very low cholesterol affinity.[24, 25] It appears that proteins with modest cholesterol affinity (namely, those characterized in Figures 1-3) would be partially filled with cholesterol in such membranes while the oligomers with high affinity and cooperativity would

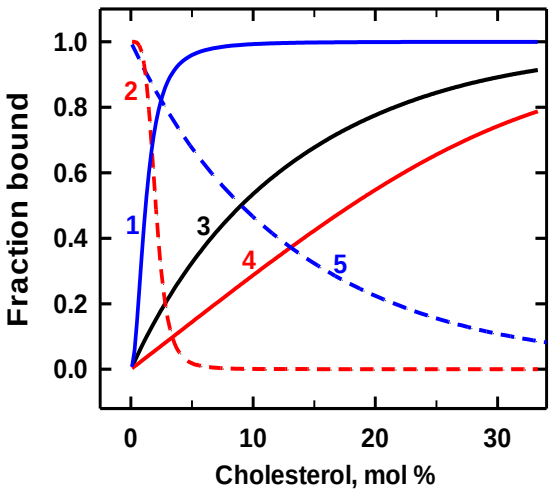

Figure 8. Simulation of the association of cholesterol with proteins in membranes with the phospholipid characteristics of hypothetical endomembranes. We used the values for K_{Ln} and n for five of the transporters studied above and endomembranes that contained a 30/70 mixture of two phospholipids with stoichiometries of C:P = 1:1 and 1:2 and sterol association constants K_P = 21 and 10, respectively.[24] The characteristics of the proteins are given in Table 8.

Table 8. Values used in Figure 8

Curve	Protein	n	K_{Ln}	Code
1	AChR	2	9×10^5	Blue line
2	BK	4	1×10^{11}	Red dash
3	GAT	1	100	Black line
4	Kir3.4*	1	35	Red line
5	Kir2	1	100	Blue dash

be essentially saturated. Of course, individual organelles such as the endoplasmic reticulum and distal Golgi membranes differ in their cholesterol content; also, the maturation and modifications of these proteins may vary among organelles. Thus, this exercise is simply heuristic.

DISCUSSION AND CONCLUSIONS

Cholesterol affects the structure and function of a variety of plasma membrane proteins (see Introduction). As illustrated by the Figures, their activities can have a deeply sigmoidal dependence on membrane cholesterol concentration. These thresholds can reflect both the cooperative binding of the sterol by oligomeric proteins and competition by membrane phospholipids for cholesterol.[46] Here, we present and apply a computational model that estimates the cholesterol association constant and binding stoichiometry of integral membrane proteins based on their competition with the membrane phospholipids for the sterol.

The approach was tested by matching simulations to the cholesterol dependence curves for the activities of six integral membrane proteins. GAT, Kir2 and Kir3.4* did not appear to bind cholesterol cooperatively; that is, n = 1 gave the best matches in Figures 1-3. Even so, these proteins could have bound multiple cholesterol molecules noncooperatively. The deep sigmoidicity of the isotherms suggests that their sterol affinities are weak (namely, K_{L1} = 35-100) compared to those of typical phospholipids, the K_P values of which are in the range of 100-5,000.[29] Figure 7 suggests that the cholesterol dependence of these proteins would have a threshold near stoichiometric equivalence with the plasma membrane phospholipids, ~41 mol%. This is the homeostatically-regulated physiologic setpoint of plasma membrane cholesterol.[24] Thus, as previously proposed, such proteins might have evolved to respond acutely to small variations in the concentration of plasma membrane cholesterol at its resting level.[30]

The data for the other three oligomers were not well fit using n = 1 (Figures 4-6). Rather, they appeared to bind the sterol cooperatively. Furthermore, their subunit association constants were roughly an order of magnitude greater than those of the three non-cooperative proteins (Table 9). Figure 7 suggests that this form of the BK channel and the acetylcholine receptor are saturated with cholesterol at the physiological setpoint of the plasma membrane; all else being equal, they might therefore be insensitive to small variations in its sterol concentration in vivo.

Table 9. Cholesterol binding characteristics estimated for six transporters

Protein	Figure	n	Subunit K_{L1}	Subunit ΔG^o, KJ/mol
GAT	1	1	100	-11.4
Kir3.4*	2	1	35	-8.8
Kir2	3	1	100	-11.4
BK	4	4	563	-15.7
Kir3.4* M182I	5	4	700	-16.2
AChR	6	2	950	-17.0

Table 9 gives the free energy changes inferred for the association of cholesterol with the six transporters. The values are in the middle of a range of estimates (from less than -5 to greater than -50 KJ/mole) computed by different methods for multiple binding sites on a variety of integral membrane proteins.[14-21, 45] Notably, the cluster of ΔG^o values obtained here (namely, -8.8 to -17.0 KJ/mol) fall in the range of those determined for G protein coupled receptors by molecular dynamics and docking methods (namely, ΔG^o = -12.9 to -20.0 KJ/mol); see the Supplement to Lee.[20]

The present analysis has limitations. (a) The experimental data sets were sparse, undermining the precise determination of sterol association constants and, especially, the binding stoichiometries which depend strongly on the contour of the isotherm. Note, however,

that the curves computed for all binding stoichiometries of a protein intersect at the same membrane cholesterol concentration and have the same K_{L1} value. Consequently, good estimates of the sterol affinity of the subunit of an oligomer can be secured even when its stoichiometry is uncertain. (b) The model may be too simple, given the complex behavior of many membrane proteins.[47] For example, a protein might not be an ideal oligomer with n values that are integers, as assumed here. Also, the model stipulates that the only effect of the bilayer on the binding of the sterol to the protein is through competition by the phospholipids while, in reality, other kinds of interactions may obtain.[4] On the other hand, the matches of simulation to data were satisfactory without the need for a more elaborate model. (c) In vitro may not replicate in vivo. For example, the nearly complete inhibition by cholesterol of the type of BK channel studied in Figure 4 might not obtain in vivo where factors such as cytoplasmic calcium, membrane voltage and accessory proteins serve as physiologic regulators.[38, 40] In this regard, the model could be used to compare values obtained for a plasma membrane protein *in vitro* and *in vivo*. (d) The analysis does not enumerate individual sterol binding sites on a protein or report on dynamics or other molecular features. Rather, it characterizes the equilibrium binding reaction that affects protein activity. (e) The K_{Ln} values obtained depend on characteristics assumed for the phospholipid mixtures. Only approximations are available for some of these.[29] This might not be a big problem, however. For example, doubling or halving the K_P value assigned to curve 3 in Figure 1 (namely, changing the K_P from 200 to 100 or 400) yielded K_{L1} values of 55 and 190 rather than the best match, K_{L1} = 100. The flanking K_{L1} estimates correspond to $\triangle G^o$ values of -9.9 and -13.0, not far from the best match: $\triangle G^o$ = -11.4 KJ/mole. Thus, the K_{Ln} values we have obtained should be useful despite their uncertain accuracy. (f) Could the experimental sigmoidal isotherms simply reflect cooperative cholesterol binding by the protein without competition by the phospholipids? Some of our simulations were consistent with this supposition (not shown). However, the extensive evidence for sterol-phospholipid complexation makes the central premise of the model secure.[23]

The model should be applicable to any integral membrane protein that binds cholesterol; for example, Patched and Scap-Insig.[20, 48] Future applications will surely deliver better estimates of K_{Ln} and n by applying more detailed sterol-dependence isotherms. Liposomes, planar bilayers, intact cells and other membrane preparations can be used. It would be best to employ synthetic bilayers containing a single phospholipid species with a stoichiometry of C:P = 1:1 and a well-determined cholesterol affinity; for example, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC).[29] For the study of plasma membrane proteins in situ, the characteristics of erythrocyte phospholipids can be used as the reference, as in Figure 3.[24, 29] The model posits that K_{Ln} and n will not vary from one bilayer environment to another; this is a testable premise. For example, the influence of charged phospholipids can be examined. In this regard, the values obtained for proteins in their native plasma membranes might reflect the influence of physiological factors—so, how they differ from those obtained using synthetic bilayers could be informative. The method not only enables the study of individual proteins in different environments but also the comparison of a variety of proteins under identical conditions. Such information might shed light on why these proteins have evolved to associate with sterols in the first place.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge. It contains a description of the model, the computational code and the derivation of Eqs.(5) and (6).

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