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

Theodore L. Steck[#], S. M. Ali Tabei[§] and Yvonne Lange[*]

[#]Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, Il 60637

[§]Department of Physics, University of Northern Iowa, Cedar Falls, Iowa 50614

[*]Department of Pathology, Rush University Medical Center, Chicago, Il 60612

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 simulation techniques. 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 model assumes competition for cholesterol between the proteins and the phospholipids. This competition makes the binding isotherms of the proteins sigmoidal with strongly-lagged thresholds. Using sterol association constants and stoichiometries for the phospholipids taken from the literature, we matched computed isotherms to experimental curves. 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 strongly and cooperatively 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 of phospholipids for which reasonable estimates of sterol affinities and stoichiometries can be assigned.

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 simulations 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 simulation and other computational methods.[4, 13-20]

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 is 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.[21-23] Using published values for the sterol affinity and stoichiometry of the membrane phospholipids, the model delivers estimates of the sterol affinity and stoichiometry of the proteins.

At one extreme, very avid proteins outcompete the phospholipids for the cholesterol and so bind it with a nearly hyperbolic isotherm. 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.[24] 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). Note that two forms of cholesterol are available to the protein: (a) that taken from membrane phospholipid complexes by strong competition; and (b) that readily available beyond the stoichiometric equivalence point of the complexes.[24]

METHODS

The model. This model simulates the binding of sterol molecules to a ligand within a phospholipid bilayer and estimates 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. [24] 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.[25, 26] For simplicity, we assume that the ligand (L) is 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.[27] 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 *Supplemental 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 %. The abundance of the protein (L_T) was set at 1×10^{-3} for biological membranes and mole liposomes and 1×10^{-5} for planar bilayers. (Variation in this small fraction of protein did not affect the results significantly and competition by extraneous proteins should not matter either.) 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.[28] We assumed that the stoichiometry of binding of phospholipid to cholesterol, r, was one or two.[23, 24, 28] The program allows membranes to contain two types of phospholipids with different cholesterol affinities and stoichiometries in different proportions. The membrane cholesterol concentration in most figures is expressed as mol% cholesterol; *i.e.*, 100

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

We matched computed isotherms to experimental data by eye by assigning various values to the cholesterol affinity and stoichiometry of the phospholipids and protein. The experimental data in Figures 1-6 were kept in their published form, scaled on the left-hand ordinates. Simulations were scaled from 0 to 1 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 so that the experimental data could be matched by various pairs of values for K_P and K_L. Consequently, the delivered association constants were not uniquely determined and the values for the phospholipids are therefore not definitive.

The model makes the simplifying assumption that the protein is a homooligomer that can bind cholesterol with perfect cooperativity. The association constant for an n-mer is therefore equal to that of one of its identical subunits raised to the n-th power; i.e., $K_{Ln} = (K_{L1})^n$. It can be shown from Eq. 4 that, for a given protein at half saturation (i.e., when $C_nL = L_f$), the free cholesterol concentration at any stoichiometry, n, is given by

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

Thus, $(C_f)_{1/2}$ is independent of the sterol binding stoichiometry, n.

Figures 2-6 suggest that all isotherms calculated for a protein intersect at essentially the same total cholesterol concentration, $(C_T)_{1/2}$, whatever the value of n. To examine this relationship, we made the reasonable assumption that (CnL) is orders of magnitude smaller than $(C_T)_{1/2}$. It then follows from Eqs. 2 and 4 that

$$(C_T)_{1/2} = (C_f)_{1/2} + K_P \times (P_f)_{1/2} / (K_{Ln})^{1/n} \quad (6)$$

$$= (C_f)_{1/2} + K_P \times (P_f)_{1/2} / K_{L1} \quad (7)$$

$$= (C_f)_{1/2} \times [1 + K_P \times (P_f)_{1/2} / \alpha] \quad (8)$$

Eq. 8 shows that the half-saturation point in an isotherm for a protein with affinity K_{Ln} is essentially independent of n. This verifies the observation that all such isotherms intersect at virtually the same $(CT)_{1/2}$.

RESULTS

Many integral plasma membrane proteins associate with cholesterol (see Introduction). However, sterol-dependence curves suitable for our analysis of these interactions are few.[29] Six such isotherms were tested here.

GAT. This family of oligomeric transporters facilitates the recovery of secreted γ-aminobutyric acid (GABA) across presynaptic plasma membranes, symported by NaCl gradients.[30] The data in Figure 1 show the cholesterol dependence of the transport activity of a purified rat brain GAT reconstituted in liposomes.[31] The overall sterol affinity of the phospholipids was not known, so we tested various K_P values for this polyunsaturated mixture.[28]

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). The other curves in Figure 1 explore the model. Curve 1 shows that when the phospholipids have a low sterol affinity, the protein binds cholesterol strongly with a nearly hyperbolic isotherm. Curves 2 and 4 show the results of doubling and halving the sterol affinity of the phospholipids assigned in

curve 3. The spread among curves 2, 3 and 4 thus indicates the sensitivity of the isotherm to the value of 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 the sharp rise of free cholesterol beyond its saturation of the phospholipid mixture at 42 mol%.

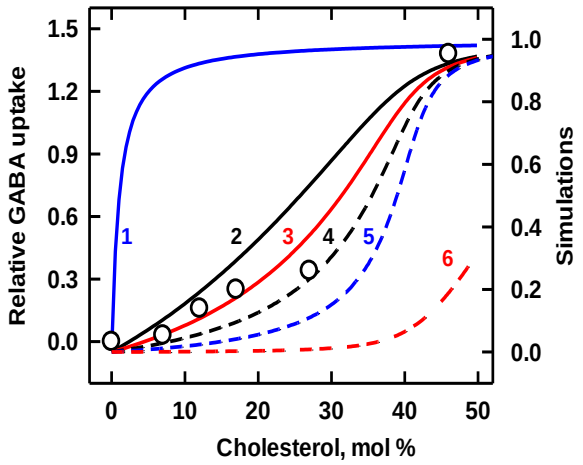

Figure 1. Simulation of the binding of cholesterol to a GAT transporter. Experimental data (symbols) depict the uptake of γ-aminobutyric acid (GABA) by liposomes containing the transporter, asolectin, brain phospholipids and varied cholesterol.[31] The simulations in curves 1-5 assigned K_{L1} = 100 and n = 1 to the protein. Lacking experimental values for this phospholipid mixture, we assumed that the bilayer contained equal proportions of two phospholipid species with stoichiometries of C:P = 1:1 and 1:2, both with the K_P association constants shown in Table 1.[28] 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 assuming that GAT has two or four identical binding sites; that is, n = 2 and 4. The affinity of the monomer was again taken to be K_{L1} = 100. These simulated binding curves had lagged thresholds and very acute ascents; they did not match the experimental data well (not shown). The simulations therefore suggest that the binding of sterol to 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.[32] 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%.

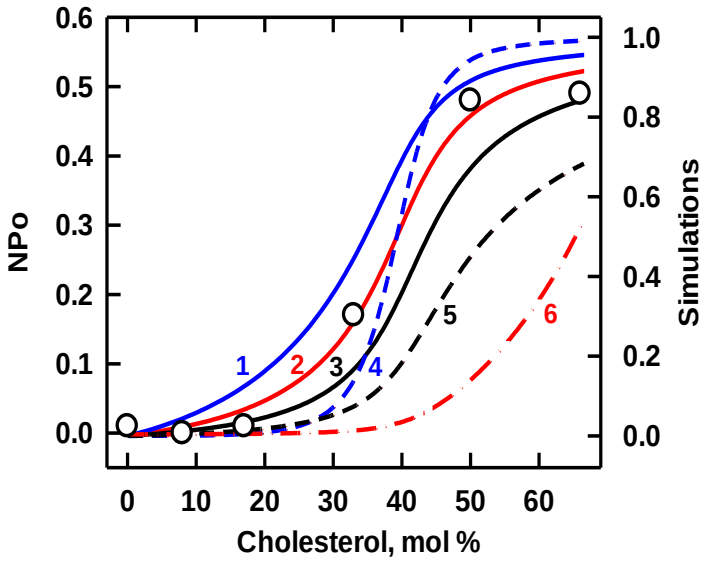

Figure 2. Simulation of the binding of cholesterol to Kir3.4*. Experimental data (open circles) represent the open probability (NPo) of single channels in a planar bilayer composed of 1:1 mixtures of 1-palmitoyl-2-oleoyl-phosphatidylserine and brain phosphatidylethanolamine.[32] The former phospholipid 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.[28] 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 intersected 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.[33, 34] The simulation of these data was slightly better

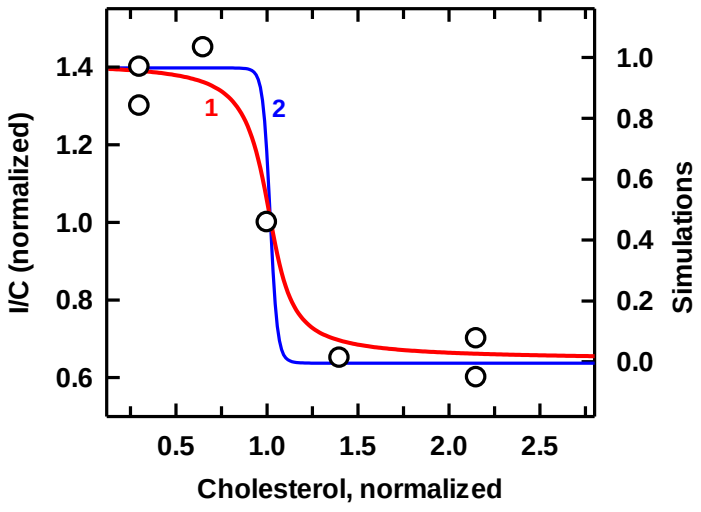

Figure 3. Simulation of the binding of cholesterol to Kir2 channels. Experimental data (open circles) give the peak current density (normalized I/C) obtained by patch clamping cultured bovine aortic endothelial cells that had been treated with methyl-β-cyclodextrin plus varied cholesterol to adjust the plasma membrane sterol concentration.[33] 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).[23] 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.[23] 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.[35] 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

for n = 1 (curve 1) than for n = 4 (curve 2), both by eye and from the value of R^2 (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 its phospholipid complexes but depends on the free cholesterol emerging at the stoichiometric equivalence point.

BK. This voltage-gated potassium channel is activated by cytoplasmic calcium ions with high positive cooperativity.[36, 37] However, as shown in Figure 4, it is inhibited by cholesterol.[37, 38] 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). A sterol affinity for the subunit of BK1 of K_{L1} = 563 gave a good match (Table 4). Thus, BK was much more avid for cholesterol than the three transporters analyzed above.

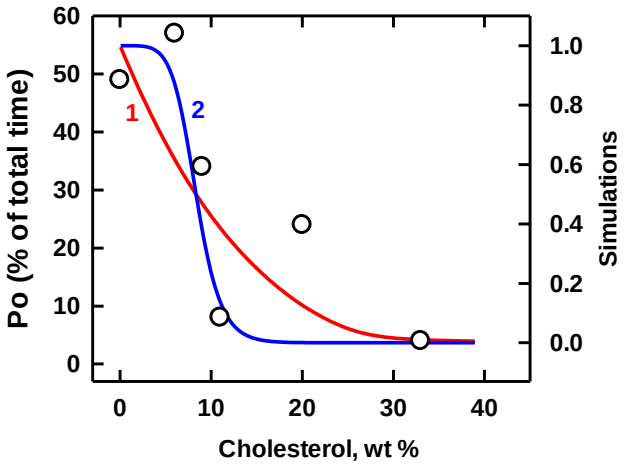

Figure 4. Simulation of the binding of cholesterol to BK channels. (Presumably, these were tetramers of the alpha subunit.[39]) Experimental data (open circles) represent the open probability (P_o) of single channels in a planar bilayer composed of a 1:1 mixture of 1-palmitoyl-2-oleoyl-phosphatidylserine plus 1-palmitoyl-2-oleoyl-phosphatidylethanolamine in the presence of 0.1 mM $CaCl_2$.[38] 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.[28] To convert the experimental cholesterol concentrations from percent of total lipid weight to mol%, we assumed molecular weights of 386 and 750 for C and P, respectively. Consequently, the midpoint of the curve, at 8.3 weight %, is equivalent to 12.7 mol% cholesterol. The values for BK listed in Table 4 were used in the calculations. The intersection of the curves at their half-saturation values is a prediction of the model (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 recombinant homotetrameric potassium channel was mutated in a recent study.[40] Our analysis showed that the cholesterol dependence of its conductance was altered in three ways. First, while cholesterol activated the wild type (Figure 2), it inhibited the variant protein (Figure 5). Second, whereas the open probability of the

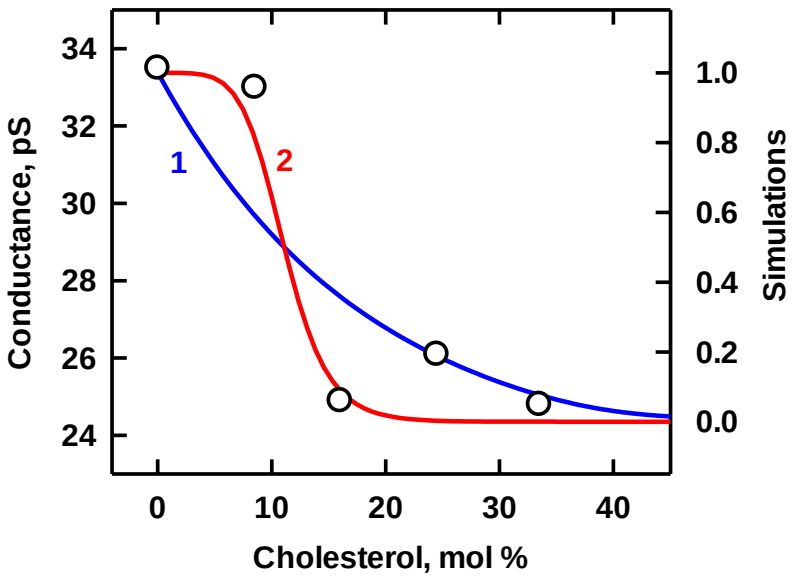

Figure 5. Simulation of the binding of cholesterol to Kir3.4* M182I channels.[40] Experimental data (open circles) represent the conductance of single channels in a planar bilayer composed of a 1:1 mixture of 1-palmitoyl-2-oleoyl-phosphatidylserine and 1-palmitoyl-2-oleoyl-phosphatidylethanolamine. We assumed that the two phospholipids had C:P stoichiometries of 1:2 and 1:1 and affinities of K_p = 210 and 130, respectively.[28] The computed curves used the protein values listed in Table 5. As predicted by the model, the curves intersected 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

unmodified channel in Figure 2 appeared to have a first-order dependence on cholesterol concentration, the data for the mutant were better matched by assuming n = 4 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 comparable bilayer environments, the striking difference in their isotherms can be attributed to the mutation in the protein.

Nicotinic acetylcholine receptor (AChR). This pentameric cation channel has complex allosteric behavior and specific binding sites for cholesterol.[41, 42] The data points in Figure 6 show the sterol-dependence of a conformational switch in the receptor from a desensitized to a resting state.[43] Simulation with n = 1 (curve 1) gave 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) provided an excellent match. Calculations using n = 3 or n = 4 gave intermediate simulations while varying the affinity of the phospholipids moved 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 isotherm.

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, with a free energy change of $\triangle G^o$ = -17.2 KJ/mole. Molecular dynamics simulation suggested the energy of interaction of cholesterol with subunits of the receptor to be -25 to -50 KJ/mole.[44] However, those simulations were performed in vacuo rather than in a bilayer.

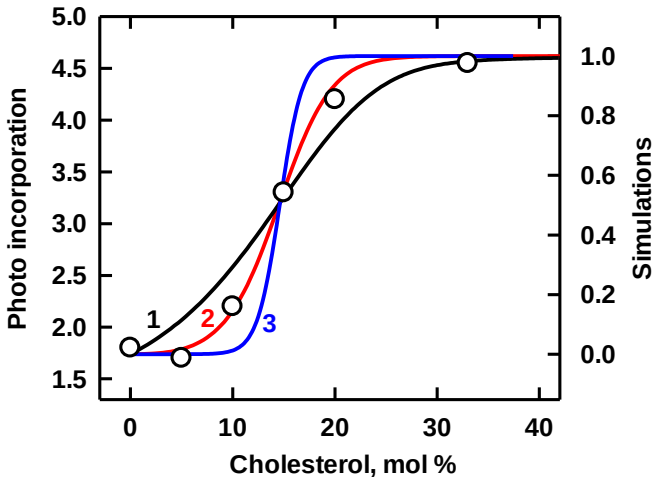

Figure 6. Simulation of the binding of cholesterol to nicotinic acetylcholine receptors. Experimental data (open circles) represent the labeling of the protein by a conformationally-sensitive radioactive diazirine probe in vesicles composed of 3:1 dioleoylphosphatidylcholine:dioleoylphosphatidic acid.[43] 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.[28] The calculations used the protein values listed in Table 6. As predicted by the model, the curves intersected 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 encouraged 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%.[23] 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 and were saturated with the sterol in the resting plasma membrane.

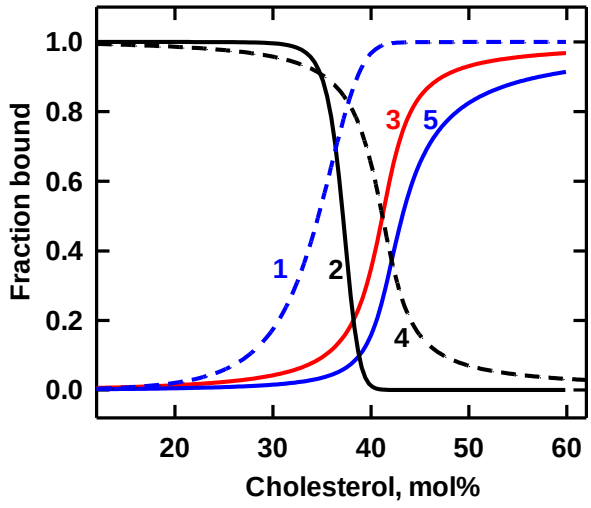

Figure 7. Simulation of the association of cholesterol with five transporters in a hypothetical plasma membrane. Using the values obtained above for K_{Ln} and n, we simulated sterol binding curves for the proteins in their native environment, the plasma membrane. The bilayer was represented by a 1:1 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.[23] 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

The cytoplasmic endomembranes, taken as a whole, appear to have a very low cholesterol content (namely, ≤ 10 mol%) and phospholipids of very low cholesterol affinity. [23, 24] In Figure 8, we simulated the association of the sterol with five of the integral membrane

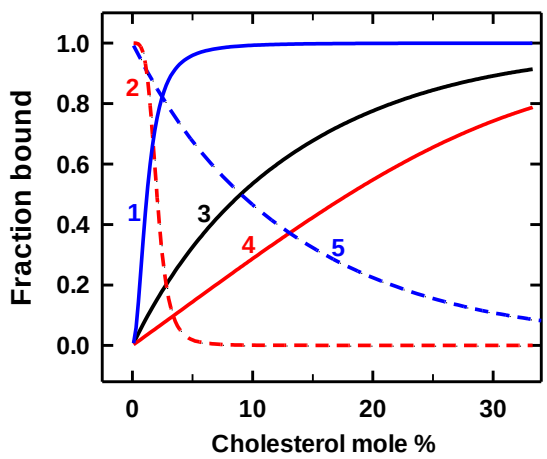

Figure 8. Simulation of the association of cholesterol with proteins in membranes with the overall phospholipid characteristics of hypothetical endomembranes. We used the values for K_{Ln} and n for five of the transporters studied above and endomembranes that contained a 30/70 mixture of two phospholipids with stoichiometries of C:P = 1:1 and 1:2 and sterol association constants K_P = 21 and 10, respectively.[23] 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

proteins in a hypothetical intracellular compartment. 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 be essentially saturated. Of course, individual organelles such as the endoplasmic reticulum and distal Golgi membranes would differ in their cholesterol contents and the proteins might differ with location in their maturation and modification. So, this hypothetical analysis 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.[45] Here, we present and apply a computational model that estimates the sterol association constant and binding stoichiometry of a protein based on its competition with the membrane phospholipids.

The model applies to any intramembrane ligand that binds cholesterol; even a third phospholipid. It was tested here 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 these 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.[28] 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.[23]

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.[29]

The data for the other three oligomers were not well fit using n = 1 (Figures 4-6). Rather, their binding of the sterol appeared to be cooperative. 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
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 for the association of cholesterol with the six transporters. The values are in the middle of a range of estimates from simulations computed by different methods for multiple binding sites on a variety of integral membrane proteins the values for which varied from less than -5 to greater than -50 KJ/mole.[14-20, 44]

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 computed curves for all binding stoichiometries of a protein intersect at the same membrane cholesterol concentration and yield 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.[46] For example, a given protein might not be an ideal oligomer with integer n values as assumed here. Also, the model assumes that the only effect of the bilayer is through the competition of phospholipids for the sterol 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 form of the BK channel studied in Figure 4 might not be observed in vivo where factors such as cytoplasmic calcium, membrane voltage and accessory proteins serve as physiologic regulators.[37, 39] 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 the sterol binding sites on a protein or report on dynamics or other molecular features. Rather, it characterizes the equilibrium binding process that affects protein activity. (e) The values obtained depend on characteristics assumed for the phospholipid mixtures. Only approximations are available for some of these.[28] 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 sigmoidal isotherms for the experimental data simply reflect cooperative cholesterol binding by the protein—that is, without competition by the phospholipids? Some of our simulations revealed that this can be the case (not shown). However, extensive evidence for sterol-phospholipid complexation makes the central premise of the model secure.[22]

Future applications will surely deliver better estimates of K_{Ln} and n by using more detailed sterol-dependence isotherms. Liposomes, planar bilayers, intact cells or other membrane preparations can be used. It would be best to use 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.[28] For the study of plasma membrane proteins in vivo, the characteristics of erythrocyte phospholipids can be used as the reference, as in Figure 3.[23, 28] The values obtained for proteins in their native plasma membranes could reflect the influence of physiological factors and how they differ from those obtained in artificial bilayers could be informative. The model posits that K_{Ln} and n will not vary from one bilayer environment to another; this is a testable premise. The method allows the comparison of a variety of proteins under identical conditions as well as a single protein in different environments without knowing the affinity and stoichiometry of the phospholipids. 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 and the computational code.

AUTHOR INFORMATION

Corresponding Author

Theodore L. Steck -- Department of Biochemistry and Molecular Biology, University of Chicago, 929 East 57th Street, Chicago, Il 60637. Phone: 773 667-8346. tsteck@bsd.uchicago.edu. ORCID: https://orcid.org/0000-0002-8391-9244

Authors

S. M. Ali Tabei -- Department of Physics, University of Northern Iowa, Cedar Falls, Iowa 50614

Yvonne Lange -- Department of Pathology, Rush University Medical Center, Chicago, Il 60612.

ORCID: http://orcid.org/0000-0003-2095-826X

Notes The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Francisco Barrantes (Pontifical Catholic University of Argentina), Anna Bukiya (University of Tennessee Health Science Center), Elena Levitan (University of Illinois at Chicago) and Eduardo Perozo (University of Chicago).

REFERENCES

[1] Song, Y. L., Kenworthy, A. K., and Sanders, C. R. (2014) Cholesterol as a co-solvent and a ligand for membrane proteins, *Protein Sci 23*, 1-22.

[2] Gimpl, G. (2016) Interaction of G protein coupled receptors and cholesterol, *Chemistry and Physics of Lipids 199*, 61-73.

[3] Fantini, J., Epand, R. M., and Barrantes, F. J. (2019) Cholesterol-Recognition Motifs in Membrane Proteins, In *Direct Mechanisms in Cholesterol Modulation of Protein Function* (RosenhouseDantsker, A., and Bukiya, A. N., Eds.), pp 3-25.

[4] Muller, M. P., Jiang, T., Sun, C., Lihan, M. Y., Pant, S., Mahinthichaichan, P., Trifan, A., and Tajkhorshid, E. (2019) Characterization of Lipid-Protein Interactions and Lipid-Mediated Modulation of Membrane Protein Function through Molecular Simulation, *Chemical Reviews 119*, 6086-6161.

[5] Duncan, A. L., Song, W., and Sansom, M. S. P. (2020) Lipid-Dependent Regulation of Ion Channels and G Protein-Coupled Receptors: Insights from Structures and Simulations, *Annu Rev Pharmacol Toxicol 60*, 31-50.

[6] Duncan, A. L., Corey, R. A., and Sansom, M. S. P. (2020) Defining how multiple lipid species interact with inward rectifier potassium (Kir2) channels, *Proc Natl Acad Sci U S A 117*, 7803-7813.

[7] Marlow, B., Kuenze, G., Li, B., Sanders, C. R., and Meiler, J. (2021) Structural determinants of cholesterol recognition in helical integral membrane proteins, *Biophysical Journal 120*, 1592-1604.

[8] Bukiya, A. N., and Rosenhouse-Dantsker, A. (2023) From Crosstalk to Synergism: The Combined Effect of Cholesterol and PI(4,5)P2 on Inwardly Rectifying Potassium Channels, In *Cholesterol and PI(4,5)P2 in Vital Biological Functions: From Coexistence to Crosstalk* (Dantsker, A. R., Ed.), pp 169-191, Springer International Publishing, Cham.

[9] Isu, U. H., Badiee, S. A., Khodadadi, E., and Moradi, M. (2023) Cholesterol in Class C GPCRs: Role, Relevance, and Localization, *Membranes 13*, 17.

[10] Levental, I., and Lyman, E. (2023) Regulation of membrane protein structure and function by their lipid nano-environment, *Nat Rev Mol Cell Biol 24*, 107-122.

[11] Song, Y., Hustedt, E. J., Brandon, S., and Sanders, C. R. (2013) Competition between homodimerization and cholesterol binding to the C99 domain of the amyloid precursor protein, *Biochemistry 52*, 5051-5064.
[12] Papadopoulos, N., Suelves, N., Perrin, F., Vadukul, D. M., Vrancx, C., Constantinescu, S. N., and Kienlen-Campard, P. (2022) Structural Determinant of β-Amyloid Formation: From Transmembrane Protein Dimerization to β-Amyloid Aggregates, *Biomedicines 10*, 2753.
[13] Lee, J. Y., and Lyman, E. (2012) Predictions for cholesterol interaction sites on the A2A adenosine receptor, *J Am Chem Soc 134*, 16512-16515.
[14] Rosenhouse-Dantsker, A., Noskov, S., Durdagi, S., Logothetis, D. E., and Levitan, I. (2013) Identification of novel cholesterol-binding regions in Kir2 channels, *J Biol Chem 288*, 31154-31164.
[15] Domicevica, L., Koldsø, H., and Biggin, P. C. (2018) Multiscale molecular dynamics simulations of lipid interactions with P-glycoprotein in a complex membrane, *Journal of Molecular Graphics and Modelling 80*, 147-156.
[16] Corey, R. A., Stansfeld, P. J., and Sansom, M. S. P. (2019) The energetics of protein–lipid interactions as viewed by molecular simulations, *Biochem Soc T 48*, 25-37.
[17] Hedger, G., Koldso, H., Chavent, M., Siebold, C., Rohatgi, R., and Sansom, M. S. P. (2019) Cholesterol Interaction Sites on the Transmembrane Domain of the Hedgehog Signal Transducer and Class F G Protein-Coupled Receptor Smoothened, *Structure 27*, 549-559 e542.
[18] Ansell, T. B., Curran, L., Horrell, M. R., Pipatpolkai, T., Letham, S. C., Song, W. L., Siebold, C., Stansfeld, P. J., Sansom, M. S. P., and Corey, R. A. (2021) Relative Affinities of Protein-Cholesterol Interactions from Equilibrium Molecular Dynamics Simulations, *J. Chem. Theory Comput. 17*, 6548-6558.
[19] Lee, A. G. (2021) Interfacial binding sites for cholesterol on GABA(A) receptors and competition with neurosteroids, *Biophysical Journal 120*, 2710-2722.
[20] Tzortzini, E., Corey, R. A., and Kolocouris, A. (2023) Comparative Study of Receptor-, Receptor State-, and Membrane- Dependent Cholesterol Binding Sites in A2A and A1 Adenosine Receptors Using Coarse-Grained Molecular Dynamics Simulations, *J. Chem Inf. Model. 63*, 928-949.
[21] Lange, Y., and Steck, T. L. (2008) Cholesterol homeostasis and the escape tendency (activity) of plasma membrane cholesterol, *Prog Lipid Res 47*, 319-332.
[22] Lange, Y., and Steck, T. L. (2020) Active cholesterol 20 years on, *Traffic 21*, 662-674.
[23] Steck, T. L., Tabei, S. M. A., and Lange, Y. (2021) A basic model for cell cholesterol homeostasis, *Traffic 22*, 471-481.
[24] Lange, Y., Tabei, S. M. A., and Steck, T. L. (2023) A basic model for the association of ligands with membrane cholesterol: application to cytolysin binding, *Journal of Lipid Research 64*, 100344.
[25] Yifrach, O., Zandany, N., and Shem-Ad, T. (2009) EXAMINING COOPERATIVE GATING PHENOMENA IN VOLTAGE-DEPENDENT POTASSIUM CHANNELS: TAKING THE ENERGETIC APPROACH, In *Methods in Enzymology, Vol 466: Biothermodynamics, Pt B* (Johnson, M. L., Holt, J. M., and Ackers, G. K., Eds.), pp 179-+, Elsevier Academic Press Inc, San Diego.
[26] Banerjee, K., Das, B., and Gangopadhyay, G. (2013) On the estimation of cooperativity in ion channel kinetics: Activation free energy and kinetic mechanism of Shaker K+ channel, *The Journal of Chemical Physics 138*.
[27] Morea, V., Angelucci, F., Tame, J. R. H., Di Cera, E., and Bellelli, A. (2022) Structural Basis of Sequential and Concerted Cooperativity, *Biomolecules 12*, 1651.

[28] Lange, Y., Tabei, S. M. A., Ye, J., and Steck, T. L. (2013) Stability and Stoichiometry of Bilayer Phospholipid–Cholesterol Complexes: Relationship to Cellular Sterol Distribution and Homeostasis, *Biochemistry 52*, 6950-6959.
[29] Lange, Y., and Steck, T. L. (2016) Active membrane cholesterol as a physiological effector, *Chem Phys Lipids 199*, 74-93.
[30] Scimemi, A. (2014) Structure, function, and plasticity of GABA transporters, *Frontiers in Cellular Neuroscience 8*.
[31] Shouffani, A., and Kanner, B. I. (1990) Cholesterol is required for the reconstruction of the sodium- and chloride-coupled, gamma-aminobutyric acid transporter from rat brain, *J Biol Chem 265*, 6002-6008.
[32] Bukiya, A. N., Osborn, C. V., Kuntamallappanavar, G., Toth, P. T., Baki, L., Kowalsky, G., Oh, M. J., Dopico, A. M., Levitan, I., and Rosenhouse-Dantsker, A. (2015) Cholesterol increases the open probability of cardiac KACh currents, *Biochim Biophys Acta 1848*, 2406-2413.
[33] Romanenko, V. G., Rothblat, G. H., and Levitan, I. (2002) Modulation of endothelial inward-rectifier K+ current by optical isomers of cholesterol, *Biophysical Journal 83*, 3211-3222.
[34] Fang, Y., Emile, R. M., Hsieh, E., Osman, H., Hashemi, S. M., Davies, P. F., Rothblat, G. H., Wilensky, R. L., and Levitan, I. (2006) Hypercholesterolemia suppresses inwardly rectifying K+ channels in aortic endothelium in vitro and in vivo, *Circulation Research 98*, 1064-1071.
[35] Lange, Y., Ye, J., and Steck, T. L. (2014) Essentially all excess fibroblast cholesterol moves from plasma membranes to intracellular compartments, *PLoS One 9*, e98482.
[36] Hite, R. K., Tao, X., and MacKinnon, R. (2017) Structural basis for gating the high-conductance Ca(2+)-activated K(+) channel, *Nature 541*, 52-57.
[37] North, K. C., Zhang, M., Singh, A. K., Zaytseva, D., Slayden, A. V., Bukiya, A. N., and Dopico, A. M. (2022) Cholesterol Inhibition of Slo1 Channels Is Calcium-Dependent and Can Be Mediated by Either High-Affinity Calcium-Sensing Site in the Slo1 Cytosolic Tail, *Molecular Pharmacology 101*, 132-143.
[38] Chang, H. M., Reitstetter, R., Mason, R. P., and Gruener, R. (1995) Attenuation of channel kinetics and conductance by cholesterol: an interpretation using structural stress as a unifying concept, *J Membr Biol 143*, 51-63.
[39] Bukiya, A. N., Leo, M. D., Jaggar, J. H., and Dopico, A. M. (2021) Cholesterol activates BK channels by increasing KCNMB1 protein levels in the plasmalemma, *J Biol Chem 296*, 100381.
[40] Corradi, V., Bukiya, A. N., Miranda, W. E., Cui, M., Plant, L. D., Logothetis, D. E., Tieleman, D. P., Noskov, S. Y., and Rosenhouse-Dantsker, A. (2022) A molecular switch controls the impact of cholesterol on a Kir channel, *Proceedings of the National Academy of Sciences of the United States of America 119*, 10.
[41] Changeux, J.-P. (2018) The nicotinic acetylcholine receptor: a typical ‘allosteric machine’, *Philosophical Transactions of the Royal Society B: Biological Sciences 373*, 20170174.
[42] Barrantes, F. J. (2021) Nanoscale interactions between the nicotinic acetylcholine receptor and cholesterol, *Biocell 45*, 1479-1484.
[43] Hamouda, A. K., Sanghvi, M., Sauls, D., Machu, T. K., and Blanton, M. P. (2006) Assessing the lipid requirements of the Torpedo californica nicotinic acetylcholine receptor, *Biochemistry 45*, 4327-4337.
[44] Baier, C. J., Fantini, J., and Barrantes, F. J. (2011) Disclosure of cholesterol recognition motifs in transmembrane domains of the human nicotinic acetylcholine receptor, *Sci Rep 1*, 69.
[45] Ferrell, J. E., Jr., and Ha, S. H. (2014) Ultrasensitivity part II: multisite phosphorylation, stoichiometric inhibitors, and positive feedback, *Trends Biochem Sci 39*, 556-569.

[46] Zerbetto De Palma, G., Recoulat Angelini, A. A., Vitali, V., González Flecha, F. L., and Alleva, K. (2023) Cooperativity in regulation of membrane protein function: phenomenological analysis of the effects of pH and phospholipids, *Biophysical Reviews*.