

Evidence that specific interactions play a role in the cholesterol sensitivity of G protein-coupled receptors

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Abstract: 2

222 G protein-coupled receptors (GPCRs) are known to be modulated by membrane
222 cholesterol levels, but whether or not the effects are caused by specific receptor-
222 cholesterol interactions or cholesterol's general effects on the membrane is not well-
222 understood. We performed coarse-grained molecular dynamics (CGMD) simulations
222 coupled with structural bioinformatics approaches on the β_2 -adrenergic receptor (β_2 AR)
222 and the cholecystokinin (CCK) receptor subfamily. The β_2 AR has been shown to be
222 sensitive to membrane cholesterol and cholesterol molecules have been clearly resolved
222 in numerous β_2 AR crystal structures. The two CCK receptors are highly homologous and
222 preserve similar cholesterol recognition motifs but despite their homology, CCK₁R shows
222 functional sensitivity to membrane cholesterol while CCK₂R does not. Our results offer
222 new insights into how cholesterol modulates GPCR function by showing cholesterol
222 interactions with β_2 AR that agree with previously published data; additionally, we
222 observe differential and specific cholesterol binding in the CCK receptor subfamily while
222 revealing a previously unreported Cholesterol Recognition Amino-acid Consensus
222 (CRAC) sequence that is also conserved across 38% of class A GPCRs. A thermal
222 denaturation assay (LCP-T_m) shows that mutation of a conserved CRAC sequence on
222 TM7 of the β_2 AR affects cholesterol stabilization of the receptor in a lipid bilayer. The
222 results of this study provide a better understanding of receptor-cholesterol interactions
222 that can contribute to novel and improved therapeutics for a variety of diseases. 2

222 Introduction

222 G protein-coupled receptors (GPCRs) are the largest family of integral membrane
222 proteins in eukaryotic cells. Characterized by a conserved seven transmembrane helical

bundle, GPCRs are classified into five distinct phylogenetic families: class A/rhodopsin, class B/secretin, class C/glutamate, class F/frizzled, and Adhesion, with the vast majority of receptors being found in class A [1] (Fig. 1A). GPCRs play essential roles in physiological pathways and cell signaling events, making them important potential drug targets [2].

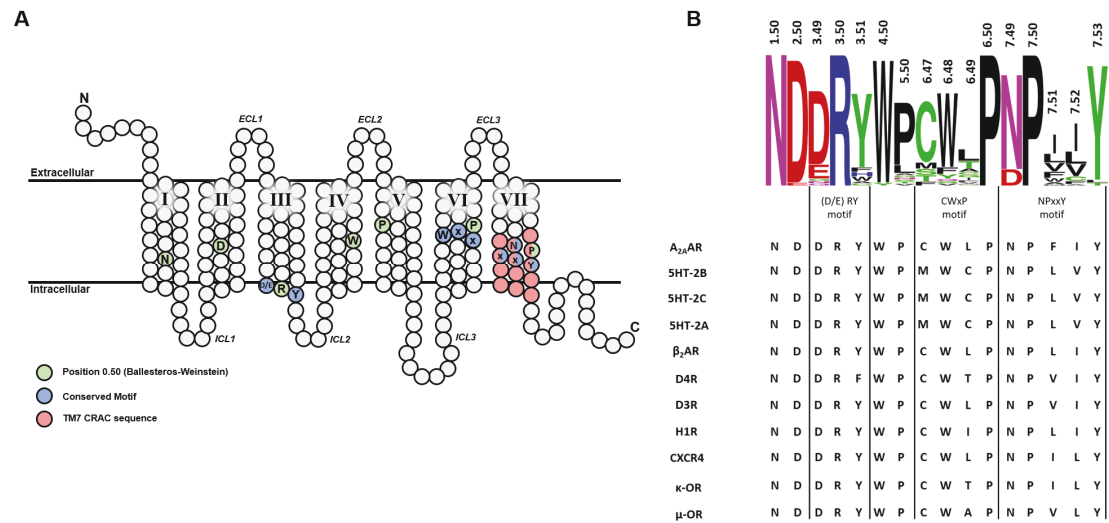


Figure 1. Location and conservation of important residues across Class A GPCRs. (A) Snake plot shows the location of well-conserved residues and motifs in class A GPCRs in the transmembrane bundle. (B) Cartoon visualization of the most conserved residues on each transmembrane helix as well as well-conserved motifs. Eleven well-studied GPCRs are shown.

GPCRs are commonly found residing in lipid rafts, which are highly ordered regions of the bilayer due to their high cholesterol content [3]. GPCR sequestration to these ordered microdomains had led to the hypothesis that cholesterol acts as an allosteric modulator for GPCR functions. Studies involving membrane cholesterol depletion showed specific altered signaling for the β₂-Adrenergic Receptor (β₂AR) [4] the cannabinoid receptor CB₁ [5], and serotonin receptor 5HT_{1A} [6, 7]; while cholesterol presence is required for the high affinity active state of the oxytocin receptor [8, 9]. Molecular spatial distribution analysis showed high density distribution of cholesterol

around Pro^{7.48} (Ballesteros-Weinstein numbering) [22] at the bottom of TM7 of rhodopsin associated with receptor activation [22] (Fig. 1A). [22]

Additional evidence suggested that other receptors (e.g. Smoothed, chemokine receptor CXCR2, and GPR183) may directly bind cholesterol through specific interactions [2222] with multiple specific GPCR-cholesterol interaction sites having been identified [2222]. The CCM motif, first characterized from a structure of the β_2 AR, is strictly conserved in 21% of class A GPCRs with 44% of class A receptors containing a slightly relaxed form of the motif [22]. CRAC sequences, defined as $-(L/V)-(X_{1-5})-Y-(X_{1-5})-(R/K)-$, where X_{1-5} represents 1-5 residues of any amino acid, are considered the most well-known cholesterol recognition motif in literature and are found on many GPCRs [22]. Cholesterol molecules were clearly resolved in the crystal structures of numerous receptors [22], but questions remain regarding whether the cholesterol molecules found in these crystal structures represent physiologically relevant and specific interactions, or if they are simply crystallization artifacts due to the abundance of cholesterol in the lipid bilayer and proximity to receptor molecules.[22]

Alternatively, there is evidence suggesting that the observed cholesterol effects arise indirectly through the physical effects of cholesterol on the lipid bilayer [22]. Cholesterol is known to alter membrane fluidity [22], thickness [22], and curvature [22]. Membrane fluidity was shown to have a direct effect on ligand binding at the 5HT_{1A} [22] and the type-1 cholecystokinin receptor (CCK₁R) [22]. Similarly, membrane thickness and curvature is known to affect the active/inactive equilibrium of Rhodopsin [22]. Taken together, there is a critical need to address and reconcile the apparent ambiguities in how cholesterol exerts its effect on GPCR functions. [22]

Here, we set out to probe the specificity of cholesterol-GPCR interactions through a combination of coarse-grained molecular dynamics (CGMD) simulations [22, 23] with structural bioinformatics and experimental validation using β_2 AR as the model system and apply the structural dynamics and bioinformatics approaches to the CCK receptor family. CGMD has been widely used to predict and elucidate detailed interactions of GPCRs with lipids [22,23], in particular the interaction of cholesterol with GPCRs [22,23,24]. The MARTINI CG model that we are using here constrains the secondary structure and prevents conformational changes [25]; therefore, our results report on cholesterol-protein interactions with protein conformations close to the experimental crystal structures or models and do not include either conformational changes induced by cholesterol or different conformational states of the receptors. All-atom MD simulations do not share the same limitations but are computationally much more expensive and thus limited in the length of achievable simulations and sampling of protein-lipid interactions [26]. Given the previous successes of CGMD simulations [22,23,24] and the need to collect statistics to obtain reasonable estimates of long cholesterol binding times, we chose CGMD as the appropriate level of approximation that generates sufficient sampling. Further, we focus the study on the CCK receptor family due to the differential effects cholesterol exerts: the CCK₁R ligand binding and downstream signaling are both modulated by membrane cholesterol levels, while the highly homologous (53% identity overall, and 69% in the transmembrane regions) gastrin receptor (CCK₂R) is unaffected by cholesterol [27]. Even though both CCK receptor subtypes share cholesterol recognition motif sites, the functional implications of membrane cholesterol composition are quite distinct. Our results show that the CCK receptors interact with cholesterol

through different motifs despite similar recognition sequences on the primary level. The CRAC motif at the bottom of TM3 has been identified as the functionally important site in CCK₁R [36]. The CCK₁R has been implicated as a potential therapeutic target to treat metabolic syndrome and obesity, with an allosteric modulator designed that can prevent the inhibitory effects of the high cholesterol environment associated with obese patients [37, 38].

Lastly, we discover a new CRAC motif near the end of transmembrane-helix seven (TM7) that is conserved across 38% of class A GPCRs (Fig. 1A). The results reported herein elucidate GPCR-cholesterol interactions in greater detail and could prove insightful in the development of additional allosteric therapeutics specifically targeting the CCK₁R.

Results:

We performed CGMD simulations of β_2 AR and the two CCK receptors in a mixed POPC:cholesterol membrane and analyzed the residency times of cholesterol at a per-residue resolution. Simulations were performed in 20% and 40% cholesterol. The 20% simulations appeared to better discriminate residues with long residency times from ones with shorter times and we therefore present the results for 20% cholesterol while results for 40% cholesterol are shown in the Supplementary Information. In the following discussion we focus on computational results that are consistent between the 20% and 40% simulations as the most robust findings. For the β_2 AR, residues from the CCM motif including I153^{4.45} and W158^{4.50} showed peaks just below the labeling cutoff of our analysis (τ_{slow} = 140 and 86 ns, respectively). I55^{1.54} also showed prolonged interaction

times with cholesterol molecules ($\tau_{\text{slow}}=84$ ns just below the labeling cutoff in Fig. 2A). In certain structure models (e.g., PDB: 2RH1) a cholesterol molecule is found within 5 Å from this residue. Some of the strongest signals arose from V157^{4,49}, T164^{4,56}, and W313^{7,40} (Fig. 2A). Though not observed in crystal structures, these residues have been implicated to interact with cholesterol as well [30, 39, 40].

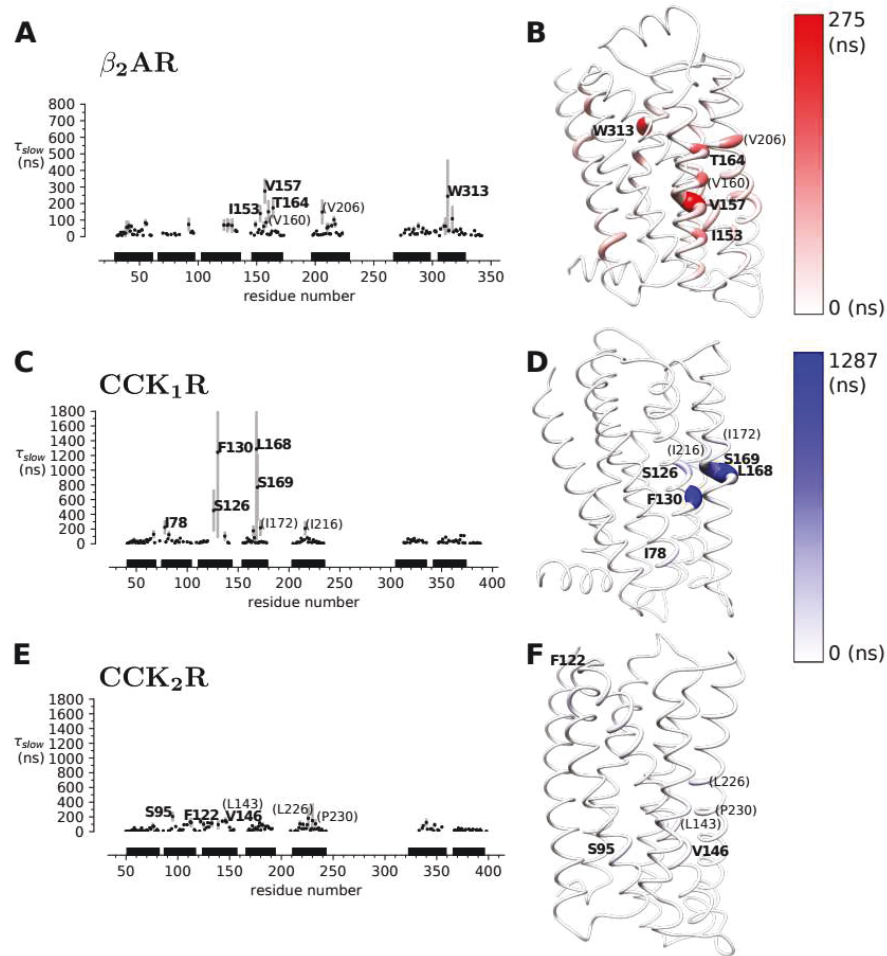


Figure 2. Cholesterol residency times τ_{slow} by residue from CGMD simulations with 20% cholesterol. (A), (C), (E): Residency times at the $\beta_2\text{AR}$, CCK_1R , and CCK_2R . Data points are the means over multiple simulation repeats and approximately 20- μs simulation blocks, with the standard deviation shown as a symmetric bar. Residues for which the mean τ_{slow} is at least three times the overall mean over all residency times in the protein are explicitly labelled. Residues that show consistently high residency times in the 20% and 40% cholesterol simulations are bold-faced whereas other residues that are only apparent in either simulation set are shown in parentheses in normal typeface. In C, the error bars of F130 and L168 are truncated at high values to focus the range of the plot on the means (which is set to be comparable between the 20% data here and the 40% data in Supplementary Fig. S2). (B), (D), (F): Structure models highlight residues with long residency times. The radius of the ribbon is scaled proportional to the average residency time and colored according to the color scale.

With the successful application of our CGMD approach to α_2 AR, we next used the same methods to study the CCKRs. On the CCK₁R, we observed strong receptor-cholesterol interactions surrounding TM3 with the highest residency time out of all of the receptor targets in this study. Furthermore, few CCK₁R residues have residency times higher than the background signal. A binding pocket at TM3 is further supported by long interactions at F130^{3,41}, L168^{4,52}, and S169^{4,53} (Fig. 2). While the CCK₁R-cholesterol interactions were mainly centered around TM3, the CCK₂R interactions were more spread throughout the receptor and residency times were all close to background. However, some interactions (such as at S95^{2,45}) suggest that CCK₂R may also interact with cholesterol molecules via the CCM motif mentioned above (Fig. 2, 3C). Direct comparison of the residency times for structurally equivalent residues between the two receptors demonstrated that residues in the CCK₁R typically bound cholesterol longer than the corresponding residues in CCK₂R (Fig. 3B). These results were consistent between the simulations with 20% and 40% cholesterol concentrations (Supplementary Fig. S3), implying that the CGMD simulations are able to differentiate which of the two homologous receptors interacts preferentially with cholesterol.

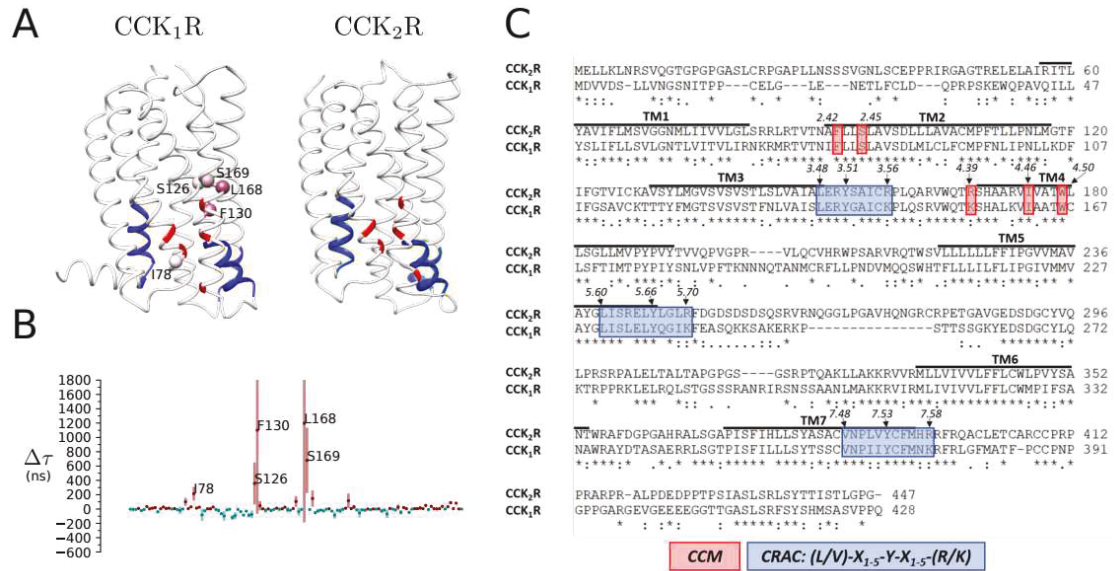


Figure 3. Differential interactions with cholesterol in the CCK subfamily. (A) Structure models of the CCK receptors. CRAC sequences are shown in blue and the CCM motif is shown in red. Spheres represent residues that showed differential cholesterol residency during the simulation and are colored by $\Delta\tau$. (B) Difference in residency times $\Delta\tau = \tau_{\text{slow}}(\text{CCK}_1\text{R}) - \tau_{\text{slow}}(\text{CCK}_2\text{R})$ for equivalent residues according to the sequence alignment. Maroon represents residues that bind cholesterol for longer times (higher τ_{slow}) in CCK₁R whereas cyan represents residues with higher τ_{slow} in CCK₂R than in CCK₁R. Error bars on the differences represent the standard deviation. (C) Sequence alignment of the two CCK receptors. The CCM motif is highlighted in red and CRAC sequences are highlighted in blue. Arrows highlight the residues that make the recognition sequence. The specific fixed residues of these motifs are annotated with their canonical residue numbers.

During our analysis of the interactions observed on the $\beta_2\text{AR}$, we noticed a CRAC sequence made up of residues L324^{7.51}, Y326^{7.53}, and R328^{7.55} on the intracellular side of TM7. This CRAC site is centered around the highly conserved NPxxY motif in class A GPCRs [41]. Thus, we aligned all class A GPCRs (excluding olfactory receptors) using the structure-based alignment tool from the GPCR database [42] to see if this new CRAC site is also present in other class A GPCRs [42]. In all, 38% of the 285 class A GPCRs that were aligned and analyzed were found to contain a CRAC sequence surrounding the NPxxY motif (Supplementary File S1). On the CCK receptors, this novel CRAC sequence consists of V365^{7.48}, Y370^{7.53}, and K374^{7.57} on the CCK₁R, and V385^{7.48}, Y390^{7.53}, and R394^{7.57} on the CCK₂R (Fig. 3C).

To better understand possible cholesterol interactions on TM7, we performed a series of mutations on the β_2 AR and used the LCP-T_m assay to quantify the stabilizing effects of cholesterol binding to the receptor [22]. We analyzed single point mutations at the newly discovered CRAC sequence along with other residues on TM7. A summary of all mutations tested is shown in Table 1. Two mutations, Y326A^{7.53} and F336A^{8.54}, nearly abolished protein expression completely. Of the β_2 AR constructs with reasonable expression, four of the seven remaining mutants were highly aggregated after purification in the presence of the high-affinity ligand timolol (Table 1). Due to poor protein expression and aggregation of most of the mutants, we were only able to collect LCP-T_m data on two mutant receptors alongside an unmutated receptor control sample. The first, F321A^{7.48} (located two helix turns near W313^{7.40}) was included as it could potentially interact with cholesterol molecules on the TM7 CRAC site. This residue has also been implicated to interact with cholesterol in previous studies [22]. Similarly, R328A^{7.55} was incorporated due to its position in the TM7 CRAC sequence (Fig. 4C). Previous studies showed the unmutated β_2 AR to be stabilized by 2.3°C when 10% (w/w) cholesterol was added to the host lipid monoolein relative to the monoolein-only sample [22]. In our experiments, the T_m of unmutated β_2 AR is 43.45 ± 0.65 °C (monoolein-only) and 45.15 ± 0.54 °C (monoolein with 10% cholesterol) resulting in a Δ T_m of 1.71 ± 0.85°C (Fig. 4A/B). The F321A and R328A mutations showed similar transition temperatures to the unmutated control in cholesterol-free assays, with T_m values of 42.65 ± 0.21 and 43.10 ± 0.74 °C, respectively. In cholesterol, the F321A mutant had a very slight increase in stability with a Δ T_m value of 0.46 ± 0.77 °C. Surprisingly, the R328A mutant had the largest Δ T_m value at 5.75 ± 1.65 °C with the addition of cholesterol into the host lipid

(Fig. 4). Given that CGMD simulations showed a clear pattern of differential cholesterol binding in the CCKRs, we performed CGMD simulations of the R328A mutant to test if the mutation would have a strong effect on cholesterol binding as measured by our residence time analysis. The difference in residence times between WT and mutant receptor were not significantly different from 0 (Supplementary Fig. 4), unlike the case for CCK1R vs CCK2R.

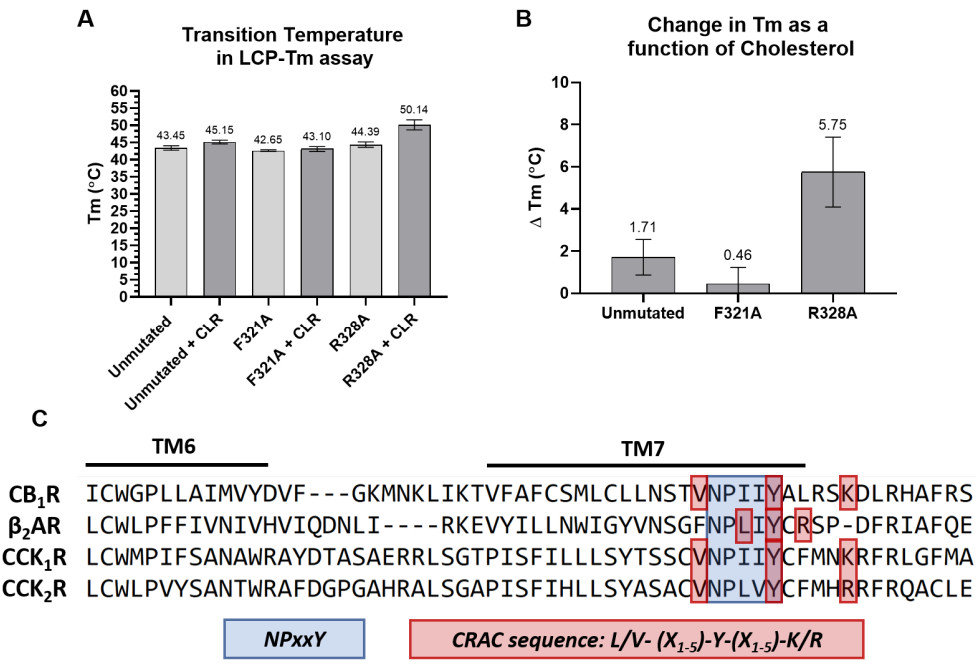


Figure 4. A conserved CRAC sequence modulates cholesterol-induced stability on the β₂AR. (A) Mean transition temperatures from LCP-Tm assay with and without cholesterol in the LCP. (B) ΔT_m values for each mutant. (C) Alignment of CB₁R, β₂AR, and the CCK receptors. The conserved NPxxY motif on helix 7 is shown in blue. Residues fulfilling the CRAC sequence are shown in red.

TABLE 1. Summary of the mutations on the β₂AR and their results.

Construct	Purpose	Behavior	ΔTm(°C)
Unmutated	Control	Good	1.71±0.85
W313A	Flagged in simulation	High Aggregation	-
S319A	Control mutation	Slight aggregation. Biphasic data could not be fit to Boltzmann	-

		curve in LCP-Tm	
I319A	Control mutation	Heavily Aggregated	-
F321A	Theoretical interaction with cholesterol on TM7	Good	0.46±0.77 %
L324A	Part of CRAC sequence on TM7	Heavily Aggregated	-
I325A	Control Mutation	Heavily Aggregated	-
Y326A	CRAC sequence	Expression Abolished	-
R328A	CRAC sequence	Good	5.75±1.65 %
F336A	Theoretical interaction with cholesterol	Expression Abolished	-

2222

2222 **Discussion:**

2222 The β_2 adrenergic receptor (β_2 AR) was initially used as the model system to
2222 initially test and validate the methods outlined in this study. The structure, function, and
2222 cholesterol sensitivity of the β_2 AR have been extensively characterized with multiple
2222 cholesterol recognition motifs (CCM, CRAC) already identified on this receptor [22].
2222 Overall, we found that our CGMD simulations on the β_2 AR revealed receptor-cholesterol
2222 interactions consistent with previously published literature results [22, 22, 22, 22].2

2222 Our simulations on the CCK receptor family showed differential cholesterol
2222 binding despite a high sequence homology between these receptors. Experiments on
2222 chimeras of the two receptors showed that the CRAC motif at the bottom of TM3 is
2222 responsible for the differential cholesterol sensitivity between the two receptors [22],
2222 consistent with our simulation data. Furthermore, the CCK₁R Y140A mutation at the
2222 bottom of TM3 within this CRAC motif has been shown to mimic the wildtype CCK₁R
2222 conformation in a high cholesterol environment along with the loss of cholesterol

sensitivity [22, 22]. Our simulations predict that the CCK₁R mainly interacts with cholesterol molecules via F130^{3.41}. Taken together, our results along with the previously published data suggest that cholesterol binds the CCK₁R on TM3. Strong cholesterol interactions around position 3.41 are intriguing as mutation of this position to tryptophan is commonly performed during receptor engineering for structural studies, and has been included in the thermo-stabilization of numerous receptors for crystallization [22222]. Initially discovered on β_2 AR, mutation of the residue at this position (3.41) decreased affinity for small molecule ligands 2-fold while increasing recombinant expression by nearly 4-fold and purified monomer yield by 5-fold [22]. Position 3.41 mutations to tryptophan have also been successfully extended to chemokine, serotonin, and dopamine receptor structural studies [22]. Taken together, the ability to affect receptor expression, yield, and stability as well as broadly being applicable to other GPCRs underscore the potential importance of this residue in GPCR physiology. Therefore, it is not unreasonable to presume that cholesterol interactions at 3.41 could be responsible for the cholesterol sensitivity of the CCK₁R (without necessarily affecting the expression levels, given that tryptophan may lead to a stronger interaction with lipid or detergent headgroups that may compensate for missing stabilizing protein-cholesterol interactions) [22, 22].

The CCK₂R seems to mainly interact with cholesterol via the CCM as one of the strongest signals was from S95^{2.45} at the bottom of TM2 but it appears to interact with cholesterol with lower specificity and affinity than CCK₁R. In the CGMD simulations, CCK₁R interacted with cholesterol for much longer and at more specific residues than its insensitive homolog CCK₂R (Fig. 3B). These results imply that specific cholesterol-

receptor binding and interaction could be responsible for the difference in physiological effects between the two CCK receptors seen in a high cholesterol environment. Although indirect effects of cholesterol on membrane curvature could be responsible for modulating receptor functions, our data suggest that CCKRs bind cholesterol specifically and differentially. Thus, the CCKRs may be insensitive to the global effects of cholesterol on lipid bilayer curvature, fluidity, and microdomain-receptor sequestration. Our simulations agree with previous literature results that TM3, and specifically F130, is responsible for the differential effect of membrane cholesterol on the CCK receptors [22, 23].

Additionally, we discovered a new CRAC site on TM7 that overlaps with the highly conserved class A GPCR NPxxY motif; subsequent sequence alignment with all 285 class A GPCRs showed that 38% share a CRAC sequence in this region (Supplementary Fig. S1). Tyr^{7.53} of the NPxxY motif plays an essential role in class A GPCR activation, as mutation of Tyr^{7.53} on numerous receptors has shown a decrease or elimination of downstream signaling [22]. Upon ligand binding, conformational changes in the transmembrane helices result in the formation of a water-mediated hydrogen-bond network that links Tyr^{7.53} to Tyr^{5.58}. This interaction helps facilitate the outward swing of helix VI which in turn allows for the G_s subunit to bind the receptor and activate the signaling cascade [22]. The importance of both the NPxxY motif and cholesterol in GPCR physiology have been extensively supported in the literature and it is well established that the NPxxY motif plays a significant role in stabilizing active state receptors [22]. Our mutations on the μ_2 AR reinforce the importance of this region in

receptor stability and function as mutations to Y326A^{7.53} and F336A^{helix8} nearly abolished expression of the protein (Table 1).

Our LCP-T_m assay on the unmutated receptor showed that incorporating 10% cholesterol into the host lipid increased the T_m by 1.71 ± 0.85°C. F321^{7.48} is located on TM7 three amino acids above the novel CRAC sequence. We expected that mutating this residue (F321A) would alter cholesterol binding at this location and would thus decrease the ΔT_m value in the LCP-T_m assay. However, our ΔT_m value of 0.46 ± 0.77 °C from the unmutated receptor was not found to be statistically significant at a 95% confidence interval ($p = 0.1310$). R328^{7.55} is a key part of the CRAC sequence outlined above. Like our F321A mutation, we hypothesized that the R328A mutation would inhibit cholesterol interactions and lower the ΔT_m value of the receptor. Surprisingly, the R328A mutant's T_m value increased from 44.39 ± 0.76 to 50.14 ± 1.47°C with the addition of cholesterol, yielding a ΔT_m 5.75 ± 1.65 °C. This result was found to be significant at a 95% confidence interval ($p=0.0194$). This result is counterintuitive as we expected that the positively charged arginine residue should interact favorably with the hydroxyl group of cholesterol molecules, and thus the R328A mutation would result in a smaller ΔT_m than the unmutated control. CGMD simulations of the mutant receptor did not show a significantly increased binding of cholesterol compared to WT, although the large standard deviations of the residence times leaves open the possibility that with much longer sampling times (well in excess of 100 μs) more subtle effects might be discovered. In the absence of reliable molecular data we may speculate that the ΔT_m of R328A could be explained by the physical impact of cholesterol on lipid bilayers [22, 22, 22]. A mismatch between the length of the hydrophobic transmembrane helix and the width of

the lipid bilayer is known to destabilize proteins [22](an effect that cannot be captured by the MARTINI CG model because of its inherent secondary structure restraints). Hypothetically, these physical changes in the bilayer upon the addition of cholesterol could place the charged arginine residue into an environment that destabilizes the receptor (i.e., deeper into the hydrophobic membrane). Therefore, mutation to alanine would remove the potentially destabilizing effect of the charged arginine in the bilayer. Nevertheless, the difference in the experimental ΔT_m values between the R328A mutant and the unmutated control were found to be statistically significant. Although our results from the biochemical assay and simulations were unexpected, and do not definitively show cholesterol binding at this CRAC, the data does suggest that R328^{7.55} may affect the receptor's behavior in a membrane with modulated cholesterol levels. Additionally, studies have shown that mutation of this CRAC sequence at the type-1 cannabinoid receptor inhibited the cholesterol sensitivity of the receptor [2], which may support the importance of this CRAC sequence in other receptors besides the μ_2 AR and the CCKRs. [2]

It should be noted that CRAC sequences, as well as CCM and other cholesterol-recognition motifs, provide little predictive value in how cholesterol will interact with the protein [22, 22]. CRAC sequences are ubiquitous in membrane proteins and the presence of a CRAC sequence does not ensure cholesterol binding. A case for this can be seen in our simulation data as the CCK receptors, though containing CRAC sequences in various locations (TM3, TM5, and TM7), were not found to bind cholesterol at all of these locations. Instead, cholesterol molecules may have an affinity for the region surrounding a CRAC sequence as the correct hydrophobic, aromatic, and polar groups are in place to

facilitate protein-cholesterol interactions [22, 22, 22], and in some cases, these interactions can have profound physiological effects. [2]

Our study has shown that cholesterol interacts stronger with specific regions of cholesterol-sensitive receptors compared to their insensitive homologs. These data imply that specific receptor-cholesterol interactions play an important role in the modulation of receptors as a function of membrane cholesterol content. More detailed experimentation to determine the conformational changes associated with cholesterol binding would be very helpful in understanding these phenomena. We have identified a CRAC sequence that is conserved at the bottom of TM7 in 38% of class A GPCRs. Due to its overlap with the NPxxY motif, it is interesting to hypothesize that receptor-cholesterol interactions at TM7 could play an important role in cholesterol-mediated modulation throughout this family and detailed studies on other cholesterol sensitive receptors could yield more information. Additionally, downstream signaling assays could shed light on how these CRAC sequences affect receptor function. Regarding the CCK subfamily, our data agrees with previously published literature and could be helpful in the development of allosteric modulators to treat metabolic syndrome and obesity.

Materials and Methods:

Model Preparation and Simulations

Models for CCK₁R and CCK₂R were generated using Modeller [22, 22] with the orexin receptors 1 and 2 (OX₁R and OX₂R) as templates (PDB codes: OX₁R: 4zj8 and 4zjc (Yin, #374); OX₂R: 4s0v [22], 5wqc and 5ws3 [22]). Fusion protein inserts were removed from the crystal structures and a multi-sequence alignment was performed using Jalview [22] with the CCK₁R and CCK₂R sequences (Uniprot (<https://www.uniprot.org>) accession

numbers P32238 and P32239, respectively) independently. Modeller was used to create twenty models for each protein and the structure with the lowest normalized DOPE score [22] for each was chosen (-0.52 and -0.40 for CCK₁R and CCK₂R, respectively). The system setup for the β_2 -adrenergic receptor (β_2 AR) began with removing the T4-lysozyme fusion from the third intracellular loop (ICL3) in the crystal structure (PDB 2rh1 (Rosenbaum, 2007 #60)). Modeller was used to fix the resulting gap in the structure by joining the short loop segments left after removing the insert. Using the CHARMM-GUI server [22], each of the three models was coarse-grained using the MARTINI force field [22, 22] with the ElNeDyn secondary structure restraints and inserted into a membrane. Two separate systems were created for each protein: one with a 1:4 cholesterol:1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane (“20% cholesterol”) and one with a 2:3 cholesterol:POPC membrane (“40% cholesterol”). Coarse-grained molecular dynamics (CGMD) was performed using Gromacs 2019 [22] at 303.15 K and 1 bar using semiisotropic Parrinello-Rahman pressure coupling [22] and stochastic velocity-rescale temperature coupling [22] with a 20 fs time step. A reaction-field with a 1.1 nm cutoff was used for the electrostatic interactions and a single cutoff of 1.215 nm was used for the van der Waal interactions [22]. Constraints were added according to the MARTINI force field [22]. Simulation lengths for each system are reported in Table 2.

Time series of the cholesterol-protein contacts were collected on a per residue basis for each trajectory using functionality in the MDAnalysis package [22], where a contact was defined with a 7 Å cutoff. Survival functions were calculated from a histogram of the

waiting times for each residue. The survival functions were least-square-fitted with relative weights to a two-term hyperexponential with the form

$$s(t) = ae^{-\alpha t} + (1 - a)e^{-\beta t} \quad (1)$$

using the *curve_fit()* function in SciPy [22]. Artifacts from the cutoff manifest themselves as spurious contacts with short waiting times, i.e., higher rates, so only the slower rates from the longer waiting times were taken to be indicative of cholesterol-protein interactions. Specifically, the slower of the two rates was used to calculate a mean waiting time τ_{slow} for each residue, termed the “residency time”, as

$$\tau_{\text{slow}} = \frac{1}{\min(\alpha, \beta)} \quad (2)$$

Further technical details on the methods will be published elsewhere (Sexton et al, in preparation). τ_{slow} was plotted against the residue number to compare mean waiting times across the protein using Matplotlib [22]. Errors for τ_{slow} values were obtained by dividing the trajectories into non-overlapping contiguous blocks of at least 20 μs and less than 21 μs and performing the contact analysis for each block. The mean and standard deviation were calculated from the τ_{slow} values over all blocks. As shown in Supplementary Figs. 6 – 13, a block length of $\sim 20 \mu\text{s}$ provided a consistent picture compared to fewer but longer blocks and was chosen for its conservative (large) error estimate while still providing a sufficient number of events for the fitting procedure to converge. When we plotted residence times we only automatically labelled residues with a residence time at least three times greater than the overall background as computed by the mean over all residence times (excluding residues without data or with insufficient data for the

hyperexponential fit to converge). The residence times for the CCK homologues were compared by performing a sequence alignment and taking the difference (CCK1R - CCK2R) of the τ_{slow} values obtained, which was called $\Delta\tau$. The standard deviation of the difference was calculated from error propagation of the individual standard deviations of the τ_{slow} values. VMD [22] and Chimera [22] were used for molecular visualizations. 2

TABLE 2. Run times for the simulations in microseconds (μs)

20% Cholesterol	$\beta_2\text{AR}$	$\beta_2\text{AR_R328A}$	CCK ₁ R	CCK ₂ R
Run 1	121.5	222.77	100.3	103.3
Run 2	120.9	222.77		
Run 3	123.8	222.77		
Total	366.2	222.77	100.3	103.3
40% Cholesterol				
Run 1	120.5	222.77	101.3	40.82
Run 2	121.9	222.77		40.3
Run 3	122.4	222.77		
Total	364.8	222.77	101.3	81.1

2222

Mutant Receptor Preparation

Site-directed mutagenesis was performed on $\beta_2\text{AR}$ using primers with internal mismatches (IDT) and Phusion polymerase (Thermo Fisher Scientific). Mutant receptors were verified via Sanger sequencing (Genewiz). Receptors were expressed and purified as previously described [22]. Receptors were analyzed for total and surface expression on a Guava flow cytometer (Millipore). Purified receptors were analyzed for purity and homogeneity via analytical size exclusion chromatography. Homogeneous receptors were further analyzed using the LCP- T_m assay. 2

2222 LCP-T_m Assay2

2222 Cholesterol binding to mutant receptors was quantified via a thermal denaturation
2222 assay in LCP as described [22]. Briefly, purified receptors were mixed in an
2222 approximately 35% (v/v) ratio with molten monoolein (Nu-check) containing either 0 or
2222 10% cholesterol. Final protein concentrations were 0.015 mg/mL for each assay.
2222 Fluorescence signal was recorded using a Cary Eclipse Emission Spectrophotometer
2222 (Agilent). Background signal from a blank LCP sample was subtracted from the protein
2222 sample and the inner filter effect was corrected for as described [22]. CPM (7-
2222 Diethylamino-3-(4'-Maleimidylphenyl)-4-Methylcoumarin (Sigma)) probe was added to
2222 the protein at a final concentration of 0.004 mg/mL and allowed to incubate on ice, and in
2222 the dark, for 30 minutes before incorporation into the LCP matrix. The excitation
2222 wavelength for fluorescence was 387 nm and emissions were recorded via scanning
2222 between 400 and 500 nm with a step size of 1 nm. Mathematical fitting and statistics
2222 were carried out in Prism version 8.3.1(GraphPad) as described [22]. The effects of
2222 cholesterol were quantified as the change in denaturation temperature as a function of the
2222 addition of cholesterol into the LCP matrix. ΔT_m values for mutant receptors were
2222 compared to an unmutated control. 2

2222 Acknowledgements:

2222 Research reported in this publication was supported by the National Institute of
2222 General Medical Sciences of the National Institutes of Health under Award Number
2222 1R15GM124623-01A (O.B.), the National Institutes of Health grants R21DA042298
2222 (W.L.) and R01GM124152 (W.L.), the STC Program of the National Science
2222 Foundation through BioXFEL (No. 1231306; W.L.), a Mayo Clinic ASU Collaborative

Seed Grant Award (W.L.), and the Flinn Foundation Seed Grant (W.L.)

The funders had no role in study design, data collection and analysis, decision to

publish, or preparation of the manuscript.

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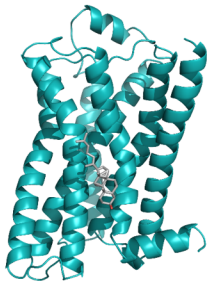
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Cholesterol Sensitivity of GPCRs



Course-Grained Molecular Dynamics

Structural Bioinformatics

Specific, differential cholesterol binding between the cholecystikinin receptors

A newly discovered cholesterol recognition motif is conserved across class-A GPCRs

