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Role of Fluctuations in Ligand Binding Cooperativity of Membrane Receptors

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Signal transduction upon binding of a ligand to a membrane protein can occur not only via allosteric conformational changes but also through fluctuations. We report a numerical study on the influence of conformational fluctuations on the cooperativity of a binding reaction in a simple model of an integral membrane receptor consisting of transmembrane helices. We find that small fluctuations lateral as well as perpendicular to the membrane can increase the cooperativity, with the former more dominant. Moreover, too much fluctuation induces negative cooperativity. Proteins with fewer than four helices do not show positive cooperativity under any circumstances. This behavior is rather robust, and independent of the receptor topology or ligand size. Fluctuations measured in all-atom molecular dynamics simulations of a *G*-protein coupled receptor fall within the predicted region of maximum cooperativity.

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The functioning of transmembrane signaling proteins such as those belonging to the *G*-protein-coupled receptors (GPCRs) is typically explained by an allosteric conformational change upon binding of a signaling molecule (e.g., a hormone), which conveys environmental information to the inside of the cell, setting off signaling cascades further downstream that ultimately lead to a cellular response [1]. Usually the allosteric conformational change is envisioned as a switch between two (meta)stable structures of the receptor protein. However, while recently detailed structures of several GPCRs have become available [2], the molecular allosteric mechanism of GPCRs still remains elusive. As proteins are flexible molecules, the allosteric signaling process might also depend on a change in conformational fluctuations [3]. Such a possibility has been explored for DNA binding proteins [4–6]. In addition to experimental evidence that supports a more dynamical mechanism of GPCRs [7], molecular dynamics (MD) simulations have ruled out previously proposed static mechanisms that consider only conformational changes [8].

Any model of the working mechanism of GPCRs will contain both a specific and a generic part. The specific part depends on the details of the molecular interactions between protein and substrate, the generic part focuses on common features of all GPCRs, for instance, that they have 7 transmembrane helices (TMs). The present Letter focuses on such generic questions. Indeed, the fact that protein receptors are almost always built from several TMs connected by flexible linkers suggests that fluctuations might be important for this generic part. The flexibility of the receptor protein would then result in fluctuations in the position of these TMs with respect to membrane and to each other. The presence or absence of signal molecules may have a pronounced effect on the amplitude of these fluctuations. The hypothesis of this work is thus that a GPCR protein's TM helices exhibit relative motion, and that it is this flexibility, rather than a conformational change, that causes signal transduction. We explore the consequences of this hypothesis by focusing on the cooperativity of the binding of the extracellular signal molecule and the intracellular guanosine diphosphate liganded *G* protein. Note that we focus on fluctuations within a single membrane receptor, not on those due to relative motions of rigid proteins in a multireceptor complex. As we are interested in generic, rather than specific, aspects of GPCR-mediated signal transduction, we consider a highly simplified model, that only takes into account ligand binding and receptor flexibility but leaves out all detail on the atomistic scale. Such simple models, while disregarding atomistic details, can still provide essential physical insights in the underlying phenomena. Moreover, these models allow tuning the different types of fluctuations explicitly and separately, which is not straightforward to implement with MD simulations. Here, we consider two types of fluctuations: (1) the lateral flexibility of TMs within the membrane; (2) the flexibility of TMs normal to the membrane.

The model consists of $N_L = 2$ ligands, one extra and one intracellular, that can both bind to an integral receptor. The membrane is modeled as a surface of thickness h_M impenetrable to ligands, positioned in the x, y plane, at the center ($z = 0$) of a cubic box with box length L. The ligands are hard spheres of radius r_L, and thus are excluded from a region $|z| < r_L + h_M/2$. To restrict the ligands to their respective volume, the simulation box is periodic only in the x, y direction. The receptor is a chain of TMs represented as N_R rigid rods connected by springs that represent the linker sequences between the TMs and other TM-TM interactions. Each rigid rod consists of $n_R = 8$ adjacent hard spheres of radius $r_R = \sigma$, and thus has a length $h_R = 2n_R\sigma$. The TMs are kept perpendicular to the

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membrane but are allowed to diffuse laterally and fluctuate perpendicularly. (In principle, rotational freedom could also be allowed, but here we keep the model as simple as possible.) Both symmetric and asymmetric geometry of intracellular or extracellular domains are considered by setting the size of ligands $r_L^{\text{in}} = r_L^{\text{ex}}$ and $r_L^{\text{in}} = 2r_L^{\text{ex}}$, respectively. The geometrical parameters of the model are chosen approximately proportional to their typical values in nature. Setting the unit of length $\sigma = 2.5$ Å, the TM radius $r_R = 2.5$ Å corresponds roughly to the radius of a real α helix. A small ligand such as a hormone is modeled by $r_L = 1.1\sigma = 2.75$ Å. The membrane thickness $h_M = 12\sigma$ and the TM length $h_R = 16\sigma$ correspond to approximately 30 and 40 Å, respectively. In the asymmetric case, the intracellular ligand is chosen to mimic the topology of a larger ligand (e.g., part of a G protein). The Hamiltonian for the above model reads $H(r^N) = \sum_{i<j}^{n_R \times N_R} U_{\text{HS}}(r_{ij}) + \sum_j^{N_L} \sum_i^{N_R} U_{RL}(r_{ij}) + \sum_i^{N_R - 1} \frac{1}{2} k_{RR}(r_{i,i+1}^{xy} - r_{RR}^0)^2 + \sum_i^{N_R} \frac{1}{2} k_{RM} z_i^2$, where U_{HS} represents the hard-core interaction among all receptor particles, with r_{ij} denoting the distance between particles i and j, U_{RL} is the binding attraction between a ligand and the terminal sphere of a TM, modeled by a square well potential with width $\Delta = 0.1\sigma$ and depth ϵ. In the third term, $r_{i,i+1}^{xy}$ denotes the lateral distance between TM_i and TM_{i+1}. The harmonic spring with force constant k_{RR} and equilibrium lateral distance $r_{RR}^0 = 2\sigma$ represents the fact that neighboring TMs are linked through extracellular and cytoplasmic loops, and nonbonded interactions, and constrained by membrane lipids. For simplicity all ligand-TM pair interactions are assumed identical. The last term restrains the normal movement z_i of the TMs with respect to membrane via a harmonic potential with spring constant k_{RM}. The ligand binding behavior is governed by the maximum total binding energy, $\beta U_{\text{tot}} = 12$, where $\beta = 1/k_B T$ is the reciprocal temperature, with k_B Boltzmann's constant, yielding a TM-ligand binding energy $\beta\epsilon = \frac{\beta U_{\text{tot}}}{2N_R} = \frac{6}{N_R}$. This ensures a reasonable value of binding energy of $6k_B T$ per ligand, large enough for sufficient binding and not too large to prevent release of the ligand. The box size is set to $L = 35\sigma$ to accommodate the most extended configurations. Figure 1 shows an example of a configuration of the model. While the GPCRs are integral signal proteins with N and C termini, in the membrane they resemble a compact ring structure due to the constraints of membrane lipids [7]. We mimic this ring structure by adding an extra link between the receptor's first and last TMs. To investigate the influence of this topological constraint, both ring and string topology are studied.

Monte Carlo simulations [9] of the model were performed with the following settings. The number of TMs of the receptor varied from $N_R = 1$–8, for symmetric and asymmetric ligands, and for ring versus string receptor topology, giving in total $2 \times 2 \times 8 = 32$ different systems.

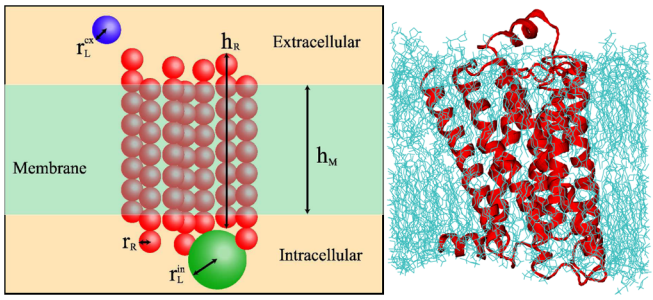

FIG. 1 (color online). Left: Communication between extracellular signal (top sphere) and G protein (bottom sphere) is mediated only by one integral receptor composed of a number of transmembrane helices. Right: (part of) a snapshot from the all-atom MD simulation of a $\beta 2AR$ receptor embedded in a POPE bilayer. The protein backbone is rendered in cartoon style and the membrane as lines. The solvent is not shown.

For each of these systems we considered 5 values for the lateral fluctuation parameter $k_{RR}\sigma^2 = [4, 40, 400, 4000, \infty]k_B T$, and 15 values for the perpendicular fluctuation parameter $k_{RM}\sigma^2$ ranging from $0.04k_B T$ to ∞. The total number of different system settings is thus $32 \times 5 \times 15 = 2400$. For each of these settings we performed parallel tempering [10] runs consisting of 13 replicas, with $1 < \beta < 6$. A single replica ran $\sim 10^8$ cycles. Each cycle consisted on average of a trial move for every particle. A replica swap was attempted every 250 cycles. The acceptance ratio of the particle move and replica swap was ~ 0.2 and ~ 0.8, respectively.

Essentially, the current system can be regarded as a four state chemical reaction $s_0 \leftrightarrow s_{1_{\text{in}}}$, $s_{1_{\text{ex}}} \leftrightarrow s_2$, where the labels 0, 1, 2 denote the number of ligands bound to the receptor, ex and in denote extra and intracellular ligand, respectively. Cooperativity in binding is defined as the difference between the sum of the standard free energy differences of the substeps (binding to one ligand only) and the standard free energy difference between reactions and products [11,12] (also known as the allosteric free energy $\Delta\Delta G$ [3,4,6]) $c = \Delta G_{01_{\text{in}}}^0 + \Delta G_{01_{\text{ex}}}^0 - \Delta G_{02}^0$ with $\Delta G_{ij}^0 = G_j^0 - G_i^0$. The standard is set by the volume (or pressure) of the simulation box. Using the relation between the free energy and the equilibrium constant $\beta\Delta G^0 = -\ln K$ it immediately follows that $c = -k_B T \ln(\frac{K_{01_{\text{in}}} K_{01_{\text{ex}}}}{K_{02}}) = k_B T \ln(\frac{p_0 p_2}{p_{1_{\text{in}}} p_{1_{\text{ex}}}})$, where $K_{ij} = p_j/p_i$ is the equilibrium constant of subreaction $s_i \leftrightarrow s_j$, and p_i denotes the population of s_i. $c > 0$ and $c < 0$ indicate positive and negative cooperativity, respectively. The populations p_0, $p_{1_{\text{ex}}}$, $p_{1_{\text{in}}}$, p_2 can be extracted directly from the MC simulations. Since the ligand-TM attraction is modeled by a square well, we can simplify the analysis by combining the number of ligand-TM bonds n for each ligand into a "binding pattern" of 2 numbers $n_{\text{ex}}, n_{\text{in}} \in [0 \ldots N_R]$. The probability to find a certain pattern can then be summarized in a $(N_R + 1) \times (N_R + 1)$ matrix Z. Here entry $Z_{i,j}$ for index

i, j denotes the probability that the extracellular ligand has i bonds with TMs established, while the intracellular has j bonds. As for $N_R \geq 5$, the peak in the Z at high β is not always at (N_R, N_R), but around a certain (n, n) with $n > \frac{N_R}{2}$, we define $p_2 = \sum_{n \leq i,j \leq N_R} Z_{i,j}$; $p_{1_{\text{ex}}} = \sum_{0 \leq i < n}^{n \leq j \leq N_R} Z_{i,j}$, $p_{1_{\text{in}}} = \sum_{n \leq i \leq N_R}^{0 \leq j < n} Z_{i,j}$, and $p_0 = \sum_{0 \leq i,j < n} Z_{i,j}$. Figure 2 exemplifies such resulting populations and corresponding free energy for a specific parameter setting.

The cooperativity $c(\beta)$, while a function of β, is relatively constant over the range of β, but much more dependent on the fluctuation parameters. Averaging the cooperativity over inverse temperature $\langle c \rangle_\beta$ allows compressing the simulation results into two-dimensional cooperativity landscapes as a function of the number of TMs N_R and the perpendicular fluctuation harmonic constant k_{RM}. In Fig. 3 we plot these landscapes for both symmetric and asymmetric ligands and three types of lateral fluctuations. For all cases, $\langle c \rangle_\beta$ is small if $N_R < 4$, suggesting that a low TM number does not lead to strong positive cooperative binding of the two ligands. In the case without lateral fluctuation (left column) $\langle c \rangle_\beta$ is positive for $N_R \geq 4$ and $0.4 k_B T < k_{RM} \sigma^2 < 40 k_B T$. These k_{RM} values correspond on average to moderate perpendicular fluctuations $0.1 \sim 1\sigma$ (0.25–2.5 Å). For higher values of $k_{RM}\sigma^2 \gg 40 k_B T$, no positive cooperativity is observed. Note that for $N_R \geq 7$ the equilibrium ring is too large for a small ligand to bind to a sufficient number leading to $p_2 = p_{1_{\text{ex}}} = 0$ and an undefined c. Allowing lateral fluctuation (middle column) shifts and increases the maximum of $\langle c \rangle_\beta$ to values of $k_{RM}\sigma^2 > 40 k_B T$, but does not alter the position of the maximum in terms of N_R. The effect of lateral fluctuations is more dominant than that of normal fluctuations; i.e., reducing lateral fluctuation decreases cooperativity, while reducing normal fluctuations increases it. Changing from the ring to the string topology (right column) allows further flexibility in the lateral movement but does not change the qualitative picture. This implies that neighbor TM distance fluctuation is much less dominant than the shape deformation of receptor from the equilibrium ring structure. Introducing asymmetry between ligands (lower row) only slightly reduces $\langle c \rangle_\beta$ with respect to the symmetric case, but induces a negative $\langle c \rangle_\beta$ for small k_{RM}, for all N_R.

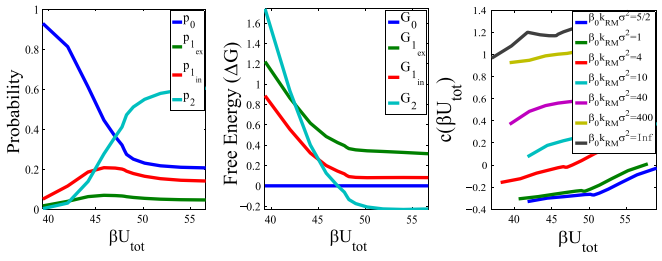

FIG. 2 (color online). Simulation results for specific model settings ($N_R = 6$, $k_{RM}\sigma^2 = 40 k_B T$, $k_{RR}\sigma^2 = 40 k_B T$) of a ring topology receptor with asymmetric ligands as a function of βU_{tot} (inverse temperature, or maximum total binding energy): (a) population probabilities p_2, $p_{1_{\text{ex}}}$, $p_{1_{\text{in}}}$, p_0; (b) free energy of binding with respect to $G_0 \equiv 0$; (c) cooperativity $c(\beta)$ for several spring constants k_{RM}.

Our results are in agreement with Ref. [3] in which cooperativity is proposed to originate from a shift in vibrational density of states that accompanies the ligand binding. Such a shift is also observed in our model, as a suppression of fluctuation upon binding. We can compare our numerical results for $N_R = 2$ and no lateral fluctuation with an analytical model along the lines given in Ref. [6]. Approximating the influence of (symmetric) ligand binding by an additional harmonic coupling k_c, the system is effectively a system of coupled harmonic oscillators. The cooperativity in this model is $c = \frac{k_B T}{2} \ln[\frac{(1+2\alpha)^2}{1+4\alpha}]$ with $\alpha = k_c / k_{RM}$. Since α is by definition non-negative, the cooperativity $c \geq 0$. Our results indeed show that the cooperativity is $c \approx 0$ for $k_{RM} \to \infty$, and is increasing for small $k_{RM} < 1$, as predicted, but is slightly negative for intermediate values of k_{RM}. This is caused by $N_R = 2$ configurations with ligands sideways bound to both receptors, which are partly excluded in the s_2 state. While this result is clearly due to the simplicity of our model, we believe that the overall features of the cooperativity landscapes are robust.

To evaluate the existence and scale of the flexibility of membrane receptors and to justify our model, we performed a 240 ns MD simulation of a $\beta 2AR$ receptor. We first deleted the T4L residues of $\beta 2AR$ (PDB entry 2RH1), capped the exposed termini of Leu-210 and Lys-263, mutated it to wild type sequences [8], and then embedded this clipped $\beta 2AR$ into a phosphatidyl ethanolamine (POPE) bilayer normal to the xy plane. Using the GROMOS87 force field, simple point charge water and Berger lipid model [13], the resulting system consists of 308 lipids, 73 Na^+, 77 Cl^-, and 12 153 water molecules and totally 55 546 atoms in a box $\approx 9.7 \times 9.7 \times 8.6$ Å (see Fig. 1 for a snapshot of the system). The production run was performed at 298 K with the v-rescale thermostat and at 1 bar with the Parrinello-Rahman barostat. In this 240 ns trajectory, the standard deviation (SD) of approximated Gaussians for x, y components of the distances between the center of mass (COM) of each linked TM-TM pair are $\langle x^2_{\text{MD}} \rangle^{1/2} \approx 0.310$ Å, $\langle y^2_{\text{MD}} \rangle^{1/2} \approx 0.225$ Å, respectively. The SD of z component of COM distance between the TMs and the whole protein is chosen to quantify the normal fluctuation and reads $\langle x^2_{\text{MD}} \rangle^{1/2} \approx 0.203$ Å. These values are then transformed into our model units and compared with corresponding k_{RM} and k_{RR} values (or equivalently the histogram of fluctuations in state s_0), since the MD simulation only represents s_0 in our simple model: $\beta k^{\text{MD}}_{RR} \sigma^2 = \sigma^2 / (\langle x^2_{\text{MD}} \rangle + \langle y^2_{\text{MD}} \rangle) \approx 40$ and $\beta k^{\text{MD}}_{RM} \sigma^2 = \sigma^2 / \langle z^2_{\text{MD}} \rangle \approx 150$. These values fall within the region of optimal positive cooperativity in Fig. 3. While the comparison is indirect, as we cannot measure the cooperativity directly in the MD

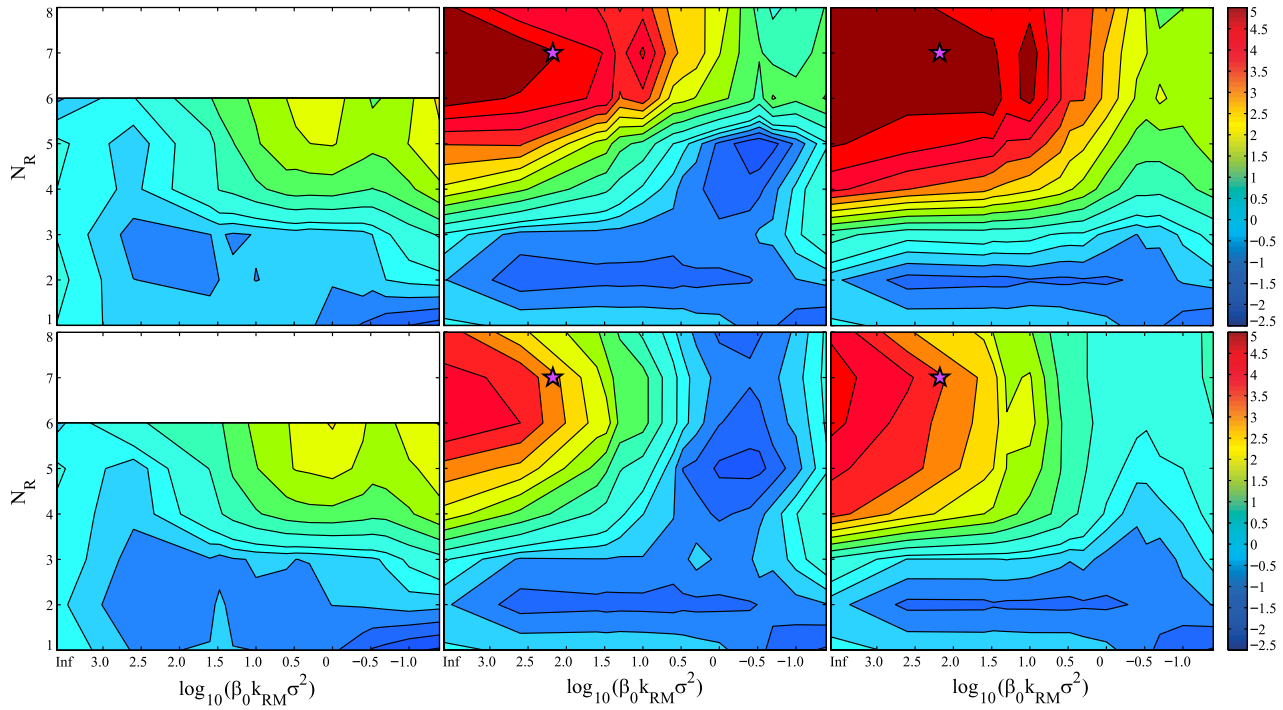

FIG. 3 (color online). Cooperativity $\langle c \rangle_\beta$ as function of $\log(\beta_0 k_{RM} \sigma^2)$ and N_R ($\beta_0 = 1$ is a reference temperature to make the log argument dimensionless). Top row: symmetric ligands; bottom row: asymmetric ligands; left column: equilibrium ring topology without lateral fluctuation ($k_{RR} = \infty$); middle column: the effect of deformation from the ring structure together with fluctuations in the neighbor TM distance ($k_{RR}\sigma^2 = 40 k_B T$); right column: the effects of removing the constraint between N- and C-terminal TMs (string topology); star: parametrization of 240 ns MD simulation of unliganded $\beta 2AR$ receptor.

simulations, these results agree with our hypothesis that naturally occurring receptors have evolved to a region of high cooperativity for optimal signal transduction.

In summary, our results suggest that cooperative binding of ligands to multiple TMs, and thus shape selectivity, might be a dominant factor underlying the working mechanism of integral membrane receptors such as GPCRs. Cooperativity is determined by the interplay between the geometry of the ligands with the modes of fluctuations (lateral and normal) of the receptor. Communication through fluctuations between extra and intracellular domains is only optimal when the shape of ligands influence the allowed modes of fluctuation. In addition, we predict that all membrane receptors in nature should have at least four TM helices to gain positive cooperativity. These predictions could be further explored via extensive all-atom MD simulations that measure TM fluctuations of both liganded and unliganded forms of GPCRs, promising NMR techniques [14,15], scattering experiments [16], or mutation studies that suppress fluctuations by, e.g., introducing chemical bonds.

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