Homology Modeling and Molecular Dynamics Simulations of the Mu Opioid Receptor in a Membrane–Aqueous System

Yan Zhang,*[a, b] Yuk Y. Sham,[c] Ramkumar Rajamani,[d] Jiali Gao,[d] and Philip S. Portoghese[a]

Three types of opioid receptors—mu, delta, and kappa—belong to the rhodopsin subfamily in the G protein-coupled receptor superfamily. With the recent characterization of the high-resolution X-ray crystal structure of bovine rhodopsin, considerable attention has been focused on molecular modeling of these transmembrane proteins. In this study, a homology model of the mu opioid receptor was constructed based on the X-ray crystal structure of bovine rhodopsin. A phospholipid bilayer was built around the receptor, and two water layers were placed on both surfaces of the lipid bilayer. Molecular-dynamics simulations were carried out by using CHARMM for the entire system, which consisted of 316 amino acid residues, 92 phospholipid molecules, 8327 water molecules, and 11 chloride counter ions—40 931 atoms altogether. The whole system was equilibrated for 250 ps followed by another 2 ns dynamic simulation. The opioid ligand naltrexone was docked into the optimized model, and the critical amino acid residues for binding were identified. The mu opioid receptor homology model optimized in a complete membrane–aqueous system should provide a good starting point for further characterization of the binding modes for opioid ligands. Furthermore, the method developed herein will be applicable to molecular model building to other opioid receptors as well as other GPCRs.

Introduction

Opioid receptors are members of the rhodopsin family of G protein-coupled receptors (GPCRs), which is characterized by a heptahelical domain spanning the plasma membrane. X-ray crystallography has confirmed the seven transmembrane (7TM) motif for rhodopsin.[1–4] The 7TM motif is believed to be an appropriate model for other GPCRs as well.[5,6]

Rhodopsin X-ray crystal-structure-based homology modeling has been carried out for GPCRs such as the dopamine D3 receptor, muscarinic M1 receptor, serotonin 5-HT_4 receptor,[7] metabotropic glutamate 5 receptor,[8] cholecystokinin CCK1 receptor,[9a] neurokinin-1 (NK1) receptor,[9b] human calcium-sensing receptor (CaR),[10] and chemokine receptor CXCR4.[11] Since the sequence identity in the TM domain of opioid receptors and rhodopsin is about 30%, it should be possible to build plausible opioid receptor models based on the rhodopsin X-ray crystal structure through homology modeling in which about 80% Cα atoms are within 3.5 Å of their correct position.[12]

At least two methods have been developed to optimize homology modeling of GPCRs. One is to build a membrane–aqueous system around the model and then conduct molecular-dynamics simulations for the entire protein–membrane–aqueous system.[11,13] The second method requires information from site-directed mutagenesis studies relating to ligand-binding affinities. Accordingly, specific amino acid residues can be constrained to counter moieties in the ligand at certain interacting distances, and then molecular dynamics can be conducted to improve the protein structure.[9]

Here we report the construction of a mu opioid receptor homology model based on the X-ray crystal structure of rhodopsin and the optimization of the model in a complete membrane–aqueous system.

Results and Discussion

Homology model of the mu opioid receptor

Construction of the mu opioid receptor initial structure involved several steps: sequence alignment, assignment of Cartesian coordinates for amino acid residues in structurally conserved regions, assignment of coordinates for amino acid residues in structurally nonconserved regions, refinement of coordinates for the backbone of the TM helices, and their correction with respect to hydrogen-bonding orientations.

[a] *Dr. Y. Zhang, Prof. P. S. Portoghese*
Department of Medicinal Chemistry, University of Minnesota
Minneapolis, MN 55455 (USA)
E-mail: yzhang2@vcu.edu

[b] *Dr. Y. Zhang*
Department of Medicinal Chemistry, School of Pharmacy
Virginia Commonwealth University, Room 534 Smith Building
410 N. 12th Street, Richmond, VA 23298-0581 (USA)
Fax: (+1) 804-828-7625

[c] *Dr. Y. Y. Sham*
Minnesota Supercomputing Institute, University of Minnesota
Minneapolis, MN 55455 (USA)

[d] *Dr. R. Rajamani, Prof. J. Gao*
Department of Chemistry, University of Minnesota
Minneapolis, MN 55455 (USA)

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Homology modeling was the main tool used to build up the helices in the TM domain. For the extracellular (EC) and intracellular (IC) regions, homology modeling and protein-database searching were combined to establish three-dimensional models. Since the N and C termini of the opioid receptors have longer chain lengths than that of rhodopsin, and no information on their conformation in the aqueous phase is directly available, these domains were partially truncated. Specifically, 35 amino acid residues at the N terminus and 47 amino acid residues at the C terminus were removed, and the new N and C termini were capped afterwards.

An analysis of the amino acid sequence identity of the three opioid receptors (mu, delta, and kappa) compared to rhodopsin was carried out, region by region, prior to homology modeling. Table 1 shows that all the TM domains of the opioid receptors, except TM3, have a higher percentage of identity to rhodopsin than the average value of the whole protein. TM3 is believed to be an important contributor in the binding of opioid ligands. On average, all other TM helices among the three receptors have a sufficient percentage of identity for conducting homology modeling.

Table 1. *Comparison of the amino acid sequence homology of opioid receptors with bovine rhodopsin by domain.*

	Sequence identity compared with bovine rhodopsin [%][a]		
Domain	Mu	Delta	Kappa
TM1	32	32	20
TM2	44	36	44
TM3	13	17	13
TM4	28	24	28
TM5	44	36	40
TM6	42	40	42
TM7	32	32	30
EL1	36	27	36
EL2	14	14	13
EL3	15	17	18
IL1	36	45	27
IL2	37	37	32
IL3	18	15	11
N terminus	6	14	10
C terminus	15	16	13
Entire protein	26	23	25

[a] Comparison and calculation of the sequence identity percentage was conducted after pair alignment of the whole protein with rhodopsin. Numbers in the columns refer to the percent of amino acid residues within a particular domain, or for the entire protein, those that are identical after a comparison of the specific opioid receptor with rhodopsin.

Since the opioid receptors have much longer N and C termini than those of rhodopsin, it is not surprising to find a very low identity in these regions for all three receptors.[13] Other regions of the opioid receptors that have significantly low percentages of sequence identity with rhodopsin domains are the extracellular loops (EL) 2 and 3, and the intracellular loop (IL) 3.

The importance of the EC regions of the receptors in the binding and recognition of opioid ligands makes it necessary to construct a model that includes those regions. On the other hand, the relatively low sequence identity between rhodopsin and opioid receptors in these regions makes it difficult to predict the exact conformation of the EC regions of the target proteins by homology modeling. At the same time, loop modeling is typically applied to isolated loop regions of globular proteins, while the loops of opioid receptors (as well as other GPCRs) have a number of unique tertiary interactions and structural characteristics. Normally, geometric constraints are required to model the tertiary folding of those regions computationally.[14] By assuming the existence of a disulfide bond between EL1 and EL2 in the opioid receptor EC region, close proximity between these two loops can be expected. The IC region provides the recognition locus for the G protein, and its conformation is very important for the activation of the receptor. One possible way to verify the tertiary structure of the IC region would be to employ the X-ray crystal structure of a heterotrimeric G protein[15] as well as the proposed G protein binding-site structure of the IC region in rhodopsin.[16–18]

The initial and possibly the most essential step in homology modeling was sequence pair-wise alignment between the mu opioid receptor and bovine rhodopsin, for which the InsightII/Homology module was using. Manual alignment of the amino acid residues was performed by comparing the chemical structures of the aligned residues in order to confirm the automatic alignment. The final alignment had 31.6% identity with the bovine rhodopsin sequence, and the gap regions comprised about 8.5% of the total sequence.

The overlapping amino acid sequences between rhodopsin and the mu receptor were divided into different lengths separated by gaps. These regions are called "structurally conserved regions". The coordinates for the amino acid residues in these regions of rhodopsin were assigned to the corresponding residues in the mu opioid receptor. Up to 80% of amino acid residues in those regions were either identical or structurally related.

The amino acid residues of the mu opioid receptor outside the structurally conserved regions constitute the "structurally nonconserved regions". The coordinates for residues in these regions were obtained by searching The Protein Database. The adopted conformations of these nonconserved regions all had close similarity to the backbone orientation of the corresponding regions in rhodopsin. If the segment was in the TM domain, the set of coordinates yielded a right-handed helix. Another critical step during this process involved filtering out the conformations that generated severe steric interactions with the structurally conserved regions.

In the process of assigning coordinates for structurally nonconserved regions, the helical backbone structures of the receptor may be kinked, bent, or distorted. Such artifacts were removed by conformational refinement with Discover/TemplateForce. Corrections for hydrogen-bond orientation were carried out with Discover/GenericDis. After the proper TemplateForce and GenericDis constraint setup for a region, energy minimization and molecular dynamics were performed, while other parts of the protein were constrained. Finally, the side-chain conformational energy of the entire protein was minimized with the backbone constrained until the energy deviation was less than 0.01 kcal mol^{-1}.

Compared with the models reported previously,[19] the mu opioid receptor homology model that we have constructed is more complete in that it contains the extracellular and intracellular loops to connect the TM helices along with a decent portion of the N- and C-terminal loops. Therefore it provides an improved starting configuration for studying the interactions between different domains of the receptor. Also, it is possible to study the roles of the EL loops of the receptor in binding affinity and ligand selectivity.[20,21] The docking of selective ligands into the opioid receptor homology model may provide greater insight into ligand selectivity.

Construction of the receptor–lipid–solvent system

A complete membrane–aqueous system surrounding the modeled receptor was constructed in order to further optimize the receptor structure. The primary reason for doing so was that the backbone of the TM domain in the receptor model was based on that of rhodopsin. Furthermore, to avoid difficulties arising from the inherent conformational flexibility of the protein, homology modeling typically requires the use of geometric constraints. Consequently, the effects of the phospholipid-bilayer–aqueous environment have been neglected in prior studies. In fact, it is important to understand the restrictions imposed on the membrane-protein structure and the effects on tertiary-structure prediction for membrane proteins by the lipid bilayers. Although the overall conformation of the TM domains with and without a membrane–aqueous environment can be similar, it is possible that refinement in such an environment might offer a better opportunity to look into the tertiary structural arrangement of the receptor model. Such understanding might afford a more "dynamic" view of the receptor–membrane–aqueous system.

Secondly, the conformation of the extra- and intracellular loops of the receptor models might be better optimized in an aqueous environment along with the transmembrane domain in a membrane matrix. In fact, as discussed in the literature, homology modeling might not be an ideal approach for predicting the loop structures of GPCRs in the gas phase or even in continuum solvent.[22] Clearly, membrane–aqueous matrix is a more desirable choice than constrained energy optimization by fixing backbone atomic positions in the TM domain.

Finally, the conformation of hydrophobic side chains on the outer surface of the transmembrane domain can be more adequately optimized through molecular-dynamics simulations in the membrane environment.

To our knowledge, there has been no literature report of a complete opioid receptor model in the membrane–aqueous system, although a recent study reported a molecular-dynamics simulation of an incomplete kappa opioid receptor in a 1,2-dipalmitoylphosphatidylcholine (DPPC) bilayer system.[13] A more relevant example is the optimized homology model of the chemokine receptor CXCR4 in a lipid-bilayer–water environment.[11]

The program we adopted to build the membrane–aqueous–protein system was CHARMM (Chemistry at HARvard Macromolecular Mechanics) version c29b2. The method we followed was developed for the purpose of constructing an initial configuration of a protein–membrane complex that resembled a real membrane–aqueous system as much as possible. We followed closely a procedure described by Woolf and Roux.[23,24] The approach represents an extension of the work of Pastor and co-workers into the investigation of lipid bilayers.[25,26]

In the setup of the membrane system, pre-equilibrated conformers for each phospholipid 1,2-dimiristoyl-SN-glycero-3-phosphorylcholine (DMPC) molecule were taken randomly from a set of 2000 that was previously generated from Monte Carlo simulations of an isolated DMPC molecule in the presence of a mean field. This molecule set was developed at 340 K, above the gel–liquid-crystal phase transition temperature. The structural characterization results calculated from molecular dynamics of the set agreed with results from solid-state NMR data of the liquid-crystalline state membrane. This was necessary because the available crystal structures of phospholipid molecules do not provide convenient configurations that can be used as building blocks to assemble the protein membrane system.[27,28]

In all, the complete membrane–aqueous system we constructed for mu opioid receptor homology model consisted of 316 amino acid residues, 92 DMPC lipid molecules, 11 chloride counterions, and 8327 water molecules. Altogether there were a total of 40931 atoms in the system. The dimensions of the central unit cell were approximately $80\times74\times60$ Å. After a 200 ps initial dynamics simulation to gradually release all the constraints in the system, another 2 ns of MD simulation were conducted. A schematic representation of the entire system from a sliced side-view snapshot of the last configuration of the molecular-dynamics simulations is shown in Figure 1.

In order to examine conformational variations of the receptor within the complex membrane–aqueous environment, the root-mean-square deviation (rmsd) of the atomic positions with respect to the starting structure was calculated. Figure 2 shows the rmsd for Cα atoms of the protein as a function of the simulation time (2 ns of the final molecular dynamics). The rmsd of Cα atoms rose to about 2 Å after 200 ps of simulation and then leveled off after 500 ps. This indicates that, after an initial increase in the magnitude of residue fluctuation, the receptor protein reached an equilibrium state characterized by the rmsd profile. In fact, the system was well equilibrated after about 700 ps of molecular dynamics simulation at 330 K based on the change of the total energy of the system. After the system had been annealed to 310 K (from 330 K), the rmsd of the Cα on the backbone of the protein was retained at about 2.6 Å relative to the starting structure.

Fluctuations of individual residues along the polypeptide chain were examined by the rmsd for the Cα atom of each residue separately (Figure 3). As expected, the TM helical domain and the EL and IL regions had markedly different dynamic behavior, in that the average rmsd value in the TM domains was about 0.5 Å, whereas the loop regions had rmsd fluctuations in the range of 1–2 Å. The largest fluctuation was observed for the N terminus. Residues in the IL1 and EL1 segments also showed a somewhat higher degree of variation. Overall, the rmsd fluctuations suggest that the 7TM helical structure of the

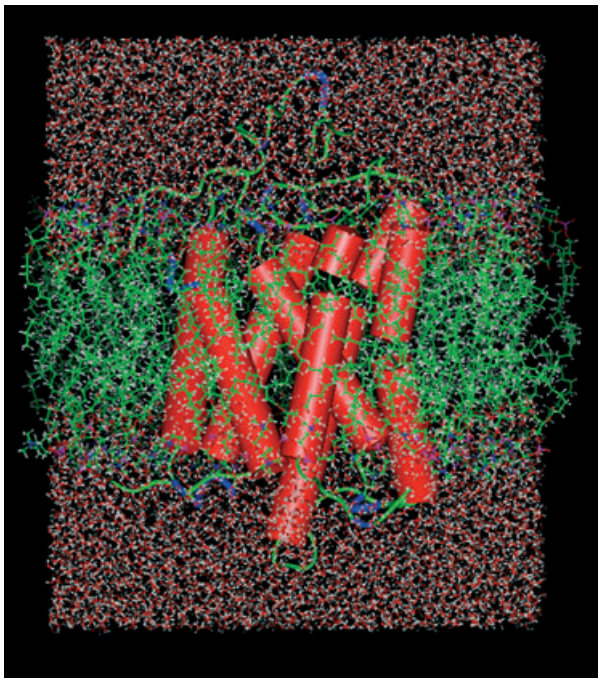


Figure 1. *Homology model of mu opioid receptor in a membrane–aqueous system.*

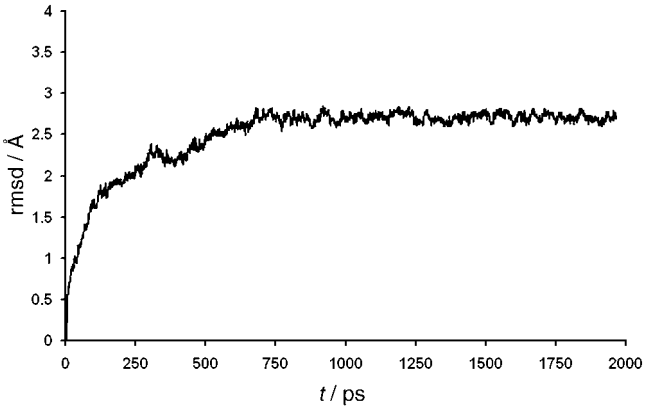


Figure 2. *Rmsd of the position of the Cα atoms of the mu opioid receptor relative to the starting structure versus simulation time.*

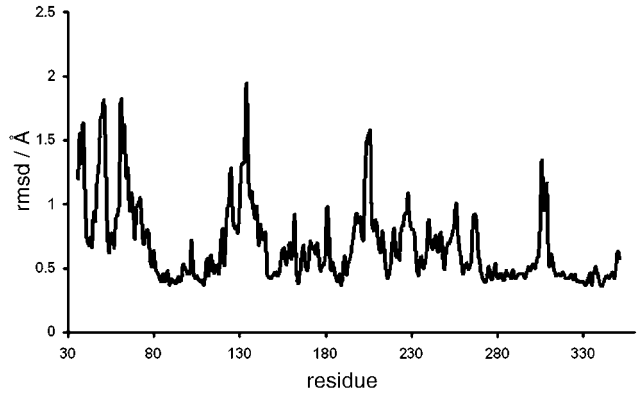


Figure 3. *Residue-by-residue Cα rms fluctuations about their average coordinates of the receptor.*

receptor protein is reasonably stable in the membrane–aqueous environment.

In summary, the mu opioid receptor homology model has been optimized in a complete membrane–aqueous matrix. After 2 ns of dynamics simulation, the conformation of TM domain and loop domains of the receptor model reached a newly equilibrated status in the matrix. Figure 4 shows the conformational changes in the homology model caused by the dynamics simulation.

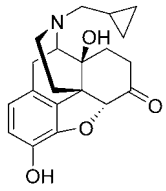
Figure 4. *Superimposition of the mu opioid receptor homology models before (gray) and after (black) the dynamic simulation in the membrane–aqueous system.*

First, the arrangement of α-helices in the TM domain became more compact; this reflected the impact exerted by the lipid bilayer. Significantly, the Cα atoms in the upper portion of TM3, TM5, and TM6—three critical helices that comprise the ligand-binding pocket—shifted inward from 0.2 to 3.8 Å relative to the starting position before dynamic simulations. This shift yielded a more compact binding pocket for opioid ligands and may provide more information about the interactions of the ligand with amino acid residues in the binding site. Secondly, the EL and IL domains of the receptor were dramatically rearranged in the aqueous layers, which could not be achieved reasonably in vacuo. This rearrangement should help us to better understand the effect of the loop domains on the structure of the whole receptor. Given these differences, the dynamics simulation of the receptor model in a membrane–aqueous system has produced a plausible starting structure for further optimization.

Molecular dynamics simulation of the mu opioid receptor docked with antagonist NTX

After an initial round of energy minimization and molecular-dynamics simulation of the receptor homology model in the membrane–aqueous environment, the universal opioid antagonist, naltrexone, was docked into the cavity that has been

identified as the binding pocket of the mu opioid receptor.[29] Energy minimization and molecular dynamics were then carried out for the ligand–receptor system to relax and optimize binding interactions between the ligand and amino acid residues in the binding cavity. Since the homology model was built based on the inactive state conformation of rhodopsin, using an antagonist in the docking process is more appropriate.



Naltrexone (NTX)

The ligand–receptor complex structure obtained after 11 ps of molecular dynamics is depicted in Figure 5. In this structure, the distance between the protonated nitrogen atom of NTX and the carboxyl group of Asp147 was initially anchored at 4.5 Å and retained at this value by a weak harmonic restraint during the molecular-dynamics simulation to represent the putative salt bridge that has been inferred from experimental studies.[30] In the final conformation of the complex, the distance (4.3 Å) was compatible with the initial setting.

Figure 5 shows that the binding pocket of mu opioid receptor has at least three binding domains to NTX. First, a polar and aromatic domain, which is composed of Asp147, Phe289, Trp293, Cys321 (not shown for clarity), and Tyr326, is in the vicinity of the protonated amino moiety and the 14-hydroxyl group of NTX. Besides the putative salt bridge between Asp147 and the protonated amino moiety of NTX, a cluster of aromatic side chains (Phe289, Trp293, and Tyr326) might bind with the amino cation through cation–π interactions.[31] At the same time, it has been suggested by site-directed mutagenesis studies that the polarity of Tyr326 might be critical for the recognition of NTX in the mu opioid receptor binding pocket, since the substitution of phenylalanine for tyrosine decreased the binding affinity of naltrexone.[32]

The second binding domain can be characterized as a number of hydrophobic interactions in the binding pocket that involve the phenolic moiety of the ligand and aromatic residues of Tyr148 from TM3, and Tyr210 and Phe221 from EL2. It has been reported that Tyr148 might have π–π interactions with the phenolic moiety of morphinane opiates.[33]

There is another hydrophobic domain that involves Trp318, Leu219, Ile322, Ile296, and Ile144 (not shown for clarity). This domain might have hydrophobic interactions with the aliphatic piperidinyl and cyclohexanonyl skeleton in NTX. As verified by site-directed mutagenesis studies, Trp318 may play a critical role in the binding of selective mu opioid receptor ligands, such as morphine and NTX, to mutant mu opioid receptor W318L and W318K for which they had lower affinity.[34]

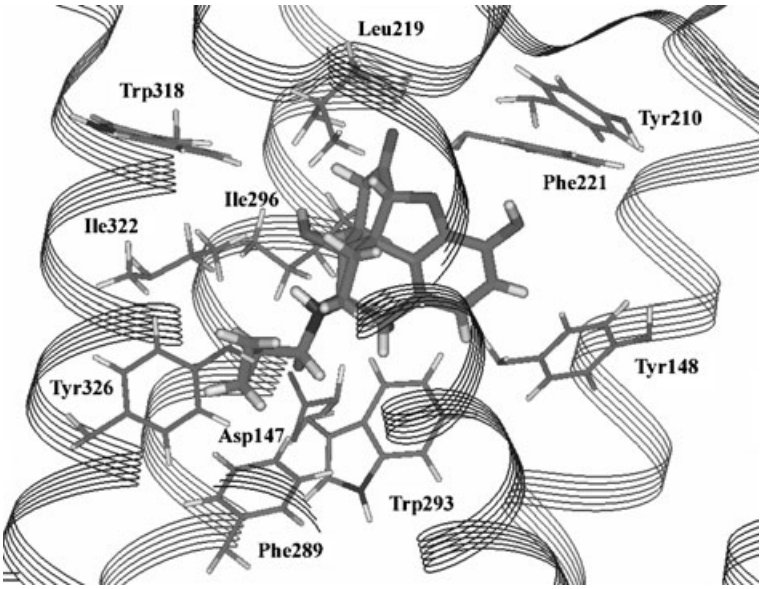


Figure 5. *Characterization of the mu opioid receptor antagonist binding site by docking of naltrexone into the binding cavity.*

Conclusion

We have constructed a homology model of the mu opioid receptor based on the X-ray crystal structure of bovine rhodopsin and further refined the model by molecular-dynamics simulations in a membrane–aqueous environment.[35] An opioid-receptor antagonist, naltrexone, was then docked into the receptor model, and specific ligand–receptor interactions were suggested after molecular-dynamics simulations. The homology model may provide a more robust model of the receptor structure for analysis of specific binding interactions with the opioid ligands. Optimization of the mu opioid receptor model in the membrane–aqueous system has provided a more compact binding pocket that accommodates a naltrexone-binding cavity. The present mu opioid receptor model might be the first step toward the construction of an interaction complex, involving the receptor, an agonist, and G protein in a membrane matrix, that will give insights into receptor activation and structural transformation.

Computational Methods

The sequence of the mu opioid receptor was obtained from the NCBI Database (ID code: AAE87936). Homology modeling was carried out by using the Homology and Discover modules in the InsightII package. All computations were performed on SGI Origin 2000 or Octane2 workstations (Silicon Graphics, Inc., Mountain View, CA), and an IBM SP system with eight processors at the Minnesota Supercomputing Institute.

The general strategy for creating a reasonable starting configuration for the protein–phospholipid system consisted of randomly selecting lipid molecules from a pre-equilibrated and prehydrated set, placing them around the protein, and finally reducing the number of core–core overleaps between heavy atoms through systematic rotations about the *Z*-axis, which is along the norm of the membrane plane, and translations in the *XY* plane of the lipids and protein. To provide the initial positions for each lipid molecule in the *XY*-plane, the whole lipid molecule was first represented by a

single effective van der Waals sphere, corresponding to the average phospholipid cross-sectional area. The packing of the effective lipid particles around the membrane protein was optimized from a molecular-dynamics simulation in which the large effective particles were harmonically restrained along the *Z* direction, but were allowed to move freely in the *XY* plane. Periodic boundary conditions were used in all calculations. The resulting Cartesian positions of the large spheres were utilized for the initial placement of the phosphate head group of each phospholipid.

The pre-equilibrated conformers for each phospholipid DMPC molecule were taken randomly from a library of 2000 that had been previously generated from Monte Carlo simulations of an isolated DMPC molecule in the presence of a mean field (this library is distributed along with the program CHARMM).[25, 26, 36] The conformers generated by the mean-field Monte-Carlo simulations, in so far as they agreed with the available experimental data, were representative of the phospholipid molecules found in a bilayer membrane in thermal equilibrium with its normal axis in the *Z* direction. In order to provide the primary hydration for the polar headgroup, approximately 20 water molecules around both the phosphate and the choline group were also included in the library; these water molecules were obtained from snapshot configurations during molecular-dynamics simulations of *o*-phosphorylcholine in bulk solution.[26]

Because the initial configuration was first assembled with totally uncorrelated phospholipid conformers, there were a large number of unrealistic overlaps. To improve the initial configuration, a global search, with systematic rigid-body rotation and translation of the lipids (along with the primary companion waters), was performed to reduce the number of bad contacts. Systematic rotations were performed with rigid-body rotations around the *Z*-axis in 10° increments for each individual lipid. Systematic translations in the *XY*-plane were performed with 0.25 Å steps around a square of side 2 Å centered on the initial position of each lipid. A "bad contact" was defined as the presence of a distance of less than 2.6 Å between two non-hydrogen atoms of the system. After the global search based on systematic rotation and translation, the remaining contacts were removed by energy minimization. For each cycle, the protein was fixed, and the van der Waals radii of the lipids were gradually increased from zero to the final force-field values.

To obtain a solvated microscopic system, the remaining bulk solvent was built in by using a previously equilibrated box of water molecules. The water box was translated along the *Z*-axis, and its position was adjusted to match the interface with the lipid bilayer. Water molecules that were within 2.5 Å of any protein or lipid non-hydrogen atom and those that penetrated into the hydrocarbon interior of the bilayer by $\geq$12 Å were deleted. After the operation, energy minimization was performed for 200 steps to remove close contacts.

The equilibration of the protein–membrane–solvent system consisted of three different stages. In the first stages, we carried out molecular-dynamics simulations using the Langevin equations of motion for 200 ps at 330 K, after the system had been gradually heated to the final temperature. Initially, the peptide backbone atoms and the centers of mass of the phospholipid head groups were restrained by harmonic forces. The harmonic restraints were gradually decreased so that, by the end of 200 ps, the full system was completely free. The Langevin dynamics were followed for additional 50 ps without any constraints. Then, Newtonian molecular-dynamics simulations were performed to further equilibrate the system for 1 ns. The first 960 ps simulation was carried out at 330 K for rapid equilibration and relaxation of the system. We then lowered the temperature from 330 K to 310 K over 20 ps, and this was followed by an equilibration of 20 ps at 310 K (making a total of 1 ns of equilibration in stage 2). The final stage consisted of a 1 ns molecular-dynamic simulation at 310 K, from which the coordinates for 1000 configurations from the trajectory were saved and used for analysis. All calculations were carried out by using the isothermal–isobaric ensemble at 1 atm and 310 K (or 330 K in the beginning).

The coordinates for the ligand molecule NTX were created by using InsightII. The conformation of the compound was subsequently optimized, relaxed, and equilibrated by molecular-dynamic simulations in aqueous solution at 300 K. The ligand was modeled in its protonated-nitrogen form. We used the final structure in water from the 2 ps molecular-dynamics calculations, which is as arbitrary as any other configuration explored during the simulation, as the initial configuration for docking into the proposed binding site of the mu opioid receptor. Experimental studies[37] suggest that the protonated nitrogen moiety interacts with the carboxyl group of Asp147 to form a putative salt bridge. The phenol ring was placed in the binding pocket to provide the most effective π–π stacking and cation–π interactions[38] with appropriate aromatic amino acids. The rest of the molecule was oriented toward the extracellular surface. Then the position of the ligand was adjusted to avoid overlap with the receptor atoms.

Molecular-dynamics simulations of the ligand and receptor complex were performed first in the gas phase with the backbone of the receptor fixed but all the side-chain atoms left unconstrained. The optimized conformation was then used as the initial configuration for the dynamics simulations including solvent, which was modeled by a 20 Å layer of water molecules above the lipid bilayer. Steepest-descent energy minimizations were performed for 2000 iterations, after which 10 000-step dynamics were conducted with 1000 steps equilibration for the initial dynamics. The total simulation time was 11 ps.

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Keywords: antagonists · binding sites · membrane proteins · molecular modeling · receptors

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