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The Role of Water and Sodium Ions in the Activation of the μ-Opioid Receptor**

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More than 800 human G-protein-coupled receptors (GPCRs) allow cells to recognize diverse extracellular stimuli (photons, odorants, hormones, lipids, etc.) and transduce the signals across the plasma membrane to regulate central physiological processes.[1] As GPCRs are implicated in many diseases, they are among the most important drug targets.[2] X-ray crystallography has delivered high-resolution seven-transmembrane helix structures of a few GPCRs before and after activation.[3] However, details of the structural and dynamic transitions within the receptors during transmembrane signaling remain to be solved and require complementary approaches to the static structure determinations. Molecular dynamics (MD) simulations have been shown to deliver important information on such processes.[4] In this context we report herein our investigation of the role of water and sodium ions during GPCR transmembrane signaling. Although there is experimental evidence that both water molecules and sodium ions are crucial for GPCR activation and signaling,[5] the mechanistic explanation of these findings is still missing.

To solve these central problems, we performed MD studies on the crystal structure of the μ-opioid receptor (μOR; PDB code: 4DKL).[6] We found distinct consecutive mobility patterns in the trajectory pathway of the sodium ions entering the receptor from the extracellular side towards the internal conserved residue $D114^{2.50}$ (Ballesteros–Weinstein helix numbering).[7] The distinct stages in the pathway of the ions are correlated with distinct local structural changes within the receptor as well as the distribution of internal water molecules. The MD simulations resolved the experimentally found dual role of sodium ions 1) to decrease the binding affinity for agonists, and 2) to facilitate G protein activation.[8]

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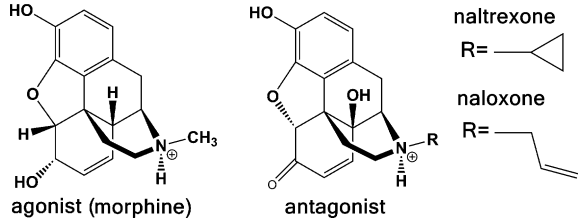
The μOR belongs to the rhodopsin-like family of GPCRs. The receptor is widely distributed in the brain, and found also in the spinal cord and digestive tract.[9] To address the role of sodium ions at the allosteric binding site[10] of μOR, and what roles sodium ions and water molecules play in μOR activation, we performed MD simulations with all atoms over a long timescale (a total of 9.4 μs; Table 1).

Table 1: Performed MD simulations.

Case	Apo	morWAT[a]	morNA[b]	naltNA[b]	naloNA[b]
ligand[d]	–	morphine	morphine	naltrexone	naloxone
time[c]	500×2	1400×2	1400×2	1400×1	1400×1

[a] Without Na^+ located in the allosteric site. [b] With Na^+ located in the allosteric site. [c] ns × number of simulations.

[d]



Most high-resolution crystal structures of GPCRs show water molecules at the allosteric site $D^{2.50}$ forming extensive hydrogen bonds.[5] However, a sodium ion was found bound to $D^{2.50}$ in recent structures of the adenosine A2A receptor[5] and a protease-activated receptor (PAR1).[11] A sodium ion was also proposed to be located in this region of dopamine D_2R.[12] Our simulations confirm that a sodium ion can access the allosteric binding site of the μOR from the extracellular side (Figure 1). During the initial 30 ns of the MD simulation (Figure 1B), the sodium ion occupied a region around the negatively charged residue $E229^{5.35}$ at TM5 of μOR, which is exposed to the extracellular phase (Figure 1A, zone I). Interestingly, residue $E^{5.35}$ was also found in δOR and κOR, implying the importance of a negative charge at this site. In the subsequent MD steps (30–160 ns, Figure 1B), the sodium ion was conducted by $E229^{5.35}$ into the receptor's orthosteric site $D147^{3.32}$ (Figure 1A, zone IIa), which is crucial for ligand binding.[13] After that, Na^+ entered zone IIb (Figure 1A) waiting for a semaphore switch to finally reach the allosteric site. A conformational change of $W293^{6.48}$ allowed the sodium ion to further into the deep pocket of μOR (Movie M1 in the Supporting Information) to be finally stabilized between residues $S154^{3.39}$ and $D114^{2.50}$ (Figure 1A, zone III), where it stayed until the end of the simulation (Figure 1B).

Our findings are in close agreement with a recent 1.8 Å resolution crystal structure of adenosine A2A receptor with

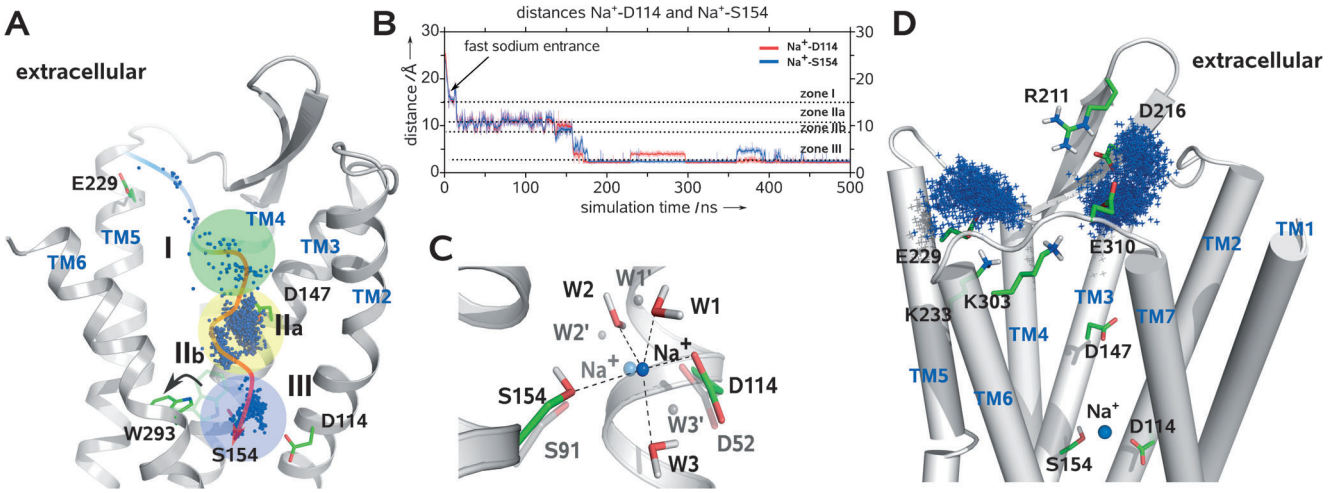


Figure 1. Pathway of the sodium ion entering the μOR. A) Cross-section of μOR shows six transmembrane helices TM1–TM6 and the trajectory (red arrow) of a particular sodium ion during the initial 200 ns of Apo μOR simulation. Blue dots are consecutive positions of the sodium ion along its pathway. A black arrow indicates a rotamer switch of W293$^{6.48}$. The sodium ion was initially captured by E229$^{5.35}$ in zone I and quickly brought to zone IIa next to D147$^{3.32}$. After a short stay in zone IIb at about 150 ns simulation, it finally stabilized in zone III near D114$^{2.50}$ and S154$^{3.39}$. B) Distances Na^+–D114$^{2.50}$ (blue) and Na^+–S154$^{3.39}$ (red) during two separate 500 ns simulations. C) Superimposed high-resolution structure of the adenosine A2A receptor (pale colors) and final MD structure for Apo μOR (bold colors). D) Positions of second sodium ion (blue crosses) during simulation of ligand-free receptor. This ion is located either at E229$^{5.35}$ or between D216^{ECL2} and E310^{ECL3} in the extracellular part of μOR.

bound agonist.[5] Superposition of the final structure of Apo μOR from our MD simulations with the crystal structure of adenosine-bound A2A receptor shows the sodium ion in both structures at nearly identical positions between D114$^{2.50}$ and S154$^{3.39}$ (Figure 1 C). Moreover, three water molecules found in the crystal structure were also observed in our simulations coordinating the sodium ion (Figure 1 C). A similar sodium ion pathway was proposed elsewhere using MD simulations of the homology model of the dopaminergic D_2 receptor,[12] but the exact position of the sodium ion was not validated because of lack of appropriate crystal structures.

After the first sodium ion was captured by D114$^{2.50}$ in the allosteric site of μOR during the simulation of the ligand-free receptor, a second sodium ion was found occupying positions either at E229$^{5.35}$ or between residues D216^{ECL2} and E310^{ECL3} (Figure 1 D). The three negatively charged residues at the extracellular side of the μOR form a triangle above the orthosteric site and they are all located beneath the positively charged residues (Figure S1). The negatively charged residues at the extracellular side were considered of great importance for the transient binding of positively charged ligands before the receptor opens the path to the binding site during thermal conformational fluctuations.[6,14] Blocking this recognition site by sodium ions could explain the observations that the ligand binding affinity is decreased with increasing sodium ion concentration.[8a,15]

Although sodium ions are known to decrease the ligand binding affinity of μOR,[8a,16] surprisingly, sodium ions were also found promoting agonist-induced activation of μOR, which was measured by binding of GTP, while inhibiting the spontaneous G_i/G_o-coupled receptor activity.[5,12] To clarify these findings, MD simulations were performed for both agonist- and antagonist-bound μOR. The binding modes for all ligands were obtained by ligand docking and optimizing the structures of the complexes. For all cases we obtained similar ligand binding conformations after MD equilibration because the ligands had similar rigid structures (Table 1, Figure S2). The phenol oxygen in each ligand forms a link to H297$^{6.52}$ through hydrogen bonds involving two water molecules on average, while a protonated nitrogen forms a salt bridge with D147$^{3.32}$. The different final influences of morphine and both antagonists on the receptor are a result of differences in their chemical structures, especially at the nitrogen. The small methyl group attached to nitrogen in morphine (Figure S2) shows nearly no interactions with the hydrophobic side chain of W293$^{6.48}$ and Y326$^{7.43}$, leaving enough space for solvent molecules to penetrate the interior of μOR. Additionally, a break of a hydrogen bond between D147$^{3.32}$ and Y326$^{7.43}$ in simulations of agonist-bound receptor enlarged the water-accessible space. Kolinski et. al also found this bond broken during the initial stages of activation of the homologous κOR.[17] In contrast, the bigger hydrophobic tails of the antagonists naltrexone and naloxone were stacked between the hydrophobic side chains of W293$^{6.48}$ and Y326$^{7.43}$ (Figure S2 C,D), preventing entrance of further water molecules.

The final structures obtained from each of the 1400 ns simulations of the antagonist-bound receptor showed distinctly fewer water molecules in the allosteric binding site (Figure S3 A,B) than in the structures of the agonist-bound receptor with Na^+ (morNA, Figure 2 A) and with water (morWAT, Figure S3 A) in the binding site. Statistics on the number of water molecules within a distance of 6 Å from D114$^{2.50}$ validated these observations (Figure S4).

The number of water molecules increased in the presence of a sodium ion at the allosteric site in morNA compared to the case without sodium ion. Furthermore, analysis of the final 50 ns for each 1400 ns simulation demonstrated that

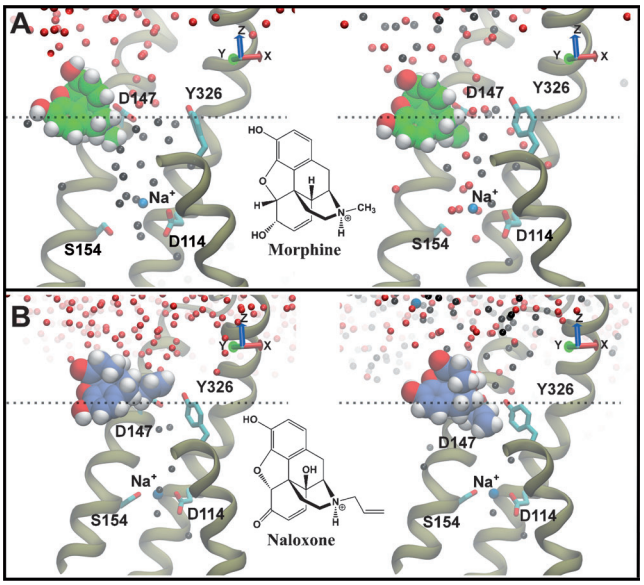


Figure 2. Water inside μOR at 1350 ns (left) and 1400 ns (right) MD simulations. Red dots: water molecules above the ligand binding site (dotted line); black dots: water molecules below the ligand binding site. A) Morphine-bound μOR with Na^+ in the allosteric site (next to $D114^{2.50}$). B) Naloxone-bound μOR with Na^+ in the allosteric site; here, extracellular water cannot exchange with water at the allosteric site. Chemical structures of morphine and naloxone are shown for clarity.

water molecules inside of the agonist-bound μOR quickly exchange with extracellular waters (Figure 2A and Figure S3A), which is not observed for the two cases of antagonist-bound μOR (Figure 2B and Figure S3B). The movement of water molecules at the conserved motifs has been shown to be important since they are associated with switches of GPCR activation.[18]

In many GPCRs the D(E)RY motif at the cytoplasmic end of TM3 forms an ionic lock between the highly conserved $R^{3.50}$ and TM6, $E^{6.30}$ in the case of rhodopsin, which retains the inactive state of the receptor.[19] In μOR the DRY motif is also present linking TM3 and TM6 by a hydrogen bond between $R165^{3.50}$ and $T279^{6.34}$. This interaction was proposed to stabilize the receptor in its inactive form[6] especially as the mutation $T279^{6.34}K$ has been found to shift the receptor to a constitutively active state.[20] During MD simulations in the equilibration phase after removal of sulfate ions from the crystal structure, the TM3–TM6 link was broken and restored later (Figure 3A). In both simulations of the morWAT a hydrogen bond between $R165^{3.50}$ and $T279^{6.34}$ was formed at 650–700 ns while the ionic interaction $R165^{3.50}$–$D164^{3.49}$ remained stable until end of the simulations. However, in the simulations of morNA a break of the ionic interaction within the DRY motif appeared nearly simultaneously with the formation of the link between TM3 and TM6 at around 1100 ns. The above-mentioned changes in the DRY motif were observed in neither the antagonist-bound μOR nor the Apo form (Figure S5). Forming a transient link by $R165^{3.50}$ between TM3 and TM6 in the initial stages of activation may help to break the intrahelical interaction $R165^{3.50}$–$D164^{3.49}$ and allow $R165^{3.50}$ to form more favorable interactions in later stages of activation, for instance with $Y^{5.58}$ in analogy to activated rhodopsin and β2-adrenergic receptor. The resulting movements of helices in morphine-bound μOR with Na^+ in the allosteric site are shown in Figure S6. They may represent very early initial stages in the activation of μOR.

In conclusion, in extended timescale MD simulations we repeatedly observed the trajectory of sodium ions entering the μOR (Figure 3B). On the one hand, experimental data indicate that sodium ions can influence the affinity of ligand binding of μOR. In the framework of our MD simulations this can be explained by the repulsive effect on positively charged ligands (protonated amines) exerted by the second sodium ion bound to the extracellular surface of the receptor. On the other hand, sodium ions can also facilitate the activation of μOR by inducing the movement of water molecules towards the allosteric site, which influences an action of the orthosteric agonist. Our results pave the way for understanding the roles of water and sodium ions involved in GPCR activation and may be applied for the virtual screening of selected

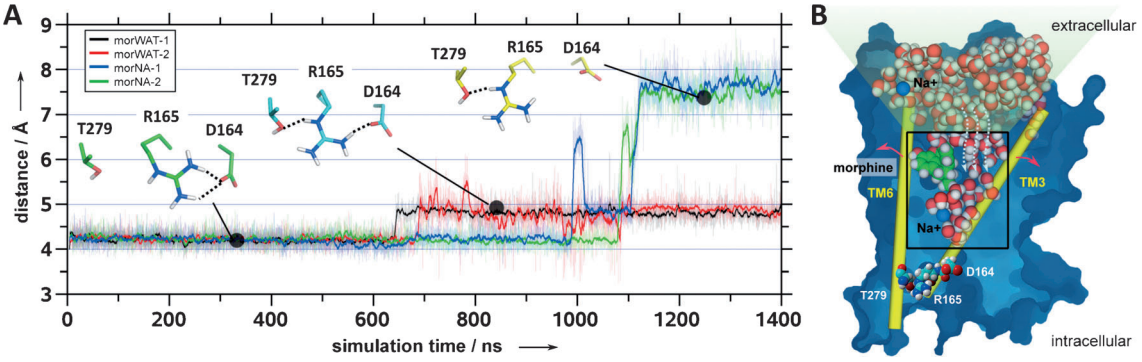


Figure 3. A) Temporal changes of the distance between $D164^{3.49}$ and $R165^{3.50}$ in DRY motif. A link to $T279^{6.34}$ by $R165^{3.50}$ was created in all simulations. When only water was present in the allosteric site no further change occurred (black and red lines), but in the presence of Na^+ in the allosteric site the ionic interaction between $D164^{3.49}$ and $R165^{3.50}$ was broken (blue and green lines). B) Role of sodium ions and water in μOR activation. In the presence of morphine there is a large excess of water in the allosteric and orthosteric sites (black rectangle). Sodium ions (blue spheres) induce additional transient movement (white arrows) of water from the extracellular side (light blue-green cone) towards the allosteric site of agonist bound μOR. Such movement is facilitated by motion of amino acid residues (red arrows) of transmembrane helices.

compounds targeting GPCRs based on their influence on water mobility inside the receptors.

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