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Ligand interaction, binding site and G protein activation of the mu opioid receptor

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Abstract

With the recently solved crystal structure of the murine mu opioid receptor, the elucidation of the structure function relationships of the human mu receptor becomes feasible. In this study, we analyzed the available structural information along with ligand binding and G protein activation of human mu receptor. Affinity determinations were performed in a HEK293 cell line stably transfected with the human mu opioid receptor for 6 different agonists (morphine, DMAGO, and herkinorn) and antagonists (naloxone, beta-Funaltrexamine, and Norbinaltorphimine) based on the method. G protein activation was investigated in membrane preparations containing human mu receptors treated with the agonist, partial agonist, or antagonist compounds. 4DKL.pdb was utilized for structural analysis and docking calculations for 28 mu receptor ligands. The predicted affinities from docking were compared with those experimentally determined. While all known ligands bind to the receptor through the same binding site that is large enough to accommodate molecules of various sizes, interaction with D147 (D149 in human mu receptor) is essential for binding. No distinguishable interaction pattern in the binding site for agonist, partial agonist, or antagonist to predict pharmacological activities was found. The failure to reconcile the predicted affinities from docking with experimental values indicates that the receptor might undergo significant conformational changes from one state to the other states upon different ligand binding. A simplified model to understand the complicated system is proposed and further study on these multiple conformations using high resolution structural approaches is suggested.

Index words

mu opioid receptor; G protein activation; Structure; function; docking; binding site

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1. Introduction

The therapeutic use and abuse of opioids has soared globally in recent years (Devi, 2011; Manchikanti and Singh, 2008; Kuehn, 2007a; b; 2009; Manchikanti, 2006). Between 1999 and 2002, the number of fatal opioid analgesic poisonings has increased by 91% while methadone-related deaths from 1999 to 2004 have increased by 390% (Paulozzi et al., 2006a; Paulozzi et al., 2006b). The White House Budget Office estimates that, in addition to the human toll, opioid abuse may contribute up to \$300 billion per year in direct healthcare costs (White et al., 2005). The pharmacological targets of opioids are opioid receptors which currently include 4 family members: mu, delta, kappa and nociception receptors (Cox, 2012). Each receptor type is responsible for different pharmacological effects and receptor-specific functional outcome despite of the high sequence homology among these receptors (67% to 76%) (Cox, 2012). The mu opioid receptor mediates pain perception and is responsible for the above mentioned problem related to opioid usage (addiction, respiratory depression and other side effects).

Crystal structures of these four receptors have been recently reported (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wu et al., 2012). Current research takes advantage of the solved crystal structure of the transmembrane portion of an engineered murine mu receptor – which displays high homology with its human counterpart (Manglik et al., 2012). The characterization of the structure of the mu opioid receptor should be part of the solution for the problems noted above as such information would further elucidate the mechanisms of these receptors. Furthermore, it would undoubtedly aid in the design and/or discovery of novel and potentially safer medication by extending structural insights and analyzing docking of other ligand in crystallized structure (Jacobson and Costanzi, 2012).

In particular, information from this crystal structure (Manglik et al., 2012) can be potentially exploited to further elucidate the structure-function relationship of the human mu receptor and thus advance our understanding of mechanisms mediating pain, addiction, and respiratory depression. Many important functional properties of the human mu opioid receptor – such as its ability to bind with various agonists, partial agonists, and antagonists, and its capacity to activate cognate guanine nucleotide-binding, G proteins, upon agonist binding – can be analyzed in a laboratory setting. G protein activation is required in most of the cases for opioid receptors and other GPCRs to trigger pharmacological events in the living organisms (Cox, 2012). It is critical to relate the receptor structure information to these downstream pharmacological effects by using a variety of modern biochemical and biophysical techniques since the co-crystallization of the receptors with G proteins and x-ray analysis remains technically very challenging.

Given the high homology between the murine and human mu opioid receptors, we investigated whether the information from the newly available crystal structure could provide insights into the biological functions of the human receptor, especially in regard to ligand binding and *in vitro* G protein activation.

2. Materials and Methods

Membrane preparations of recombinant human mu opioid receptor expressed in the mammalian cell line Chem-5 and used for G protein activation studies were obtained from Millipore (Billerica, MA, USA). All opioid ligands were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were reagent grade or higher. Herkinorin was purchased from Ascent Scientific LLC (Princeton, NJ, USA). All chemicals were used without further purification.

Although the crystal structure of the human mu opioid receptor is not available, a sequence analysis of the human (uniprot accession number P35372, <http://www.uniprot.org/>) and

mouse (uniprot accession number P42866) μ opioid receptors shows a sequence identity of 94% for the entire sequence. The similarity of the sequences in the region solved in the crystal structure (PDB access code: 4DKL(Manglik et al., 2012)) is 99%. Since differences between these sequences occur outside of the binding pocket, results from binding pocket analysis and docking experiments will be equally relevant for human mu opioid receptor.

2.1. Binding pocket volume and area determination

The binding pocket volume and area information was analyzed using CASTp (<http://sts.bioengr.uic.edu/castp/calculation.php>), an online binding pocket analysis tool (Liang et al., 1998). The default value of 1.4 Å was used for calculation. The binding pocket image was generated using PyMOL (Version 1.3, Schrödinger, LLC.; <http://www.pymol.org/>) along with a CASTp PyMOL plugin (CASTpyMOL v2.0, <http://sts.bioengr.uic.edu/castp/pymol.php>)

2.2. Docking calculations

Docking calculations for the structure of the murine mu receptor (PDB access code: 4DKL(Manglik et al., 2012)) were carried out using DockingServer (<http://www.dockingserver.com>) (Bikadi and Hazai, 2009) as previously described(Liu et al., 2012). Semi-empirical charges calculated by MOPAC2009 were added to the ligand atoms (<http://openmopac.net/MOPAC2009.html>) (Stewart, 1990). Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added to the receptor using AutoDock tools provided by the server. Grid maps of 30×30×30 Å grid points with 0.375 Å spacing centered at the known ligand binding site were generated using the Autogrid program (Morris et al., 1996; Morris et al., 2009). Opioid agonist, partial agonist, and antagonist searches were performed using the Solis and Wets local search method with a Lamarckian genetic algorithm (Solis and Wets, 1981). Initial position, orientation, and torsions of the ligand molecules were set randomly. The predicted site with a dominant energy was chosen for subsequent analysis. The estimated binding constant (K_i) was derived from the equation $\Delta G = -RT \ln K$, where ΔG is directly calculated during docking runs using the Autodock scoring function.

A total of 26 ligands for the opioid receptor – which included full agonists, partial agonists and antagonists – were selected for docking calculations based on affinities experimentally obtained in this study, by using the same methodology for affinity determination from a study published recently(Volpe et al., 2011) (see Table 1). The three-dimensional coordinates of the tested opioids were obtained from the PubChem database (<http://pubchem.ncbi.nlm.nih.gov/>). The residues interacting with the ligands were analyzed in an attempt to find potential patterns for ligand binding. PyMOL was used to render the graphics for presentation.

2.3. Affinity determinations and correlation analysis

Affinity determinations for agonists (morphine, DAMGO, and herkinorn) and antagonists (naloxone, beta-Funaltrexamine, and Norbinaltorphimine) were performed in a HEK293 cell line stably transfected with the human mu opioid receptor as previously described (Roth et al., 1981). Although most of the affinity data are available in literature, we chose some of the typical agonists from different categories (morphine, small peptide, non-opioid mu opioid receptor) and antagonists to determine the affinity using the same methodology to avoid technical variances. The determined affinities are compared with those from the docking prediction. The affinities available for 16 full agonists or partial agonists from the same study published recently (Volpe et al., 2011) were compared with those predicted by docking. Potential correlations were determined using GraphPad Prism (V5.04, GraphPad Software, La Jolla, CA).

2.4. G Protein Activation by receptor treated with agonists, partial agonists and antagonists

The effects of specific mu opioid receptor ligands on the activation of the recombinant receptor expressed in mammalian cell membranes were investigated by measuring G protein activation *in vitro*. The full agonist DAMGO ([D-Ala², N-MePhe⁴, Gly-ol]-enkephalin), partial agonist nalbuphine, and antagonist naloxone were utilized.

The assay reports the initial rates of activation of heterotrimeric G proteins ($G_{\alpha i1}\beta_1\gamma_2$) on an agonist-bound receptor by measuring the accumulation of [³⁵S]-GTP γ S (non-hydrolyzable analog of GTP) bound to the activated $G_{\alpha i1}$ subunit. Myristoylated $G_{\alpha i1}$ was expressed in *E. coli* and purified as previously described (Mumby and Linder, 1994). Recombinant human $\beta_1\gamma_2$ subunits of G protein were expressed in baculovirus-infected Sf9 cells and purified as previously described (Wildman et al., 1993). The G protein activation assay was conducted as follows (final concentrations in 50 μ l reaction mixture are given in parentheses): the membrane sample was diluted into ice-cold 10 mM MOPS buffer to reach a protein concentration of 40 ng/ μ l. Ten μ l of the diluted dispersion were dispensed into pre-siliconized glass tubes and mixed with the ligand in MOPS buffer containing 0.1% (w/v) BSA. Upon addition of a mixture of $G_{\alpha i1}$ (100 nM) and $G_{\beta_1\gamma_2}$ (500 nM), the tubes were incubated on ice for 30 minutes. The reaction was started by addition of MOPS buffer pH=7.5 (50 mM), EDTA (1 mM), $MgCl_2$ (3 mM), GDP (4 μ M), BSA (0.3% w/v), NaCl (100 mM), DTT (1 mM), and [³⁵S]-GTP γ S (5 nM, 1250 Ci/mmol) followed by rapid transfer of the tubes to a water bath at 30 °C. The incubation continued for 45 minutes. The reaction was terminated by addition of 2 ml of ice-cold stop solution, TNMg (20 mM Tris-HCl pH=8.0, 100 mM NaCl, and 25 mM $MgCl_2$). The reaction mixture was rapidly filtered through nitrocellulose filters (Millipore, Billerica, MA). Filters were washed four times with 2 ml each of cold TNMg buffer, dried, placed in scintillation vials filled with ScintiSafe Econo F scintillation liquid (Fisher, Waltham, MA), and the radioactivity counted. Duplicate samples corresponding to every ligand concentration point were counted, and results were analyzed using GraphPad Prism.

2.5. Statistical Analysis

Linear regression for K_i values was performed using a GraphPad software. A P value less than 0.05 was considered statistically significant for a linear correlation.

3. Results

3.1. Binding pocket characterization

The opioid binding site is a well-packed pocket with a total molecular surface volume of 2173 $\text{\AA}^3$ and an area of 1095 $\text{\AA}^2$. The binding pocket has a wide open mouth with a solvent accessible area of 237 $\text{\AA}^2$ extending deeply into the protein core (591 $\text{\AA}$ from mouth opening to the top of the pocket) (Fig 1A and 1B). The residues lining the protein binding pocket are shown in Fig 1C, and include 37% of hydrophobic residues (A,I,L,F,W,V) out of the all residues in the binding pocket; 37% hydrophilic residues (N,C,Q,S,T,Y); 11.1% basic(+) amino acids (K,R); 11.1% acidic(−) amino acids (D,E).

3.2. Residue interactions with agonists and antagonists

All the tested opioid agonists, partial agonists and antagonists interacted with D147 (D149 in human mu receptor) (Table 1) through hydrogen bonding. Herkinorin was the sole exception as it interacted with D147 without hydrogen bond formation. No specific and distinguishable patterns of interacting residues were detected for full agonists, partial agonists, and antagonists. Strikingly, the only significant difference between the interacting residues for morphine (full agonist) and naloxone (antagonist) was a non-specific interaction

between naloxone and N150 and its absence during morphine binding. However, this interaction was not found in the case of another antagonist (naltrexone) or partial antagonists (patazocine or nalbuphine). The analysis of interacting residues as well as the pattern of interaction fails to predict the efficacy of these ligands or their pharmacological effects as agonist, inverse agonist or antagonist.

3.3. Affinity determination and prediction

Furthermore, no significant correlation was found between the experimentally determined K_i values and the affinities predicted from docking experiments (Fig 2A, $P=0.17$). Likewise, there was no significant correlation between the experimentally measured K_i values for 19 agonists previously published (Volpe et al., 2011) and the K_i predicted from docking (Fig 1B, $P=0.5$).

3.4. G protein activation

According to the computer-assisted simulation, agonist, partial agonist, and antagonist occupy the same binding site on the mu opioid receptor binding pocket (Fig 3A). There is a very significant overlap between the space in the binding pocket occupied by nalbuphine (partial agonist) and naloxone (antagonist). However, nalbuphine produces a sigmoidal-type activation curve with an estimated EC_{50} of 7 nM while naloxone fails to activate the cognate G proteins in this assay (Fig 3B). As expected, treatment of the mu receptor with DAMGO, a full agonist, produces much higher levels of G protein activation with EC_{50} of 83 nM. At experimental conditions we observed only very low levels of binding of the γ - ^{35}S -GTP on membranes without addition of purified subunits of G proteins (results not shown).

4. Discussion

4.1. The binding pocket and ligand selectivity

The average volume of a drug-binding cavity was found to be around $610\text{\AA}^3$ or $930\text{\AA}^3$ depending on the calculation method utilized (Nayal and Honig, 2006; Perot et al., 2010). Although these values cannot be compared directly due to the use of different computational methodologies and lack of uniform standards, they nonetheless indicate that dimensions of the cavity in the binding pocket are relatively large for a mu receptor with a volume of $2173\text{\AA}^3$. The large open mouth at the extracellular side of this cavity may explain why the mu receptor could bind to a wide array of molecules of different sizes including large endogenous polypeptides (endorphins). Indeed, this large mouth has been previously used to explain why most of the opioid ligands have short dissociation half-lives (Manglik et al., 2012).

Although the presence of nitrogen atoms in the structures of traditional opioids has been considered essential for significant interaction with mu receptors, herkinorin has been found to be the first ligand without any nitrogen that has strong affinity with the receptor (Harding et al., 2005; Xu et al., 2007). The lining residues of the binding pocket include a number of hydrophobic, hydrophilic as well as basic or acidic amino acid residues. Thus, it is not surprising that different modes of interaction – such as hydrogen bonding, polar and hydrophobic interactions – could occur with various ligands.

4.2. Agonists, antagonists and interacting residues

Although the murine mu receptor structure was solved in an inactive, antagonist-bound state, all the tested agonists and antagonists (except herkinorin) were found to interact with the acidic residue D147 (D149 in the human homolog) through hydrogen bonding. These findings support several molecular modeling and site-directed mutagenesis based studies that had concluded that D147 played an important role in opioid ligand recognition and

hydrogen bond formation with various opioid ligands (Li et al., 1999; Tang et al., 1996; Xu et al., 1999). Thus future *de novo* design of new mu receptor ligands should take this prevalent interaction into account. However, the lack of hydrogen bond with D147 for herkinorin suggests that this specific form of interaction is not an absolute requirement for an opioid ligand to activate the receptor.

Considering that our finding were consistent with agonist interactions with functionally important residues of the receptor, we sought to identify residues specifically involved in interaction with only one class of ligands but not the other. However, our analysis failed to identify any specific residues or combinations of residues that could be used to differentiate between agonists and antagonists binding. For example, the only significant difference for the residues involved in interaction with morphine (full agonist) or naloxone (antagonist) was a non-specific interaction between naloxone and N150, not found in the case of morphine. However, such an interaction was not found in the case of another antagonist (naltrexone) or partial antagonist (patazocine or nalbuphine).

4.3. Predicted and experimental affinities

Autodock has been successfully used to predict binding affinity or energy values that are comparable with experimental affinities or clinical potencies in various biological systems (Adinarayana and Devi, 2011; Liu et al., 2012). For example, in the case of general anesthetics, the predicted docking affinities correlated well with affinities determined by isothermal titration calorimetry of horse apoferritin, a natural mammalian 4 helix bundle protein. Furthermore, the predicted affinities of a pentameric ligand-gated ion channel also correlated well with the EC50s of the tested clinical anesthetics ($R^2=0.98$, $P < 0.0001$) (Liu et al., 2012).

Despite these past successes, the affinities predicted for the mu opioid receptor by docking failed to significantly correlate with the affinities for various agonists and antagonist determined in this study and the affinities for various agonists available in literature. Explanations for this include: 1) Although the sequences for the transmembrane portions of the mouse and human mu receptors are essentially identical, the solved murine structure represents a heavily engineered protein stabilized by the replacement of a small part of the third intracellular loop (residues 264–269) with T4 lysozyme (residues 2–161). Although the engineered receptor demonstrates an identical affinity for the antagonist diprenorphine, it is unclear whether the affinities to other opioid ligands, especially for agonists, have been affected. 2) Multiple conformations representing different functional states of the mu receptor can be induced upon binding of different ligands; each of these states results in different pharmacological consequences. 3) Unlike the relatively simple structures of most anesthetics, the more complex configuration of opioids may result in larger degrees of uncertainties when performing docking studies. Since a significant uncertainty exists in predicted three-dimensional structures of opioid ligands, large deviations from the predicted affinities can also be expected. 4) The crystal structure covalently coupled with β - funaltrexamine, which also may impact the overall structure significantly as compared to other ligands in which no covalent bond could be formed.

4.4. G protein activation

Although opioid ligands occupy significantly overlapping space and may interact with the same residues within the binding pocket, their pharmacological effects may be very different. As noted above, analysis of the newly available high resolution structure of the receptor failed to identify the “key” elements in the chemical structures of opioid ligands that determine their pharmacological effects as agonist, partial agonist, or antagonist. This

perhaps may have been due to the limitations of the tool used in this docking study. However, the G protein activation studies support the notion of differential conformational states of the mu receptor resulting from the binding of interacting ligand. DAMGO, a full agonist, triggered a remarkable G protein activation, while nalbuphine, a partial agonist, elicited only partial activation of the G protein. No appreciable G protein activation or inhibition of the basal signal was observed for naloxone, an antagonist. The existence of basal signaling of human mu receptor in addition to the inactive state has been previously demonstrated (Divin et al., 2009; Wang et al., 2001). Taken together, our experimental results and available data in the literature^{30,31} suggest that there are at least four pharmacologically distinct states of the mu receptor: active, partially active, basal and inactive states. These dynamic states could shift from one to another resulting in different conformational intermediates and pharmacological consequences. These proposed states are summarized in Fig 4. It is important to note that this model may be overly simplified since no possible homo- or hetero-oligomeric arrangement/state is considered here. In the recently reported crystal structures of three opioid receptors (mu, delta, kappa), the receptors crystalize as either parallel- (Manglik et al., 2012; Wu et al., 2012) or antiparallel dimers (Granier et al., 2012). Although the currently available structural information is not sufficient to understand the potential determinants that cause shifts between such states, it is likely that the binding of ligand as well as several specific residues located in the binding site may play a critical role in triggering a cascade of conformational change on the receptor and modulating its subsequent interactions with G protein. As presented in the recent analysis of the 4 opioid receptor structures bound with respective antagonists, one conserved residue (D3.32) involved in the ionic interaction with antagonist plays a critical role in stabilizing an antagonist bound state for all 4 opioid receptors (D147 in murine μ receptor, D128 in murine delta receptor, D138 in human kappa receptor, and D130 in human nociception receptor) (Cox, 2012). High resolution structural studies of the receptor in multiple conformational (functional) states are warranted, i.e. using either crystallography or nuclear magnetic resonance to determine key residues specific for agonist bound states.

In summary, the recently solved crystal structure of the murine mu receptor has made it possible for the first time to analyze the structure-function relationship of the receptor using ligand binding and G protein activation assays as well as high resolution structural data. While all known ligands bind to the receptor through the same binding site that is large enough to accommodate molecules of various sizes, interaction with D147 (D149 in human mu receptor) is essential for binding. No distinguishable interaction pattern in the binding site for agonist, partial agonist, or antagonist to predict pharmacological activities was found. However, G protein activation studies clearly distinguished between the agonist, partial agonist, and antagonist ligands despite overlapping binding pockets (i.e. naloxone failed to induce any, and nalbuphine, a partial agonist, induced much weaker G protein activation than DMAGO, a full agonist). The failure to reconcile the predicted affinities from docking with experimental values indicates that the receptor might undergo significant conformational changes from one state to the other states upon different ligand binding. Further study on these multiple conformations using high resolution structural approaches is warranted.

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Fig 1.

Binding pocket of the mu opioid receptor. 1A demonstrates the location and size of the binding pocket, which extends into the interior core of the receptor; 1B demonstrates the wide opening of the mouth of the binding pocket with an antagonist sitting in the binding site; 1C shows the sequence of the receptor with the residues lining the binding pocket highlighted.

Fig 2.

Relationship between experimental affinities and docking predictions. 2A demonstrates the lack of correlation between the affinities derived experimentally in this study and the affinities predicted by docking calculation for various agonists, partial agonists, and antagonists; Fig 2B, which demonstrates the lack of correlation between the affinities experimentally determined in a published study (Volpe et al., 2011) and the affinities predicted by docking calculation for various agonists. FNA, beta-Funaltrexamine; Non-BNI, Norbinaltorphimine.

Fig 3. Binding and G protein activation. 3A demonstrates naloxone (antagonist in green) and nalbuphine (partial agonist in red) overlap well in the mu receptor binding pocket; 3B demonstrates that naloxone failed to induce any G protein activation, and nalbuphine induced G protein activation, but is much weaker than DAMGO, a full agonist.

Fig 4.

Schematic of the dynamic states of the mu opioid receptor. Each state can shift to another state based on the availability of the ligand which may result in different conformational intermediates and pharmacological consequences. The protein structures are generated from the same crystal structure with the PDB code of 4DKL. The structures are pictured in different orientations and color coded to represent the different states. A ligand is docking with each state of the receptor except the basal state; morphine for the fully active state, nalbuphine for the partially active state, and naloxone for the inactive state.

Table 1

Interacting residues for opioids in mouse μ receptor

	GLN	ASN	TYR	VAL	ILE	ASP	TYR	ASN	MET	ILE	HIS	ASP	CYS	THR	LEU	PHE	GLU	LEU	LYS	VAL	PHE	TRP	ILE	HIS	VAL	ILE	TRP	HIS	ILE	TYR
	124	127	128	143	144	147	148	150	151	196	197	216	217	218	219	221	229	232	233	236	237	293	296	297	300	301	318	319	322	326
Alfentanil	+			+		+	+		+			+	+						+	+		+	+	+	+			+	+	+
B-funaltrexamine						+	+		+						+	+	+	+	+	+		+	+	+	+			+	+	+
Carfentanil					+	+	+								+	+	+	+	+	+		+	+	+	+	+		+	+	+
DAMGO	+		+		+	+	+		+			+	+		+				+	+					+				+	+
Etorphine					+	+	+		+										+	+		+	+	+				+	+	+
Fentanyl				+	+	+	+		+	+			+									+			+				+	+
Herkinorin					+	+	+		+			+							+	+	+		+	+	+	+				+
Heroin	+	+	+			+	+		+										+	+					+					+
Hydrocodone									+										+	+					+					+
Hydromorphone						+			+										+	+		+	+	+	+			+	+	+
Levorphanol						+	+		+										+	+		+	+	+	+			+	+	+
Meperidine						+	+		+										+	+		+	+	+	+				+	+
Metadone						+	+		+										+	+			+	+	+				+	+
Morphine						+			+										+	+		+	+	+	+			+	+	+
Nalbuphine						+	+		+										+	+		+	+	+	+			+	+	+
Naltridole						+	+		+										+	+		+	+	+	+	+			+	+
Naloxone						+		+	+										+	+		+	+	+	+				+	+
Natexone						+			+										+	+		+	+	+	+				+	+
Norbinaltorphimine					+	+	+		+					+	+				+	+		+	+	+	+	+	+		+	+
Oxymorphone						+			+										+	+		+	+	+				+	+	+
Pentazocine						+	+		+										+	+		+	+	+	+	+		+	+	+
Propoxyphene						+	+		+										+	+		+	+	+	+	+		+	+	+
Remifentanyl					+	+	+		+										+	+		+	+	+	+	+	+			
Sulfentanil	+				+	+	+						+							+		+						+	+	+
Tramadol						+			+													+	+	+	+	+		+	+	+