Molecular Simulation of Dynorphin A-(1−10) Binding to Extracellular Loop 2 of the κ-Opioid Receptor. A Model for Receptor Activation

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The structure of the second extracellular loop region (EL2) of the κ-opioid receptor has been explored in an effort to understand the structural basis for dynorphin A binding and selectivity. Application of secondary structure prediction methods and homology modeling resulted in a turn-helix motif for the N-terminal region of κ-EL2. A similar motif was not predicted for EL2 of either the δ or μ opioid receptors. The EL2 helix was further shown to be amphiphilic and complementary to the helical component of dynorphin A. Using a model of the κ-receptor (Metzger et al. *Neurochem. Res.* **1996**, *21*, 1287−1294), including the newly predicted EL2 turn-helix domain, a binding mode is proposed based on helix−helix interactions between hydrophobic residues of EL2 and the helical component of dynorphin A-(1−10). Molecular simulations of the receptor−ligand complex yielded structures in which the tyramine moiety or opioid "message" of dynorphin is bound within a conserved aromatic pocket in the transmembrane domain while the helical portion contacted residues in EL2 and in the extracellular end of transmembrane helices 6 and 7. The model is in general agreement with site-directed mutagenesis data and chimera studies that have identified binding domains in both the EL2 and transmembrane regions to dynorphin A. The results confirm the importance of the opioid "message" displayed by many opioid ligands but also suggest a potential mechanism of receptor activation that may be mediated by EL2 through interactions with the "address" component of dynorphin A.

Introduction

Since the cloning of opioid receptors[1] a large body of work has been dedicated to the identification of binding sites for opioid ligands and of regions of the receptors responsible for selectivity. Analysis of the site-directed mutagenesis studies of residues conserved within the opioid family has shown that the seven-transmembrane (TM) region may contain a similar binding pocket in the three opioids receptors types κ, μ, and δ.[2] Chimera studies, on the other hand, have suggested that selectivity is mostly imparted by the three extracellular loop regions (EL1−EL3). In this regard, the negatively charged EL2 of the κ receptor has been associated with its selectivity for the endogenous dynorphin A (dynA),[3,4] a peptide with six positively charged residues at physiological pH. Chimeric constructs in which residues in the TM4−EL2−TM5 region of μ are exchanged with those of κ exhibit K_i values similar to those of wild-type κ, while the K_i values of the mirror image chimera are typical of the μ-receptor.[4] In another chimera construct, exchange of EL2 of μ with that of κ resulted in a 100-fold greater affinity of dynA.[3] Significantly, while even a high concentration of dynA could not produce more than 20% cyclase inhibition at the μ receptor, dynA is as effective as morphine at the chimera.[3] These results indicate that κ-EL2 is not only responsible for dynA selectivity but that it may also contribute to activation of the κ receptor.

The involvement of EL2 in receptor activation has been postulated for some G protein-coupled receptors (GPCRs). Antibodies targeted to EL2 of the bradykinin B2 receptor were shown to have agonist activity and to interfere with the binding of both agonists and antagonists.[5] Substitution of an EL2 fragment of the *Xenopus* thrombin receptor in the human thrombin receptor caused constitutive activity,[6] while the same fragment was also implicated in binding of thrombin agonists.[7] Activating autoantibodies to EL2 have also been shown to exists for the β_1- and α_1-adrenergic receptors.[8,9] It is unlikely that antibodies can activate these receptors by binding in the TM region. Considering that structural changes in the helical regions of GPCRs have been observed upon activation,[10−12] the antibody studies suggest that the EL regions may mediate such changes through interactions with either agonist ligands or antibodies.

Given the importance of κ-EL2 in dynA binding, we have investigated the secondary structure of this region and its relation to both selectivity and receptor activation. An understanding of the κ-selectivity of dynA may be obtained by considering the properties of this peptide. While either dynA-(1−17) or dynA-(1−13) are in a random-coil conformation in aqueous solution, both peptides are partly helical when bound to neutral lecithin membranes[13] or micelles.[14] These data partly support a theoretical model of dynA proposing a lipid-embedded α-helix from residues 1−10 and a solvent-exposed environment for residues 11−17.[15] The biophysical studies on dynA, however, have not been analyzed in connection with the molecular biological studies described above. In particular, we have investigated the possibility that the selectivity and affinity of dynA is imparted by specific structural elements of EL2 of the κ receptor which may interact with the helical portion of dynA at the water-membrane interface. In the following, a structural model for the binding of dynA to the κ-receptor is presented and the results

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compared with reported mutagenesis data. The modeling results suggest a binding mechanism that explains the structure−function relationship of dynA with the κ-receptor. The model proposed extends the "message−address" concept[16,17] to rationalize the selectivity of dynA and suggests a possible mechanism which contributes to receptor activation.

Methods

The molecular simulations were preformed using the AMBER 4.1 suite of programs[18] and the Cornell et al. force field.[19] Computations were performed using a constant dielectric of 4 with a nonbonded cutoff distance of 8.0 Å. The initial coordinates of the TM region of the κ-receptor were taken from a model previously developed in our group to rationalize the binding of naltrexone-based ligands.[2,21] This model is unique in that secondary structures are taken from nonsequential alignments to the helical domains of bacteriorhodopsin (BR).[21] This allows the conformational effects of the conserved prolines to be retained in the opioid model which is not possible through direct sequence alignment to BR. Helix packing orientations and lengths were initially predicted using Fourier transform methods with the PERSCAN suite of programs[22] (see techniques in ref 22 for example) and further refined using the sequence analysis reported by Baldwin and the projection structure of rhodopsin.[23,24] Our sequence analysis has also been updated to include all recent site-directed mutagenesis and biophysical data describing probable interhelical interactions among the GPCRs.[20] A more detailed description of the model, including the coordinates and site-directed mutagenesis data used in model building, is available on our internet site at http://www.opioid.umn.edu. The TM structure was further modified here by adding residues 197−220 (EL2) of the κ-receptor. Secondary structure predictions of this segment were performed using the Chou−Fasman[25] and Garnier *et al.*[26] methods as implemented in the Biology Workbench 1.5 available through the National Center for Supercomputing Applications at the University of Illinois at Urbana−Champaign (http://biology.ncsa.uiuc.edu). The BLAST search algorithm was used to search the EL2 sequence for homologous structures in the PDB database.[27] Default parameters (as provided by the Biology Workbench) where used in the search.

The coordinates of dynorphin A-(1−10) were taken from the two-dimensional NMR study reported by Tessmer and Kallick.[14] The peptide was *N*-methyl capped at the carboxy terminus and docked to the receptor structure via interactive graphics using MidasPlus.[28] On the basis of the secondary structure predictions of EL2 (see Table 1), the structural refinement was performed in two stages. In the first stage, the C-terminal residues of EL2 (211−220) were excluded from the simulations. The C_α atoms of both receptor and ligand were constrained using a harmonic potential with initial constraints of 5 kcal/mol for residues in TM1−TM3 and TM5−TM7 helices, and 2 kcal/mol for C_α atoms in the TM4 helix and residues 4−10 of dynA. Residues 1−3 of dynA were not constrained. The harmonic constraints were gradually lowered during a 500 ps run to a final value of 0.05 kcal/mol for the entire structure. In the second stage of refinement, the receptor coordinates were fixed using the BELLY option of AMBER, while residues 211−220 were added to close EL2. This was accomplished using a series of short molecular dynamics simulations at 300 K during which the ϕ/ψ angles of residues 211−220 were manually adjusted until bond closure could be effected between residues S220 and W221. The structure was further refined using a short simulated annealing protocol by which the structure was heated and cooled from 1200 to 0 K over a 50 ps interval.

The final structure was analyzed using the program PROCHECK.[29] This program evaluates the quality of the protein structure by analyzing the values of the dihedral angles of the peptide backbone and side chains. All residues were found in allowed regions of the Ramachandran plot with side chain dihedrals in ideal regions.

Results and Discussion

Modeling of the Second Extracellular Loop of the κ-Opioid Receptor. A 2-D representation of the κ-receptor is given in Figure 1. Although modeling studies of ligand binding to TM regions of GPCRs[2,30,31,32] have been reported in the past, very few studies have focused on modeling of the extracellular loop (EL) or intracellular loop (IL) regions.[33] An analysis of the second EL segment (EL2) shows this region can be divided into two domains separated by a putative disulfide linkage between C210 and C121. This bond creates an additional loop connecting TM4 with TM3, while the residues from C210 to TM5 can be thought as joining TM5 with TM3. The TM3 and TM4 helices are therefore connected at both extracellular (EL2) and intracellular ends (IL2).

The two fragments of EL2, denoted here as N-EL2 (residues 197−210) and C-EL2 (residues 211−220) were characterized using secondary-structure prediction methods[25,26] and homology modeling to proteins of known crystallographic structure. Application of the Chou−Fasman algorithm[25] to C-EL2 of the κ-receptor resulted in an extended conformation with a turn at P215 (Table 1). A similar analysis of the N-EL2 fragment, however, predicted an α-helical structure with a turn at G197. This 14-residue segment was further characterized by searching for homologous structures using a sequence comparison against structures in the Brookhaven Protein Data Bank (PDB).[34] The search resulted in four unique protein sequences, shown in Table 1. Two of these sequences (PDB entries 1php and 1opr) have a turn-helix motif, while the fragments from 1chm and 1oel fold into a turn or extended conformation. The Chou−Fasman algorithm correctly predicted the X-ray conformation for three out of the four homologous structures (Table 1). On the basis of the results from secondary structure prediction and the sequence homology, we have modeled residues 199−207 of N-EL2 as an α-helix.

The sequence characteristics of the N-EL2 fragment are also consistent with those found in secondary structures of globular proteins. The two glycines at the end of TM4 (G197, G198) may signify helix termination while the remaining residues present the a−g heptapeptidic repeat characteristic of globular α-helices.[35] Hydrophobic residues are located at the a and d position of the helical repeat (V201, V205, and I208),[35] while charged amino acids are located three to four residue apart, where they may form intrachain hydrogen bonds (for example K200 with E203, R202 with D206). The region near Gly197−198 was further examined to determine the possibility of a turn at this location, which may orient the N-EL2 helix away from TM4. Glycines are often found at the C-terminal ends of α-helices of globular and membrane proteins with the effect of directing the polypeptide chain in a different direction.[36,37] A previous study has shown that glycines at helix C-terminals are often found in either a Schellman-turn conformation or a left-handed helix.[36] The backbone dihedral angles of L196 and G197 were then rotated to allow for a helix break in this region. Additionally, we have assumed that the hydrophobic face of the N-EL2 helix (formed by V201, V205, V207, and I208) should be oriented toward the receptor's interior, while the polar residues (K200, R202, E203,

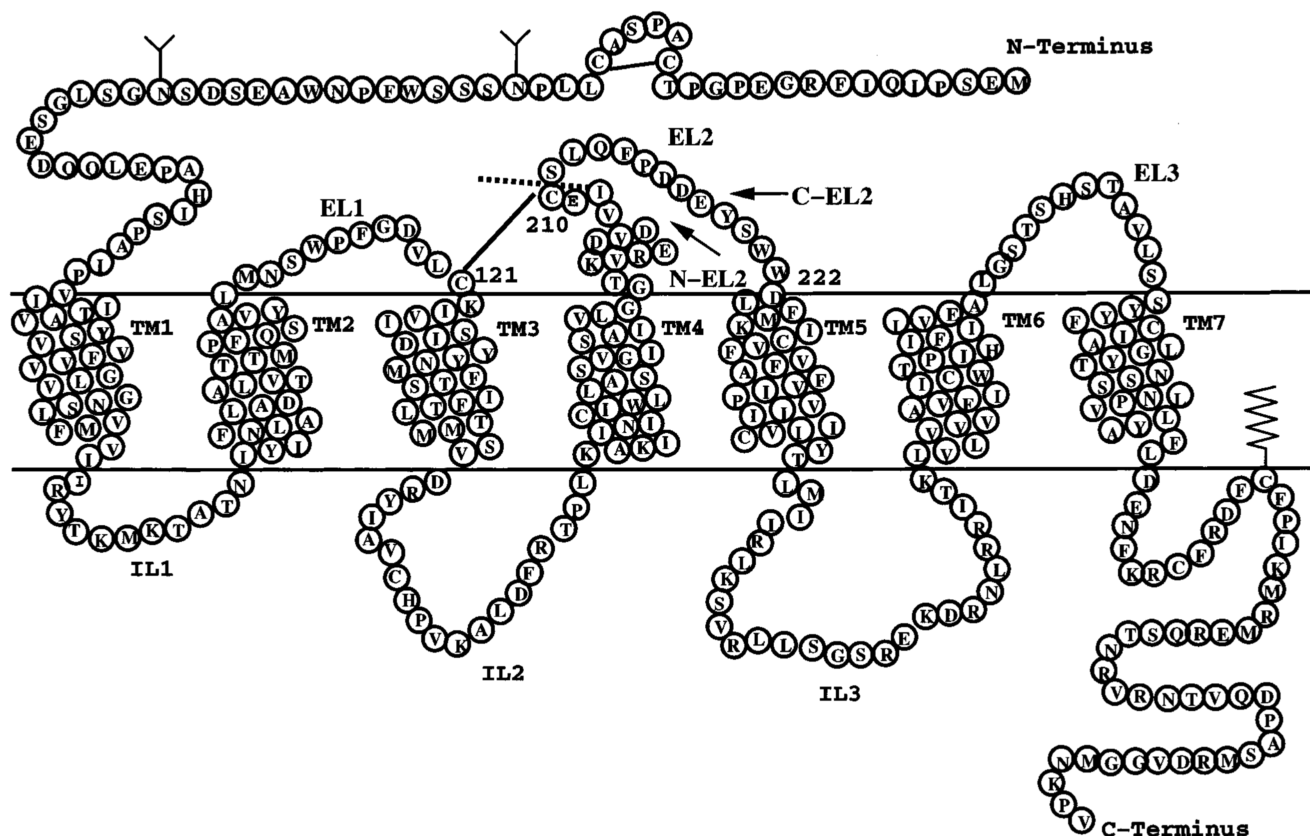

Figure 1. Serpentine model of the *κ*-receptor. The horizontal lines represent the membrane boundary, the dashed lines separate the N-EL2 and C-EL2 regions. Glycosylation sites on the N-terminus and palmitoylation site on the C-terminus are also shown. TM = transmembrane region, EL = extracellular loop, IL = intracellular loop; N-El2 = fragment 197−210; C-EL2 = fragment 211−222.

Table 1. Secondary Structure and Sequence Homology Analyses of the Second Extracellular Loop Region of the *κ*-, *μ*- and *δ*-Opioid Receptors[a]

protein[b]	sequence	motif	prediction[c]
	κ-Opioid Receptor		
N-EL2	GGTKVREDVDVIEC		turn+helix
1php	GGAKVKDKIGVID	turn+helix	turn+helix
1opr	GTAIRESMEIIQ	turn+helix	turn+helix
1chm	GLELREDIDTV	loop	turn+helix
1oel	GTGLQDELDVVE	loop+extended	extended
C-EL2	SLQFPDDEYSWW		extended+turn
1fxa	IEVPDDEY	extended+turn	extended+turn
1pss	SLEPPAPEY	loop	turn
	μ-Opioid Receptor		
N-EL2	MATTKYRQGSIDC	−[d]	extended+turn
C-EL2	TLTFSHPTWYW		extended+turn
1cyg	VVYSYPTWY	loop	extended+turn
1prh	VVYQYPTWYY	loop	extended+turn
	δ-Opioid Receptor		
N-EL2	MAVTQPRDGAVVC		extended+turn
1cid	MKVTQPDSNTLTC	extended+turn	extended+turn
1edb	VTQPADG	loop	turn
C-EL2	MLQFPSPSWYS		extended+turn
1cov	QKTSPGWWW	loop	extended+turn

[a] Sequences are shown using the amino acid one-letter code. Proteins are identified by their PDB entry names. [b] PDB entry names correspond to the following proteins: 1php, 2-phosphoglycerate kinase; 1opr, orotate phosphoribosyltransferase; 1chm, creatine amidinohydrolase; 1oel GROEL; 1fxa, [2*Fe-2*S]-ferrodoxin; 1pss photosynthetic reaction center; 1cyg, cyclodextrin glucanotransferase; 1prh, prostaglandin H2 synthase-1; 1cid, CD4 (domain 3 and 4); 1edb, haloalkane dehalogenase; 1cov, coxsackievirus B3 coat protein. [c] Secondary structure predition obtained using the method of Chou and Fasman.[28] [d] Homologous structures were not found in the PDB.

D204, D206, and E209) should be exposed to the more polar environment of the membrane−water interface, where N-EL2 is likely to be located. The resulting conformation can loosely be classified as a Schellman motif, characterized by a backbone hydrogen-bond between the carbonyl of I194 and amino proton of T199 and by a van der Waals contact between the side chains of these two residues.[36] Lastly, the backbone dihedral angles of I208, E209, and C210 were rotated to satisfy the disulfide bridge constraint between C210 and C121. Residues 197−210 (GGTKVREDVDVIEC) of N-EL2 and residues 211−220 of C-EL2 were then added to the previously built model of the *κ*-receptor.[2] The structure of the receptor is illustrated in Figure 2.

A comparison analysis of the N-EL2 and C-EL2 sequences of the *δ*- and *μ*-receptors was also performed. Application of the Chou−Fasman algorithm to these sequences resulted in an extended conformation with a turn at Pro (*δ*-P205 and *μ*-P214) for the C-EL2 fragment. An extended conformation was also predicted for N-EL2 of both receptors. These predictions were further supported by searching for homologous structures using sequence comparisons to structures in the Brookhaven PDB (Table 1). Thus, it appears that the helical motif of N-EL2 is unique to the *κ*-receptor.

Docking of Dynorphin A-(1−10) to the *κ*-Receptor. The starting conformation for dynA was taken from the two-dimensional NMR data reported by Tessmer and Kallick[14] performed in dodecylphosphocholine micelles. The model system applied in that study is thought to provide a good mimic of the membrane bilayer environment, where dynA and the receptor most likely interact. The structure displays well-ordered helical secondary structure from residues 4 to 10. The

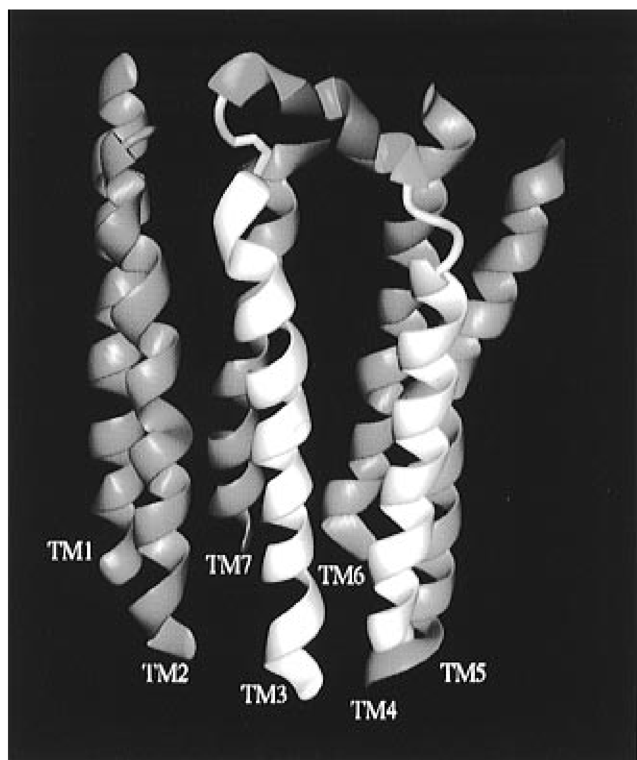

Figure 2. Ribbon representation of the κ-receptor which includes residues in the putative transmembrane region and second extracellular loop. Helices in TM3 and TM4 are shown in white, all others in gray. Hydrophobic residues V101, V105, V107, and I108 are shown in green, acidic residues in red (E203, D204, D206 and E209), and basic residues in cyan (K200 and R202). The disulfide linkage between C121 and C210 is shown in yellow.

N-terminal residues, comprising the opioid "message", and residues following the helical domain show conformational variability. In this study, we have limited our docking to residues 1−10 of dynA since it has been previously shown that dynA-(1−8), dynA-(1−13), and dynA-(1−17) peptides bind to the wild-type κ-receptor with comparable affinity.[38] This series also shows similar changes in binding using chimeric constructs.[3,4,39] The 9−17 region of dynA is therefore not likely to contribute significantly to either selectivity or affinity and simply adds computational complexity to the docking problem. It is interesting to note that these residues (i.e. 11−17) are also reported to sample a wide range of conformations (as shown by NMR studies[14]) which may in part explain the weaker contribution of dynA-(9−17) to opioid binding.

The docking mode of dynA-(1−10) was determined using sequence analysis and structural comparisons to the helical secondary structure of N-EL2. Both helices are amphiphilic. The hydrophobic residues of dynA (Phe4, Leu5, Ile8) span a 100° arc on the helical wheel, while the charged residues (Arg6, Arg7, Arg9) are located on the opposite face of the helix, as shown in Figure 3. In addition, N-EL2 is negatively charged, while dynA-(1−10) has three positively charged side chains. It is therefore reasonable to postulate a complementarity between the helical components of dynA and that of N-EL2. Recognition of dynA by the κ-receptor would then occur, in part, through helix−helix interactions between residues 4−10 of dynA and κ-N-EL2. The hydrophobic face of dynA (residues 4, 5, and 8) was docked into the corresponding hydrophobic face of N-EL2 (V201, V205, V207, and I208) and the orientation of the two helices was adjusted to correspond to a knob-and-hole type of interaction, as found in both globular and membrane proteins.[40,41] This initial docking mode was then used as the starting structure for subsequent energy minimization and molecular dynamics simulations of the dynA−κ-receptor complex as described in the Methods. The helical conformation of N-EL2 was maintained throughout the simulation, with a final root mean square deviation of 1.2 Å.

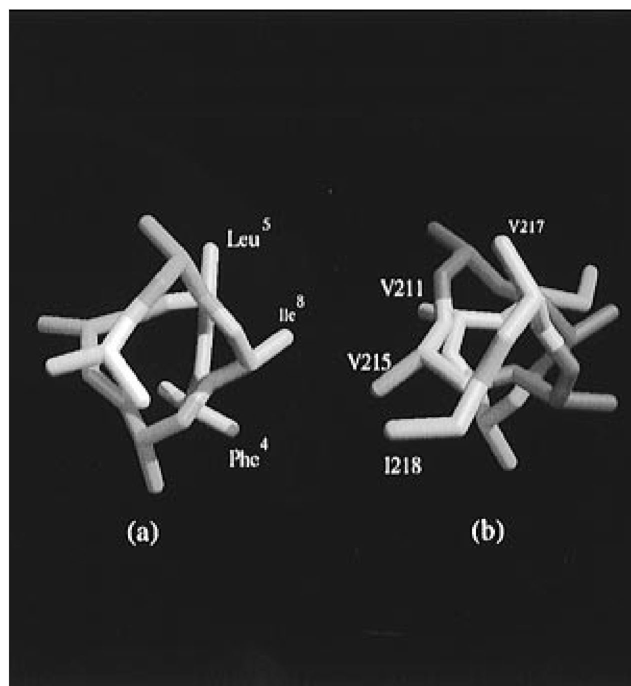

Figure 3. (a) Structure of dynorphin A-(1−10) as determined by NMR.[14] (b) Modeled structure of residues 197−207 of the second extracellular loop of the κ-receptor. Hydrophobic residues are shown in yellow, positively charged residues in cyan, and negatively charged residues in red. All other amino acids are in white.

The refined structure of dynA bound to the κ-receptor is illustrated in Figures 4 and 5, and a list of residue−residue contacts is given in Table 2. The hydrophobic residues of dynA (Phe4, Leu5, Ile8) make van der Waal contacts with V201 and V205 of N-EL2 as well as several other hydrophobic and aromatic residues at the extracellular ends of TM5, TM6, and TM7. One of these residues, I294, has been implicated in dynA binding in past studies. Mutation of I294 to bulkier side chains has been shown to cause a loss of binding of dynA to the κ-receptor,[42] suggesting that this residue is in close proximity to the dynA binding site. Structural replacement of I294 with Met or Trp in the κ-model (not shown) resulted in side-chain overlap with Phe4 of dynA, thus reinforcing the hypothesis of a steric effect for this residue. Residue Y312 in TM7 is also found near Phe4. Although this may indicate a potential aromatic interaction with dynA, a Y312A mutation did not affect binding.[42] This tends to rule out any direct binding interactions between Phe4 and Y312 but does not rule out nonspecific binding or contact since Phe4 makes several other contacts with neighboring residues.

Potential interhelical ion pairs are also seen between residues of the κ-receptor and dynA (Arg6 with κ-E297, Arg7 with κ-E209, and Arg9 with κ-D204 in Figure 4). Since the cloning of the opioid receptors, ion pairing

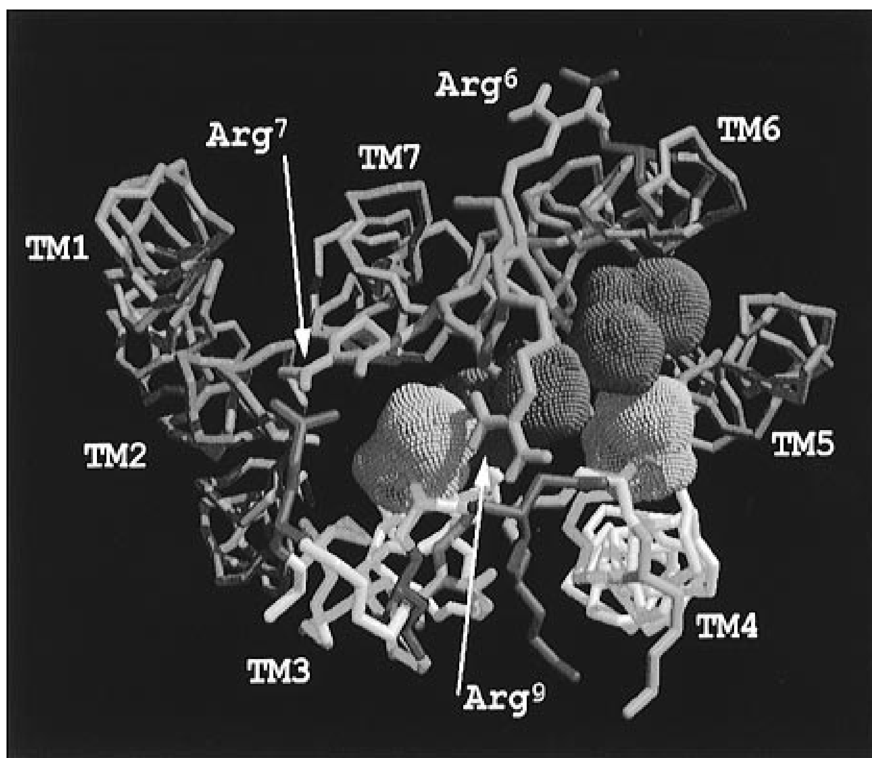

Figure 4. Structure of dynorphin A docked to the κ-receptor. Residues V201 and V205 (in yellow) in N-EL2 contact Leu5 and Ile8 of dynA-(1−10) (orange), as shown by the van der Waals surface representation. Residues in the κ-model and in dynA that interact through salt linkages are shown in red (acidic) and cyan (basic). The backbone atoms of dynA are in green.

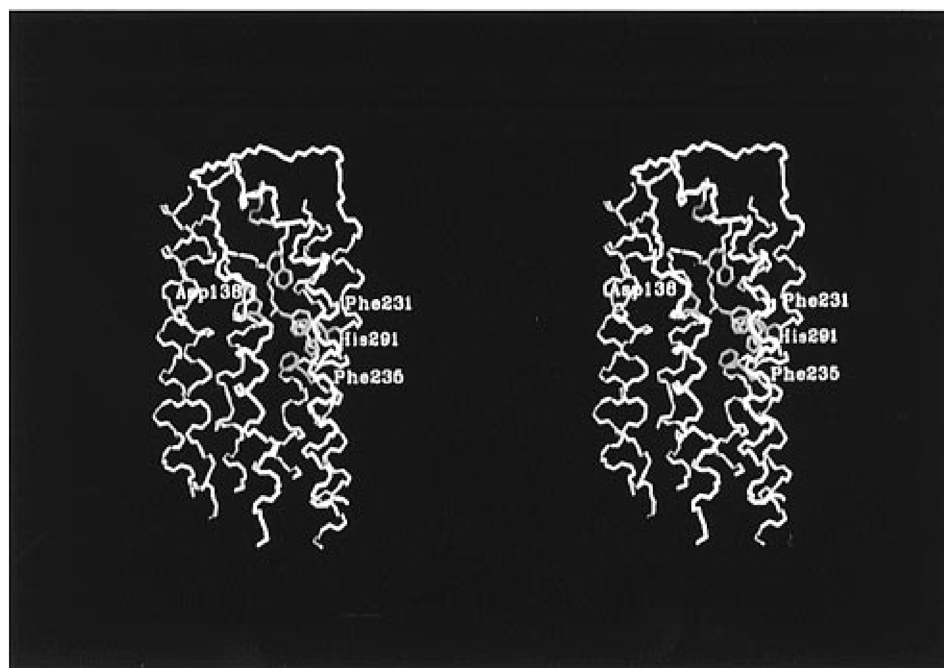

Figure 5. Stereoview of dynorphin A-(1−10) bound in the transmembrane domain of the κ-receptor. The backbone, Tyr1 and Phe4 heavy atoms of dynA are shown in orange. Conserved amino acids which interact with Tyr1 are shown in green (Phe231 and 235 in TM5 and His291 in TM6). Helices in TM3 and TM4 are in a lighter color. The disulfide linkage between C121 and C210 is shown in yellow.

interactions have been postulated to be an important recognition element of dynA binding to EL2 of the κ-receptor. Single point mutations of the acidic residues in EL2 to asparagine, however, failed to produce a significant change in dynA affinity for the κ-receptor.[43,44] A triple point mutant involving residues in the carboxy domain of EL2, κ-D216N, D217N, E218N, has also been constructed.[43,44] Although this mutant once again failed to produce changes in dynA binding, it is important to point out that the binding mode proposed here does not require ion pairing to this domain. To the best of our knowledge, a similar experiment has not been performed on the charged cluster in N-EL2. Nevertheless, the limited effect of single point mutations to the acidic residues of N-EL2 on dynA binding is surprising and may, in part, be explained by the thermodynamics of ion pairing in solution. It has become fairly well-established that salt links in protein structures are not

Table 2. Residues of the κ-Receptor within 5 Å of Dynorphin A-(1−10)[a]

dynA	TM helix	κ-opioid residue	dynA	TM helix	κ-opioid residue
Tyr1	TM3	D138,M142	Phe4	TM6	I294,L295
	TM4	G190,I194		TM7	Y312,I316
	TM5	F231,F235	Leu5	N-EL2	V201
	TM7	Y320		TM5	L224
Gly2	TM3	D138		TM6	A298
	TM4	I194,G198	Arg6	TM6	E297
	TM7	Y312		TM7	S311,F314
Gly3	TM4	G198	Arg7	N-EL2	E209
	N-EL2	V201, R202		TM7	Y312,Y313
	TM7	Y312	Ile8	N-EL2	V201,R202,D204,V205
Phe4	N-El2	V201		TM7	Y313
	TM5	L224	Arg9	N-El2	D204,V205

[a] Distances were calculated for all heavy atoms of dynorphin A-(1−10) to all heavy atoms of the κ opioid receptor.

the primary force stabilizing the native state[45] but may be more important in "lining up" the structural domains. Since ions must be partially or totally desolvated to effectively pair, the net gain in free energy may be small, especially when compared to hydrophobic collapse. The acidic residues in N-EL2 may therefore be secondary in stabilizing dynA binding to the κ-receptor. The primary forces, based on this analysis, would stem from the hydrophobic knob in hole interactions at the helical interface (as shown in Figure 4) as well as aromatic interactions within the receptor cavity (vide infra).

The predicted docking arrangement also places the opioid "message" of dynA (YGGF) within the cavity of the receptor (Figure 5). Although no attempt was made to dock the message residues in particular locations within the cavity, molecular dynamics simulations yielded orientations of the critical tyramine moiety consistent with reported site directed mutagenesis data. In particular, the charged amino terminus of dynA forms an ion pair with D138 in TM3. This interaction has been implicated in previous binding studies that show a loss in affinity following either a κ-D138A or a κ-D138N mutation.[42,43] The phenolic ring of Tyr1 is also directed toward a region rich in aromatic residues that is fairly well-conserved across the opioid receptor types. In this regard, the finding that Tyr1 is in the most conserved environment relative to other residues in dynA is consistent with the key role played by the tyramine moiety in all of the opioid peptides as well as many rigid ligand analogs. Potential contacts include a cluster of residues within the receptor cavity that may define an aromatic binding pocket for the tyramine message.[2] Indirect evidence that dynA may bind to aromatic residues within the receptor cavity comes from site-directed mutagenesis data on both the κ- and δ-receptors. In this connection, results from a κ-H291A mutation resulted in 8-fold loss in affinity for dynA.[42] Although Tyr1 and H291 are not in direct contact in the model, a simple rotation of the two side chains easily brings these two residues within van der Waals contact distance. The importance of this histidine is further reinforced by site-directed mutagensis data on the orphanin ORL1 receptor. This receptor, even though highly homologous to opioid receptors, binds dynA only with micromolar affinity.[46] A Q208H mutation in TM6 of ORL1 (identical to position H291 in κ), however, produced a 10-fold gain in dynA affinity,[47,48] suggesting that this residue may be directly involved in dynA binding, perhaps through hydrogen-bonding with its phenolic hydroxyl group.

The model also shows potential contacts between Tyr1 and F231 and F235 of TM5. Single point mutations of these conserved residues to alanine in the δ-receptor decreased dynA affinity 4−9-fold.[49] Two tyrosine side chains, Y139 and Y320, are also shown to contact the tyramine moiety. These groups are in fairly close proximity to the positively charged amino terminus. Although cation−π interactions contribute favorably to ligand binding in some systems,[50] this contribution is expected to be modest here. Mutation of these two residues to either Phe or Ala at identical positions in the δ-receptor resulted in only an 8−14-fold loss in affinity for dynA,[49] suggesting that aromaticity at Y139 and Y320 is not a major determinant for binding.[49] Another highly conserved aromatic residue in this general location, W278 (W274 in the δ-receptor), has been shown to effect dynA binding as well. While the tyramine moiety of dynA does not directly contact this residue, its proximity to the aromatic pocket may infer indirect participation in stabilization of the aromatic cluster.[49]

Conclusions

This study has proposed a novel docking mode of dynA to the κ-receptor based on the complementarity of helical domains within EL2 and the peptide structure. Our sequence analyses have revealed that the secondary structure predicted in EL2 is unique to the κ-receptor, which may, in part, explain the selectivity of dynA for this opioid receptor type. Although κ-selectivity has, in the past, been linked to the presence of basic residues on dynA and rigid analogs, our results show that potential ion pairs may only represent one "digit" of the κ-address. For amphiphilic peptides, such as dynA, the primary recognition sites, and driving force for binding, may depend more on the alignment of hydrophobic contacts at the helix−helix interface. This may explain the limited effect that single point mutations to acidic residues in EL2 have on dynA binding. While ion pairing may in fact be critical to determining the selectivity of many ligands to the κ-receptor, including dynA, it is important to point out that the free energy gained upon ion pairing may be minimal. This may be especially true in some cases where numerous hydrophobic contacts are possible. Conversely, such interactions may be magnified for ligands that make few contacts with the receptor. This latter point is best exemplified by the binding of norbinaltorphimine (norBNI) to the κ-receptor, which is known to depend heavily on the presence of an acidic residue at the top of TM6 (E297).[51] This rigid antagonist has a limited number of hydrophobic groups to contact the receptor and may therefore depend more on this ion pair for binding affinity. This hypothesis could be tested, for example, by mutagenesis experiments in which the hydrophobic contacts of N-EL2 (shown in Figure 3) are replaced with small polar or neutral amino acid residues. While dynA could still bind to such constructs, its efficacy as an agonist would be decreased. A second set of experiments could also be performed in which multiple point mutants of N-EL2 are made (e.g. E203A, D204A, D206A, E209A) to drastically modify the net charge on this segment. In this case we would not only expect a

decrease in efficacy but quite possibly a change in selectivity as well.

The helical-docking mode proposed is also supported by structure−activity relationship (SAR) studies of dynA analogs.[52−54] The promotion of helicity by cyclization of either the "message" or "address" components of dynA has been shown to increase affinity for the κ-receptor.[52,53] Our model indicates that the inherent helicity of the address in the amphiliphilic environment may predispose the message to interact with key residues in the receptor cavity. Given the NMR data and the simulation results presented here, the YGGF fragment of dynA may not be prefolded by the membrane but most likely optimizes the bound conformation by a zipper mechanism within the TM region.[55] The binding mode predicted places the opioid message within the receptor cavity and is consistent with site-directed mutagenesis results of dynA binding to the κ- and δ-receptors[42,43,49] as well as prior modeling studies of naltrexone-based antagonists.[2] The amino terminus is shown to be close to a highly conserved aspartate in TM3 that has been implicated in binding a wide variety of ligands with basic groups. The opioid core or YGGF message is also positioned to interact in an aromatic pocket previously implicated in ligand binding to the opioid receptors. The potential aromatic interactions proposed may not only provide favorable interaction energy to fold the "message" component into the pocket, but may also provide additional driving forces for dynA binding to the opioid receptors.

Finally, the proposed docking mode highlights the importance of EL2 interactions to the potential mechanism of signal transduction. The ability of EL2 to activate GPCRs through interactions with antibodies to this region has been shown in several receptors.[5−9] One possible mechanism for κ-receptor activation may similarly involve association of N-EL2 with dynA, as predicted here. Given the connectivity between TM3 and TM4 at both the intracellular and extracellular ends, it is reasonable to postulate that structural changes in TM4 brought by dynA binding to N-EL2 may affect the conformation of the second intracellular loop and thus G-protein coupling. An indication of how changes at the extracellular end may be transferred to the intracellular regions may come from data on rhodopsin.[10,11] Rigid-body motions of the TM3 and TM4 helices upon receptor activation have been observed in rhodopsin.[10] Rotations of other helices have been observed as well.[11] It may therefore be postulated that a rigid motion of TM4 resulting in helix rotation could be mediated by EL2 following dynA binding. Such a rotation would also produce changes in TM3 and TM5 that may expose residues important for G-protein coupling. In particular, we have noted that a rotation of TM3 may effect the accessability of several residues at the base of TM6 that have been implicated in G-protein coupling in the interleukin-8[56] and muscarinic receptors.[57] A schematic view of this mechanism is given in Figure 6. According to this model, the "address" component of dynA (residues 4−10) not only determines selectivity but may also be involved in receptor activation. This may, in part, explain the antagonist behavior of some of the rigid alkaloids that are thought to bind primarily within or near the receptor cavity. In this regard a ligand, such as norBNI, may not provide enough contacts to stabilize

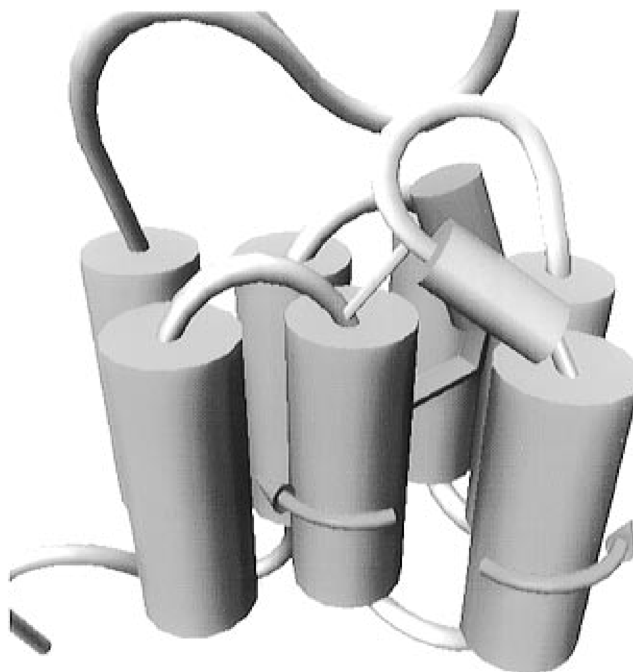

Figure 6. Postulated mechanism of receptor activation by dynorphin A. Interaction between dynorphin A and N-EL2 is depicted here by the orange and gray cylinders, respectively. Arrows signify possible rotation of the TM3 and TM4 helices upon binding of dynorphin A which in turn expose regions of intracellular loop 2 required for G protein-coupling.

the activated state or conformation of the receptor. This does not, however, explain the agonist behavior of ethylketocyclazocine[58] or other small opioid agonists.[1] Nevertheless, the mechanism is provocative and suggests that receptor activation may also be mediated through favorable interactions with the address which may have important ramifications for structure-based drug design.

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