Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 12, pp 2151–2166, 1996
Copyright © 1996 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
PII: S0968-0896(96)00219-2
0968-0896/96 $15.00 + 0.00

Ligand Recognition in μ Opioid Receptor: Experimentally Based Modeling of μ Opioid Receptor Binding Sites and Their Testing by Ligand Docking

Takeshi Sagara,[a] Hiromu Egashira,[a] Mikako Okamura,[a] Ikuo Fujii,[b] Yasuyuki Shimohigashi,[c] and Ken Kanematsu[a,*]

[a]*Institute of Synthetic Organic Chemistry, Faculty of Pharmaceutical Sciences, Kyushu University 62, Fukuoka 812-82, Japan*

[b]*Protein Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565, Japan*

[c]*Laboratory of Biochemistry, Department of Chemistry, Faculty of Science, Kyushu University 33, Fukuoka 812-81, Japan*

Abstract—For three-dimensional understanding of the mechanisms that control potency and selectivity of the ligand binding at the atomic level, we have analysed opioid receptor–ligand interaction based on the receptor's 3D model. As a first step, we have constructed molecular models for the multiple opioid receptor subtypes using bacteriorhodopsin as a template. The *S*-activated dihydromorphine derivatives should serve as powerful tools in mapping the three-dimensional structure of the μ opioid receptor, including the nature of the agonist-mediated conformational change that permits G protein-coupling to 'second messenger' effector molecules, and in identifying specific ligand-binding contacts with the μ opioid receptor. The analyses of the interactions of some opioid ligands with the predicted ligand binding sites are consistent with the results of the affinity labeling experiments.
Copyright © 1996 Elsevier Science Ltd

Introduction

The opioid receptors are the primary sites of action of opioid drugs, and transduce such information by activating G proteins that in turn alter membrane conductance for K^+ and Ca^{2+} and levels of second messengers such as cAMP and inositol 1,4,5-trisphosphate. Pharmacological studies suggest that multiple opioid receptor subtypes, namely μ, δ and κ receptors might be responsible for the actions of the opioid drugs.[1,2] Over the past year, substantial progress has been made in elucidating the molecular features underlying these multiple opioid receptor subtypes through cloning their cDNA and genes, and the distinct genes for these multiple opioid receptor subtypes have been cloned.[3]. Analysis of the deduced amino acid sequences showed that these receptors possess seven putative transmembrane domains (TM I–VII), which is the major characteristic structural feature of G-protein-coupled receptors, and have high amino acid sequence identities (~60%) to each other. In opioid receptor subtypes, the amino acid residue (μ: Asp147; δ: Asp128; κ: Asp138) in TM III was predicted to be involved in ligand-binding pocket by the studies of the mutant opioid receptors.[4,5] Site-directed mutagenesis of the μ opioid receptor indicate that the Asp 147 is probably the primary binding site as the counter ion for the ammonium head group of the opioid ligands in analogy with many other GPCRs. Some groups reported that to elucidate which portions of the opioid receptor molecules are involved in the ligand selectivity, they have expressed chimeric receptors among their subtypes and analysed their ligand binding properties. They demonstrate that the major binding determinants for the selective opioid ligands reside within the opioid receptors.[6–12] In this connection, Fukuda *et al.* have expressed chimeric receptors between the δ- and μ-opioid receptors from cDNAs and analysed their ligand binding properties, and demonstrated that the major binding determinant for the δ-selective enkephalin-related peptide, [D-Pen^2,D-Pen^5]enkephalin, resides within the region comprising TM V–VII and intervening loop regions. On the other hand, the region spanning from the intracellular loop I to the amino terminal half of TM III is shown to be involved in determining high affinity binding of the μ-selective enkephalin related peptides, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin and [D-Ala^2,MePhe^4,Met-ol^5]enkephalin, whereas the major determinant for binding of the μ-selective alkaloids, morphine and codeine, is demonstrated to exist in the region spanning TM V–VII.[10] From these data, distinct regions of the opioid receptor determine the selectivity for the δ- and the μ-selective enkephalin-related peptides and that the binding determinant for the μ-selective alkaloids is distinct from that for μ-selective enkephalin-related peptides. The results obtained in these investigations would provide insights into the mechanism for ligand selectivity of the opioid receptor. However, further studies will be necessary to identify

*Present address: National Institution for Academic Degrees, Nagatsuta, Midori, Yokohama 226, Japan.
Key words; opioid receptor, morphine, molecular modeling, affinity labelling, ligand recognition.

specific amino acid residues that interact directly with agonist molecules. Thus three-dimensional understanding of the features of the opioid receptor–ligand interaction at the atomic level is now essential for the development of the opioid drugs that provide analgesics free from abuse potential and the adverse side effects of morphine.

Recently, molecular modeling of GPCRs has been reported from a few groups.[13–25] Identifications of the ligand binding sites in these models have been made from the site-directed mutagenesis and studies of the chimeric receptors, and the mode of the receptor–ligand interaction has also been analysed by docking ligands into the receptor models. These approaches would be very useful for the functional analysis of the receptors and drug design. However, at present, biological analysis of the cloned opioid receptors has not been described well; the mutational data for the opioid receptors are considerably less than that for the other GPCRs. The involvement of the sulfhydryl (SH) groups in maintenance of structure and function has been observed for some GPCRs, including acetylcholine and β adrenergic receptors.[23–25] The interaction of the opioid ligands with their receptors has also been proposed to be regulated by sulfhydryl groups. The binding of the opioid ligands with opioid receptors in rat brain is effectively inhibited by reagents for the thiol groups such as *N*-ethylmaleimide (NEM).[26–28] Larsen *et al.* suggested that in opioid receptors there are at least two different types of thiol groups sensitive to NEM. Both of the thiols are originated from the cysteine residues: one is the cysteine β-thiol in the GTP-binding regulatory protein Gi, which exists inside the plasma membrane and couples with the opioid receptor to let the receptor react with agonists, and the other is in the ligand binding site of receptor protein.[29]

From these observations, it was assumed that information about the location of the cysteine residue in the ligand binding pocket was obtained from direct labeling of the cysteine residues by the opioid receptor probes. The affinity probes containing the functional group that can specifically label the thiol group of the cysteine residue in the binding site must be developed for this purpose.

This paper describes the full details of the work including some previously published.[30–31]

Results and Discussion

Molecular modeling of multiple opioid receptor subtypes

First, we investigated the homology of amino acid primary sequences of each receptor. The amino acid sequences of the μ, δ and κ opioid receptors were aligned based upon the studies by Fukuda et al.,[32] Yasuda et al.[33] and Minami et al.,[34] respectively (Fig. 1).

For the identification of the hydrophobic helical regions, the parameter sets of Kyte–Doolittle were used,[35] and is shown in Figure 2.

Primary amino acid sequence alignment clearly defined seven highly conserved hydrophobic sequences corresponding to the transmembrane regions. Because it was not possible to localize precisely the starting and ending amino acids of the transmembrane regions from hydropathy analyses, we predicted the putative transmembrane regions of the three opioid receptors according to the report of Yasuda et al.[33] A detailed comparison of the transmembrane regions showed the high percentage (69–72%) of sequence identity within the opioid receptor subtypes. On the other hand, the extracellular domains of the opioid receptors showed a low percentage (25–33%) of sequence identity. High identity (64–68%) of intracellular loops I–III among the three receptors were observed, suggesting that these regions may couple with G proteins. In the intracellular domain near the end of TM III, all three receptors include the conserved sequence Asp-Arg-Tyr, which is a characteristic feature in GPCRs.[18]

The studies of the chimeric opioid receptors indicated that nonpeptidic opioid ligands such as morphine derivatives have the differential binding sites from the opioid peptide ligands.[6–8,10–12] The binding sites for the opioid peptide ligands were assumed to contain not only the transmembrane region but also the extracellular loop region. Because of the difficulties of the deducing the three-dimensional structures of extracellular domains, we could not deal with the binding features of opioid peptide ligands. On the other hand, only the transmembrane regions were assumed to be required to bind nonpeptidic opioid ligands. Using this assumption, we attempted to analyze only the transmembrane regions as the binding sites for nonpeptidic opioid ligands. The extremely high homologies observed in the transmembrane regions among the opioid receptor subtypes correlate very well with the traditional structure–activity relationships of opioid ligands. Thus, medicinal chemists have failed until now to design highly selective opioid receptor ligands.

To construct three-dimensional structures of the transmembrane domains of the aforementioned three opioid receptors, the structures of the membrane spanning helices in the bacteriorhodopsin protein (PDB code 1BRD) were assumed.[36]

The high degree of similarity within these hydrophobic stretches leads to the assumption that the homologous transmembrane regions in all opioid receptors have the same secondary structures and folds in the same way. The distribution of the conserved and charged amino acids on the same face of the α-helices imply that the assumptions made above are correct. For the relative positioning of axes of the α-helices, the model derived from the cryoelectron microscopic study of bacteriorhodopsin[36] was used as a template. Using the arrangement of the α-helices found in bacteriorhodopsin, it was possible to construct models possessing a number

of features that we believe to be essential for this class of membrane-embedded receptors: the seven α-helices are tightly placed and define a central narrow, dihedral cleft. The orientation of the transmembrane helices was determined based on the analysis of the hydrophobic moment, where each helix of the three subtypes of opioid receptor was rotated around the helical axis so that the direction of the hydrophobic moment would agree with that of bacteriorhodopsin (Fig. 3).

Before further analysis of our models, we have examined the ligand binding sites by the affinity labeling experiments.

Affinity labeling of μ opioid receptors in guinea-pig ileum

There have been many reports on the affinity labeling of the opioid receptors, and some opioid ligands that were attached the electrophilic group were found to label the opioid receptors by binding covalently with the nucleophilic groups in the ligand binding pocket,[37] but it has not been proved that these nucleophilic groups in the ligand binding pocket were the thiol groups. Portoghese et al. suggested that electrophilic affinity labels are involved in two consecutive recognition processes that lead to the covalent binding of its receptor.[37] The first recognition step is reflected by receptor affinity, and the second recognition step involves the proper alignment of the electrophilic center (attached to the reversible bound ligand) with a compatible, proximal, receptor-based nucleophile. Because two recognition steps rather than one lead to the covalent binding of the affinity label, enhanced receptor selectivity (recognition amplification) is attainable. Thus, the specific covalent binding is dependent upon the nature and orientation of the electrophilic center in the affinity ligand. Based on this assumption, we have designed an *S*-activated dihydromorphine derivative (**1**) that was expected to label the thiol groups specifically by forming a disulfide bridge.[38] The sulfhydryl group was activated by the 5-nitro-2-pyridinesulfenyl (Npys) group expecting its moderate reactivity and the Npys-activated sulfhydryl group was attached to the 6β-position of the morphine skeleton according to the structure–activity relationships in the affinity labeling experiments of the μ opioid receptor.

```
ROR-B(μ)  MDSSTGPGNTSDCSDPLAQASCS-PAPGSWLNLSHVDGNQSDPCGLNRTGLGGNDSLCPQTGSPSMVT  67
DOR1(δ)   MELVPSARAELQSS-PL-------------------VNLSDAFPSAFPSAGANASGSPGARSAS-SLAL  48
pKOPR(κ)  ME-SPIQIFRGEPGPTCAPSACLLPNSSSWFP----NWAES-----DSNGSLGSEDQQLEPAHISPAI  58

          TM-1                                TM-2
ROR-B(μ)  AITIMALYSIVCVVGLFGNFLVMYVIVRYTKMKTATNIYIFNLALADALATSTLPFQSVNYLMGTWPFG 136
DOR1(δ)   AIAITALYSAVCAVGLLGNVLVMFGIVRYTKLKTATNIYIFNLALADALATSTLPFQSAKYLMETWPFG 117
pKOPR(κ)  PVIITAVYSVVFVVGLVGNSLVMFVIIRYTKMKTATNIYIFNLALADALVTTTMPFQSAVYLMNSWPFG 127

          TM-3                                TM-4
ROR-B(μ)  TILCKIVISIDYYNMFTSIFTLCTMSVDRYIAVCHPVKALDFRTPRNAKIVNVCNWILSSAIGLPVMFM 205
DOR1(δ)   ELLCKAVLSIDYYNMFTSIFTLTMMSVDRYIAVCHPVKALDFRTPAKAKLINICIWVLASGVGVPIMVM 186
pKOPR(κ)  DVLCKIVISIDYYNMFTSIFTLTMMSVDRYIAVCHPVKALDFRTPLKAKIINICIWLLASSVGISAIVL 196

                    TM-5
ROR-B(μ)  ATTKYRQGS--IDCTLTFSHP--TWYWENLLKICVFIFAFIMPVLIITVCYGLMILRLKSVRMLSGSKE 270
DOR1(δ)   AVTQPRDGA--VVCMLQFPSP--SWYWDTVTKICVFLFAFVVPILIITVCYGLMLLRLRSVRLLSGSKE 251
pKOPR(κ)  GGTKVREDVDVIECSLQFPDDEYSW-WDLFMKICVFVFAFVIPVLIIIVCYTLMILRLKSVRLLSGSRE 264

          TM-6                                TM-7
ROR-B(μ)  KDRNLRRITRMVLVVVAVFIVCWTPIHIYVIIKALITI-PETTFQTVSWHFCIALGYTNSCLNPVL   335
DOR1(δ)   KDRSLRRITRMVLVVVGAFVVCWAPIHIFVIVWTLVDINRRDPLVVAALHLCIALGYANSSLNPVL   317
pKOPR(κ)  KDRNLRRITKLVLVVVAVFIICWTPIHIFILVEALGSTSHSTA-VLSSYYFCIALGYTNSSLNPVL   329

ROR-B(μ)  YAFLDENFKRCFREFCIPTSSTIEQQNSTRVRQNT-REHPSTANTVDRTNHQLENLEAETAPLP     398
DOR1(δ)   YAFLDENFKRCFRQLCRTPCGRQEPGSLRRPRQATTRERVTACTPSD---------GPGGGAAA     372
pKOPR(κ)  YAFLDENFKRCFRDFCFPIKMRMERQSTNRVRN-TVQDPAS-----------MRDVGGMNKPV-     380
```

Figure 1. Alignment of the amino acid sequences of μ, δ and κ opioid receptors. The one-letter amino acid notion is used. Gaps (–) have been inserted to achieve maximum homology. The predicted transmembrane segments (TM 1–7) are underlined.

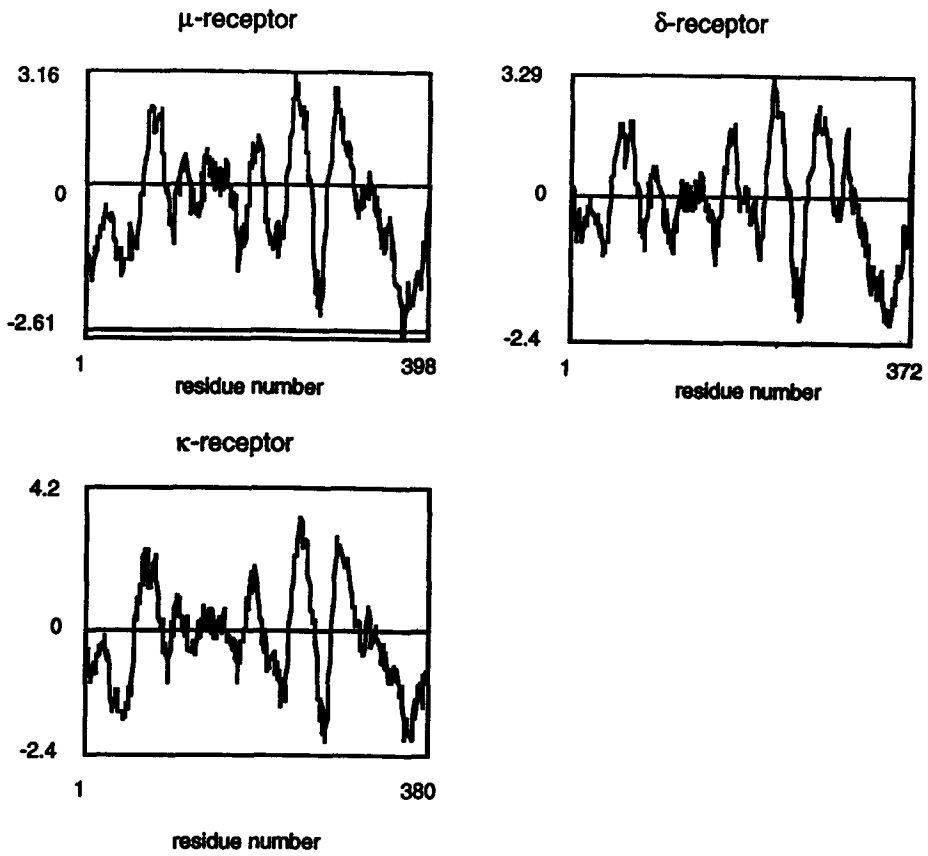


Figure 2. Hydrophilicity/hydrophobicity plots of opioid receptors. Parameter: Kyte & Doolittle. Range to average: 15.

Compound **1** was prepared by the route shown in Scheme 1.[31] 3-Acetoxy dihydromorphine (**3**) was obtained from morphine in two steps in high yield. Treatment of **3** with thioacetic acid in the presence of triphenylphosphine and diisopropyl azodicarboxylate in dry THF at 0 °C (Mitsunobu reaction) produced the 6β-thioester (**4**) in 76% yield selectively. Then the treatment of the 6β-thioester (**4**) with excess sodium borohydride in EtOH gave 6β-sulfhydryl-dihydromorphine (**5**), which was readily treated with 5-nitro-2-pyridinesulfenyl chloride in dichloromethane at 0 °C to give 6β-(5′-nitro-2′-pyridyldithio) deoxydihydromorphine (**1**) (Fig. 4) in 84% yield from **4**.

The analgesic activity of compound **1** was found by the inhibition of the electrically stimulated contraction of guinea pig ileum (GPI) and mouse vas deferens (MVD).[39] These results indicate that compound **1** reacted with a thiol in opioid receptors near the position of compound **1** through a disulfide linkage and that this linkage was cleaved reductively by DTT as illustrated in Figure 5. This is the evidence of compound **1** linking to the μ opioid receptor through the thiol–disulfide exchange.

Affinity labeling of the opioid receptors in guinea pig brain

Since the covalent linkage of compound **1** to the peripheral opioid receptors was proved, we have prepared the *S*-activated (−)-8β-sulfhydryl-dihydromorphine analogue (**2**), expecting the differential regiochemical effect between **1** and **2** on predominant affinity labeling of opioid receptors. To evaluate the binding abilities for the opioid receptors in detail, we tested compounds **1** and **2** by radiolabeled ligand binding assays using membrane preparations from guinea pig brain.[31]

The synthesis of compound **2** is shown in Scheme 2. Swern oxidation of 3-acetylmorphine (**6**) gave the 3-acetyl morphinone (**7**), which was treated with thioacetic acid in the presence of 2,6-lutidine to produce stereoselectively β-thioester isomer (**8**). Then the thioester (**8**) was treated with excess sodium borohydride in EtOH to give a mixture of epimers quantitatively (6α:6β = 7:3). Finally, the mixture was treated with 5-nitro-2-pyridinesulfenyl chloride in dichloromethane at 0 °C to afford a mixture of epimers in 92% yield (6α:6β = 7:3). The 6α epimer (**2**) was separated from the mixture by column chromatography in 65% yield.

Specific binding affinities of compounds **1** and **2** for μ opioid receptor in guinea pig brain were determined by evaluating their ability to displace [^{3}H]-naloxone.[40] Figure 6 shows the dose–response curves analysed by the computer program ALLFIT,[37] which constructs the least-square estimates of the logistic curves relating binding of labeled ligand to concentrations of unlab-

eled ligand. Both **1** and **2** are considerably potent to interaction with the μ receptor. The IC_{50} values, the half-maximal concentration of unlabeled ligands for inhibition of binding of labeled ligand, were 14.2 and 13.4 nM for **1** and **2**, respectively (Table 1). Binding affinity with the δ opioid receptor was evaluated using [^{3}H]-[D-Ser^2, Leu^5]enkephalyl-Thr^6 ([^{3}H]DSLET) as a tracer.[39] Compounds **1** and **2** exhibited IC_{50} values of 5.7 and 26.8 nM, respectively. Binding affinity with the κ opioid receptor was evaluated using [^{3}H]U-69593 as a tracer.[41] Compounds **1** and **2** exhibited IC_{50} values of 56.7 and 136.9 nM, respectively.

The ability of compounds **1** and **2** to label irreversibly opioid receptors can be examined by their incubation with membranes and subsequent assays for biological activity or receptor binding. When **1** or **2** was incubated with guinea pig brain membranes, they would first bind to the ligand binding site of the receptors. However, if the thiol group of the receptor was present near the ligand bound in the receptor, the activated thiol group of the ligand reacted with this free thiol, resulting in the formation of a covalent disulfide bond. Such affinity labeling of receptors would substantially reduce the number of receptors available for binding of the

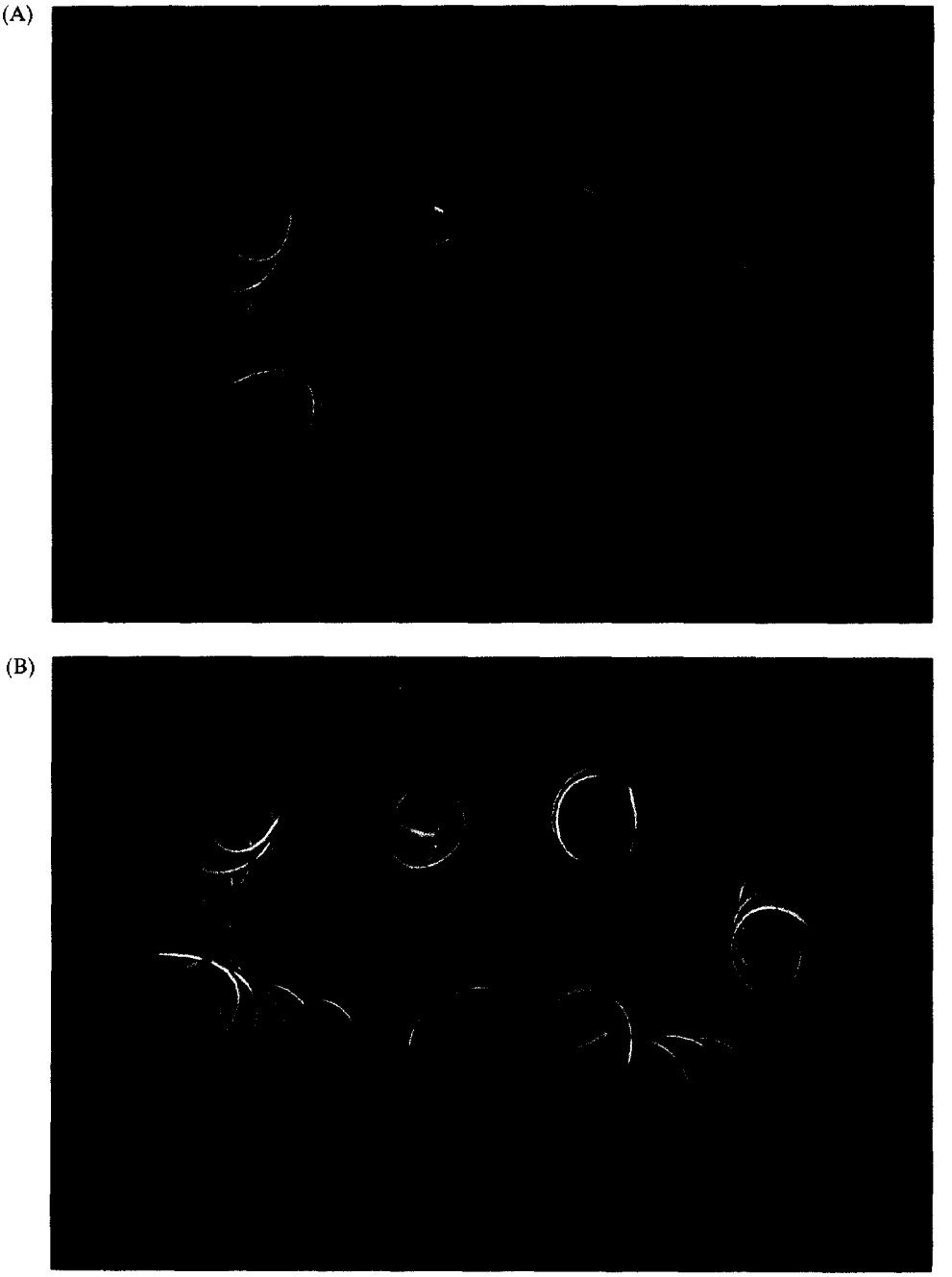


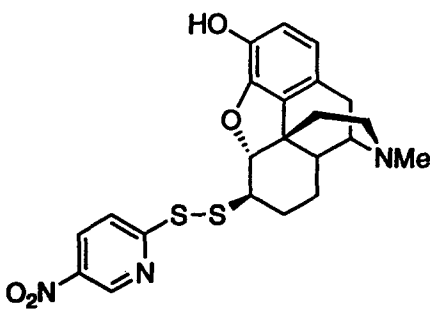
Figure 3. (A) View of the μ-opioid receptor model (extracellular view). Transmembrane helices 1–7 are depicted by coils (white). Side chains of amino acids of possible importance for ligand interaction are indicated. The other side chains are indicated in dark blue. (B) View of the δ-opioid receptor model (extracellular view). (C) View of the κ-opioid receptor model (extracellular view).

Figure 3. *continued*

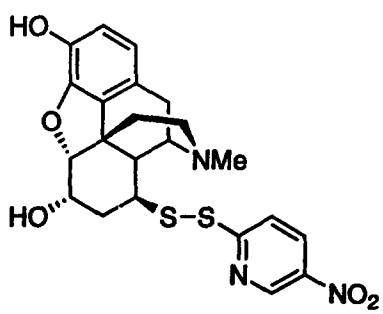
ligands added afterwards. Thus, after incubation of membranes with *S*-activated ligands, the ordinary receptor binding assay would estimate the amount of receptors unlabeled and consequently the amount of labeled receptors. To estimate the total amount of the free receptors, the amount of naloxone that displaces radiolabeled [^{3}H]naloxone was measured. This binding assay evaluates the extent of the affinity labeling of the μ receptor, because naloxone binds exclusively to the μ receptor. For the δ opioid receptor, to estimate the total amount of the free receptors, the amount of DSLET that displaces [^{3}H]DSLET was measured. For the κ opioid receptor, to estimate the total amount of the free receptors, the amount of U-69593 that displaces [^{3}H]U-69593 was measured. The affinity labeling experiment was carried out essentially as described previously.[42]

When **1** was incubated with the guinea pig brain membranes, it was found that the amount of free receptors diminished sharply, depending upon the concentrations of **1**. When the extent (percentage) of affinity-labeling was plotted against the concentrations of *S*-activated ligands incubated, the typical sigmoidal curves were depicted as shown in Figure 7. Figure 7A indicates, for example, that when the guinea pig brain membrane is incubated with 1 μM of the 6β-*S*-activated ligand **1**, it occupies approximately 60% of the μ receptor, while the 8β-*S*-activated isomer **2** cross-links only about 25% of the μ receptor. The concentration-dependent curves of affinity-labeling of δ and κ receptors are depicted as in Figure 7B and C, respectively. The effective concentrations (EC_{50}) of compound **1** to occupy the half-maximal amounts of receptors were estimated from Figure 7, these were 420.2 nM, 7.2 μM and 1.2 μM for μ, δ and κ receptors, respectively. It should be noted that **2** was difficult to affinity-label the opioid receptors completely (Table 2).

Since the IC_{50} and EC_{50} values of compound **1** for μ receptor are almost comparable to each other (14.2 and 420.2 nM, respectively), it appears that compound

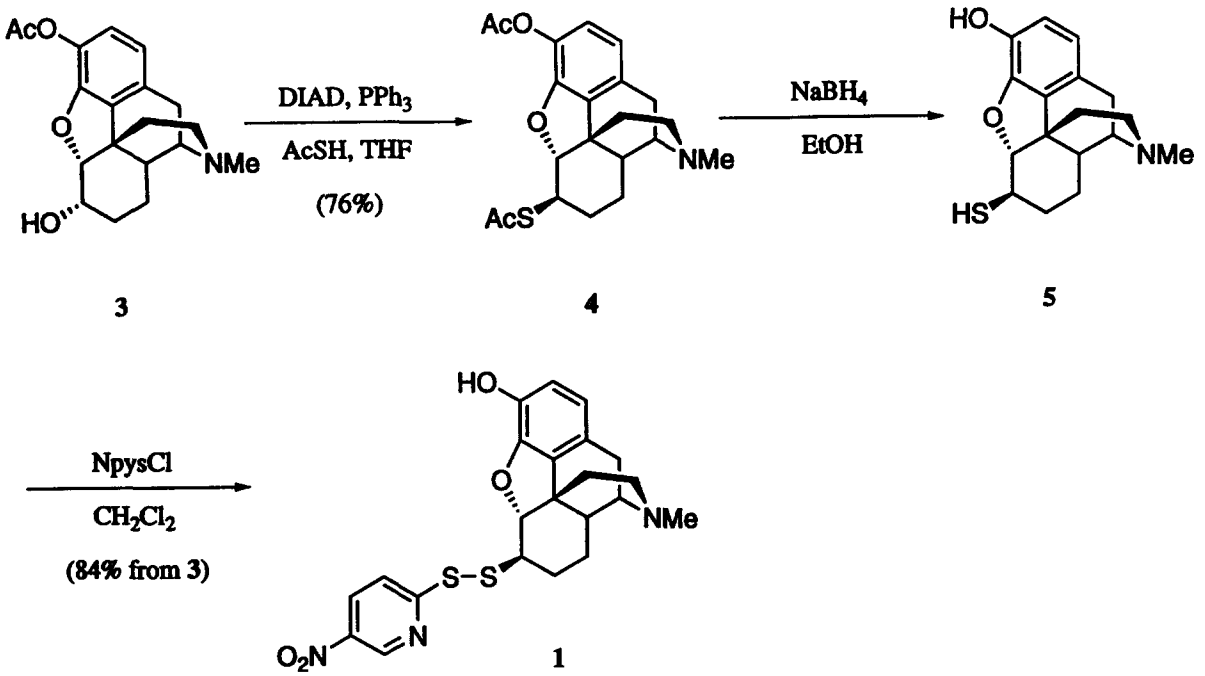


6β-[5-Nitro-2-pyridinesulfenyl] thio-dihydromorphine 1



8β-[5-Nitro-2-pyridinesulfenyl] thio-dihydromorphine 2

Figure 4. Structure of 6β-[5-nitro-2-pyridinesulfenyl] thio-dihydromorphine and 8β-[5-nitro-2-pyridinesulfenyl] thio-dihydromorphine.



Scheme 1

1 bound to the binding site almost inevitably forms a cross-link with the receptor molecule by a disulfide bonding. The structure of the 6β-*S*-activated ligand **1** appears to fit preferentially the binding site of the μ receptor in which the thiol group is located close to the 6β-*S*-activated thiol of compound **1**. In sharp contrast, compound **2** lacks the potential ability to affinity-label the opioid receptor. In particular, in spite of high binding affinity with the opioid receptor, its ability to form a cross-link with the receptor molecule was found to be extremely weak. This is certainly the reflection of regiochemistry of thiols activated by the 5-nitro-2-pyridinesulfenyl group, which is attached to position C_6 or C_8. The 8β thiol group of **2** is in much less favored regiochemistry to interact with the receptor thiol group. The receptor thiol group seemed to be present near the portion where the morphine 6β-substituent is located. The spatial proximity is expected to cause a thiol–disulfide exchange reaction between compound **1** and the receptor thiol group. Although the role of the thiol group in the molecular mechanism of receptor responses has not been clarified yet, the present results indicate that the opioid receptor protein contains a distinct free thiol group, which was originated from the cysteine residue, in the ligand binding site.

Prediction of the ligand binding sites in the opioid receptor subtypes

We attempted to estimate the ligand recognition sites in the molecular models of the opioid receptors. Hibert et al. have reported modeling of some GPCRs.[16,18] They made primary sequence comparison for amino acid sequences of transmembrane regions of cationic neurotransmitter receptors, and constructed three-dimensional models of these receptors. They also predicted the ligand binding sites from the results of the site-directed mutagenesis. Then they docked each neurotransmitter into the predicted binding sites, and investigated the steric and electrostatic interactions between the ligands and the receptors. They docked each neurotransmitter into the hydrophobic pocket of the receptor molecule so that the Asp in the middle of TM III interacts electrostatically with cationic nitrogen of the neurotransmitter. They found three highly conserved aromatic residues (Trp in TM III, Trp in TM VI and Phe in TM VI) form a hydrophobic cluster around the aspartate–ammonium ion pair and thus strongly stabilize the interaction. Asn in TM VI of the m_2 receptor plays a critical role by interacting specifically with the ester group of acetylcholine. Ser504 and Ser507 in TM V are conserved as a pair only in catecholamine receptors. These two residues can form hydrogen bonds to the catechol moiety of noradrenaline, adrenaline and dopamine. Ser504 in 5-HT receptors can interact with the 5-hydroxy substituent of serotonin. Another important finding is that Ser413 can form a stereospecific interaction with the β-hydroxyl group of noradrenaline and adrenaline, whereas Ser409 can form a hydrogen bond with the indole nitrogen atom of serotonin. For the aromatic neurotransmitters, an additional stabilizing T-shaped interaction is provided by Phe617, which is substituted by Asn617 in muscarinic receptors. Their model can explain some features of ligand–receptor interactions of GPCRs.

We attempted to estimate the ligand recognition sites in the opioid receptors from the results of our affinity labeling experiments and the homology between the opioid receptors and other GPCRs. To suggest amino acid residues participating in the ligand binding, our basic idea was that the amino acids invariant within all

subtypes, which are involved in the ligand binding sites, play an important role in the overall folding and function of the receptors. On the other hand, the amino acids specific to the receptor subtype might be responsible for the binding of the corresponding ligands and for some of the specific triggering mechanisms.

Conserved aspartic acid residues

All subtypes of opioid receptor possess three invariant acidic residues. One aspartic acid (μ: Asp114; δ: Asp95; κ: Asp105) is highly conserved across the whole GPCR family, indicating that this residue probably plays an essential role in the folding and/or the function of the receptor. The second aspartic acid (μ: Asp164; δ: Asp145; κ: Asp155) might be a member of the sequence coupling with G proteins. On the other hand, the conserved aspartic acid (μ: Asp147; δ: Asp128; κ: Asp138), which is located in the middle of TM III near the extracellular domain, is conserved in opioid receptors as well as cationic neurotransmitter receptors, but is absent in all other GPCRs. Recently published mutagenesis experiments of μ opioid receptor indicate that the aspartate residue in TM II is of importance for the binding of agonists, and the aspartate residue in TM III is of importance for both agonists and antagonists.[6] The aspartate residue in TM III is probably the primary binding site for opioid ligands having a protonated nitrogen. The aspartate residue in TM II may have an allosteric effect on agonist affinity in accordance with opioid receptors where the corresponding residue has mutated. This resulted in an impaired sodium effect when binding agonist. Considering this result, it was suggested that the binding site of opioid

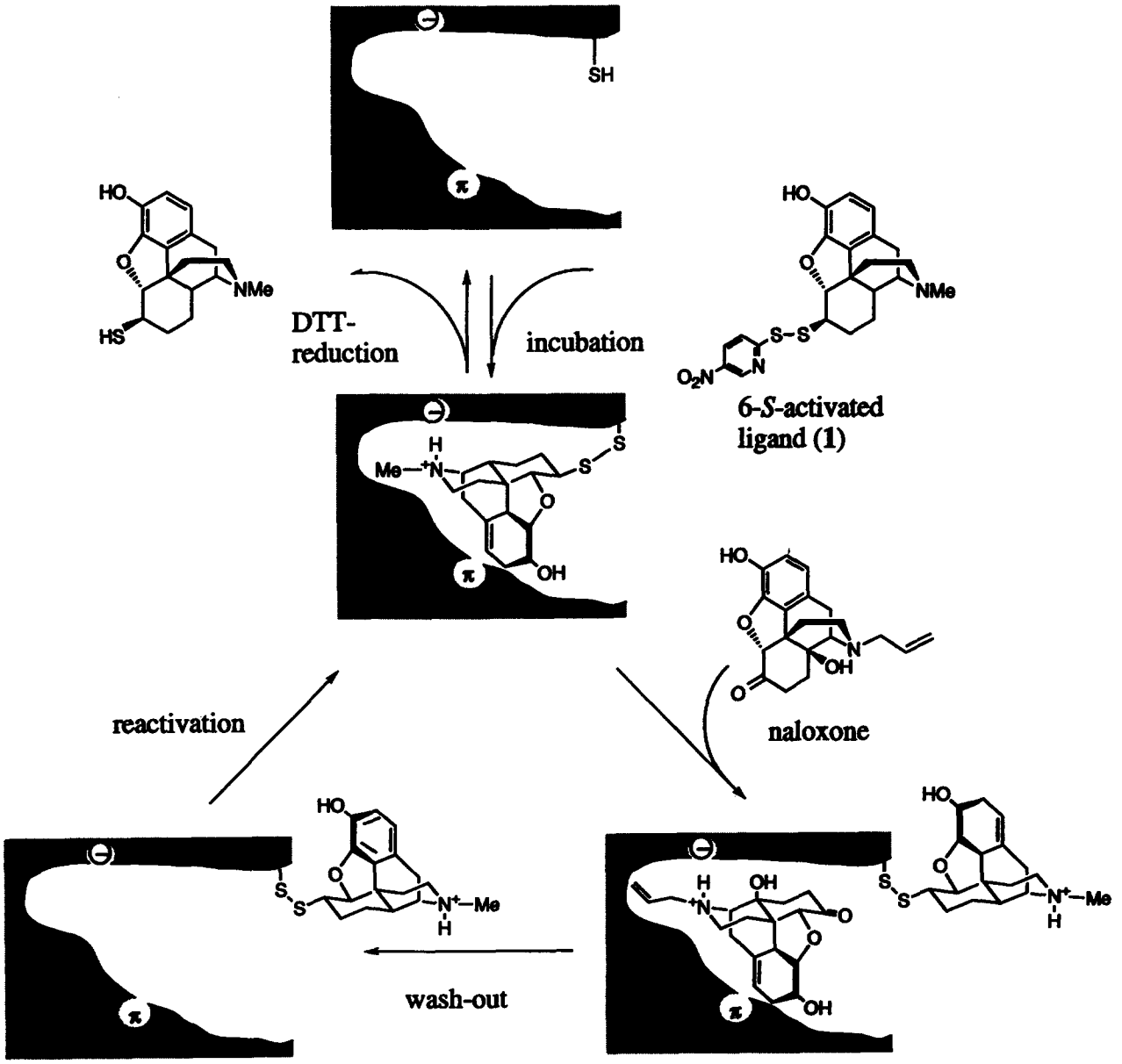
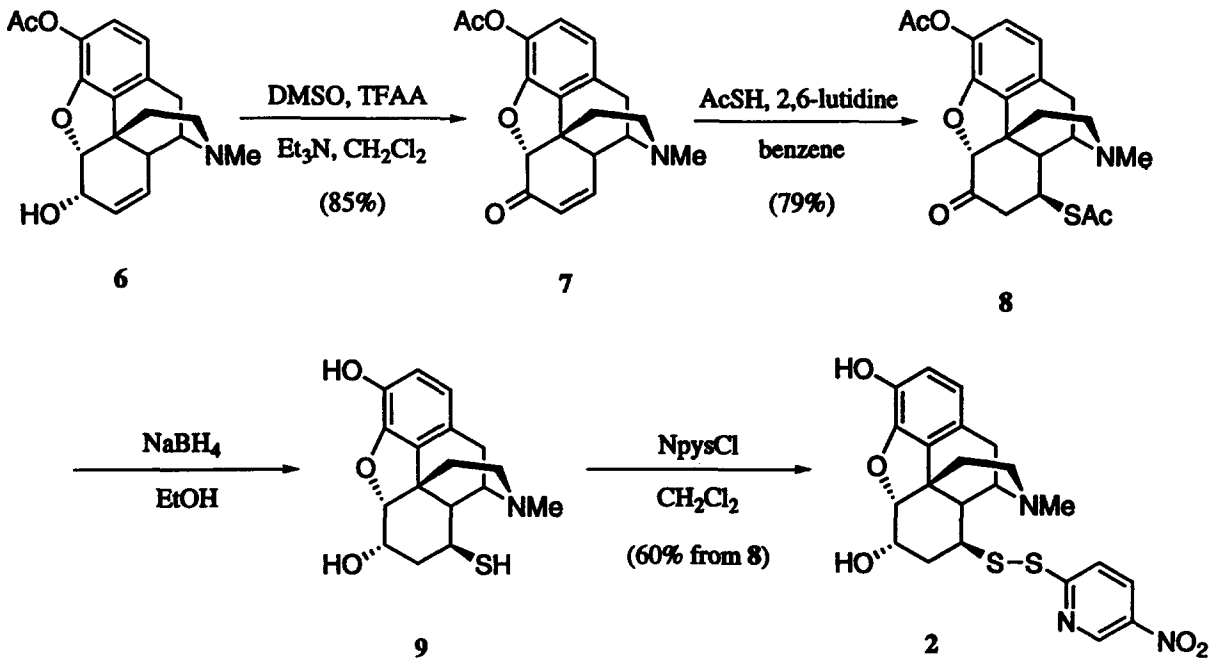


Figure 5. Schematic illustration of compound **1** with opioid receptor.



Scheme 2

receptor might be in the cleft centered in the aspartate residue in TM III.

Conserved cysteine residues

As described above, our results of affinity labeling experiments using *S*-activated dihydromorphine derivatives suggested that the opioid receptor protein contains at least one Cys residue, in the ligand binding site and the thiol group of the Cys residue seemed to be present near the portion where the morphine 6β-substituent was located.The conserved Cys residues might participate in the ligand binding in the opioid receptors. The primary sequence analysis defined several conserved Cys residues within the three types of opioid receptors, but did not suggest which Cys residues could be in the ligand binding sites and labeled by our *S*-activated ligands. However, our receptor's 3D models indicated that conserved cysteine residues (μ: Cys79; δ: Cys60 in TM I or μ: Cys321; δ: Cys303; κ: Cys315 in TM VII) could be involved in the pocket locating Asp residue in the center, and interact with the activated disulfide bond of *S*-activated ligands. These Cys residues are conserved within the opioid receptor subtypes and are absent in other cationic neurotransmitter receptors.

When *S*-activated ligand **1** was manually docked into the receptor model so that a salt bridge could be made between the cationic nitrogen of the ligand and Asp residue in TM III, the thiol group of the residue in the TM VII (μ: Cys321; δ: Cys303; κ: Cys315) or the residue in TM I (μ: Cys79; δ: Cys60) is close enough to the activated disulfide bond of **1** to undergo the thiol–disulfide exchange reaction. So we presumed that the clefts containing these Cys residues might be the binding sites for the opioid ligand, and defined the pocket involving the Asp residue in TM III (μ: Asp147; δ: Asp128) and the Cys residue in TM I (μ: Cys79; δ: Cys60) as the binding site 1, and the pocket involving the Asp residue in TM III (μ: Asp147; δ: Asp128; κ: Asp138) and the Cys residue in TM VII (μ: Cys321; δ: Cys303; κ: Cys315) as binding site 2 (Fig. 8).

Conserved aromatic residues in the predicted binding sites

Next, we examined the binding sites 1 and 2 in detail. As mentioned in the primary sequence alignment, all opioid receptors contain many conserved aromatic residues. It is generally known that the aromatic residues can be involved in important internal cross-linking hydrogen bonds and conformational changes. In particular, the Tyr residues in TM III, the Phe residues in TM VI (Tyr in μ-receptor) and the Tyr residues in TM VII that are located in the predicted ligand binding sites are conserved within all opioid receptor subtypes and are absent in the other cationic neurotransmitter receptors. The conserved Tyr residue in TM III (μ: Tyr148; δ: Tyr129; κ: Tyr139) is located at the position adjacent to the aspartate, which may be involved in electrostatic interactions with an ammonium cation head group of the opioid ligand. When a morphine molecule was manually docked into the predicted binding sites 1 and 2, where the quaternary ammonium cationic head group was placed to form electrostatic interactions with the aspartate in TM III, respectively, the phenyl ring of the morphine in both of the binding sites 1 and 2 were located close to the phenyl ring of the Tyr residue (μ: Tyr325; δ: Tyr308; κ: Tyr320) in TM VII and the Phe or Tyr residue (μ: Tyr299; δ: Phe280; κ: Phe293) in TM VI, respectively. These aromatic interactions might stabilize the ligand–receptor binding.

Variant amino acid residues in the predicted binding sites

As described above, the primary sequence analysis of the three subtypes indicates high homology in the predicted transmembrane regions, and a number of conserved amino acid residues that play an important role in the ligand-binding were defined. In contrast to this analysis, our idea is that a variant amino acid residue involved in the ligand-binding sites could be characteristic for the ligand-binding of each subtype of opioid receptor. A comparison of the 3-D models of opioid receptors defined the variant amino acid residues, which are different in charge and hydrophobic properties, within the three subtypes. In the upper region of TM VI, the μ, δ and κ receptors possess Lys303, Trp284 and Glu297, respectively. It is likely that the variant amino acid residues are able to participate in the mechanism that controls the selec-

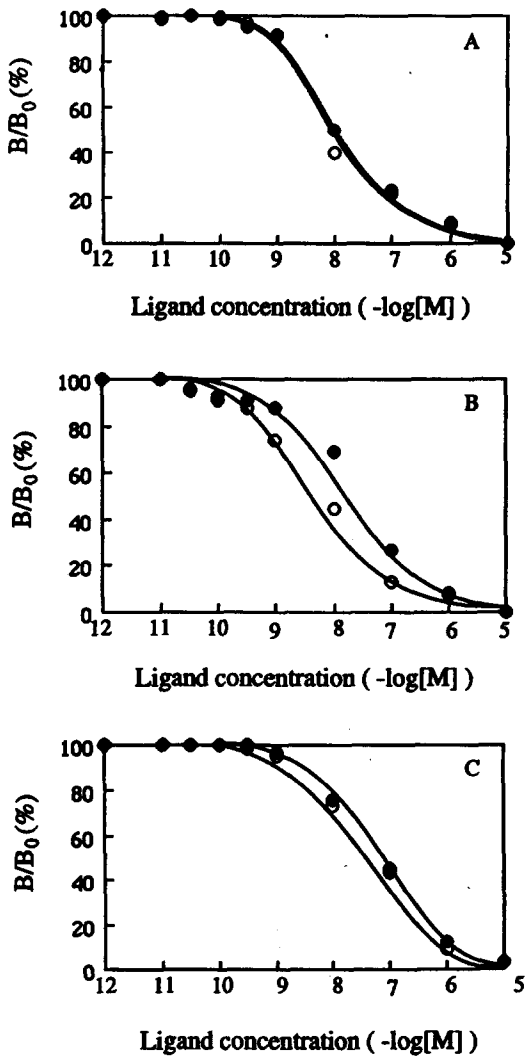


Figure 6. Dose–receptor curves of *S*-activated ligands in guinea pigs brain membrane preparations using [^{3}H]naloxone. [^{3}H]DSLET and [^{3}H]U-69593 for μ(A), δ(B) and κ(C) opioid receptors, respectively. Compounds **1** (○——○) and **2** (●——●). B/B_0(%) = relative binding activities (%) of the remained radio-labeled ligand over total specific binding.

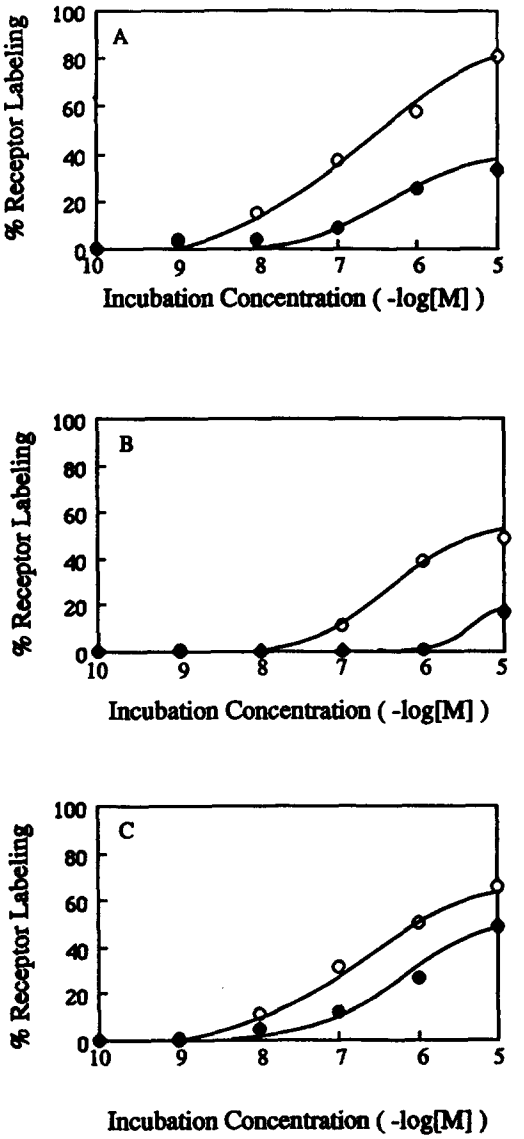


Figure 7. The concentration-dependent affinity labeling of μ (A), δ (B) and κ (C) opioid receptors by *S*-activated ligands. Compounds **1** (○——○) and **2** (●——●).

Table 1. IC_{50} values of compounds **1** and **2**

Compound	μ-sites (nM)	δ-sites (nM)	κ-sites (nM)
1	14.2	5.7	56.7
2	13.4	26.8	136.9

Table 2. EC_{50} values of compounds **1** and **2**

Compound	μ-sites (nM)	δ-sites (nM)	κ-sites (nM)
1	420.2	7.2×10^3	1.2×10^3
2	7.8×10^4	[a]N.D.	1.1×10^4

[a]Not determined.

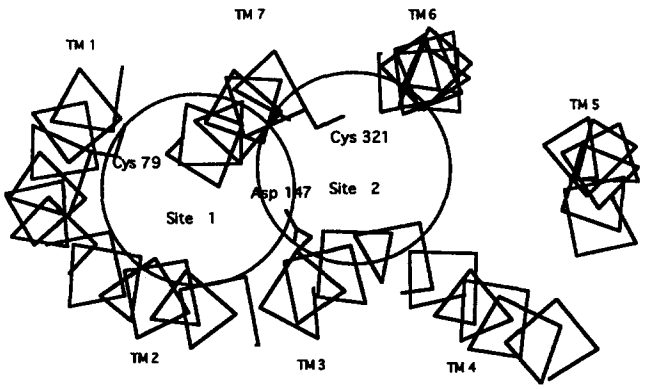


Figure 8. View of the μ-opioid receptor model (extracellular view). Putative binding sites 1 and 2 are indicated.

tivity of the ligand binding. The structural determinants for the selective binding of the nonpeptide opioid receptor antagonist norbinaltorphimine (nor-BNI) to the κ-opioid receptor were characterized using a systematic series of chimeras between the κ receptor and the homologous μ-opioid receptor. The exchange of a single residue, Glu297, for lysine, the corresponding residue from the μ receptor, reduced the binding affinity of nor-BNI 142-fold, without affecting the binding of the nonselective compounds (−)-naloxone and diprenorphine.[9]

From these observations, we presumed the ligand recognition mechanisms in the binding site 1 as: (1) the carboxylate of the aspartate residue in TM III (μ: Asp147; δ: Asp128) electrostatically interact with the quaternary ammonium cation in the opioid ligand. (2) The tyrosine residue in TM VII (μ: Tyr325; δ: Tyr308) interact with the phenol ring of opioid ligands with aromatic interaction. (3) The cysteine residue in TM I (μ: Cys79; δ: Cys60) is the second recognition site that undergoes the thiol–disulfide exchange reaction with the *S*-activated dihydromorphine derivatives. In the binding site 2 as: (1) the carboxylate of the aspartate residue in TM III (μ: Asp147; δ: Asp128; κ: Asp138) electrostatically interact with the quaternary ammonium cation in the opioid ligand. (2) The phenylalanine or tyrosine residue in TM VI (μ: Tyr299; δ: Phe280; κ: Phe293) interact with the phenol ring of opioid ligands with aromatic interaction. 3) The cysteine residue in TM VII (μ: Cys321; δ: Cys303; κ: Cys315) is the second recognition site that undergoes the thiol–disulfide exchange reaction with the *S*-activated dihydromorphine derivatives.

Analyses of the predicted ligand binding sites by ligand docking tests

We tested the predicted binding sites 1 and 2 by ligand docking analyses. The structures of ligands, whose crystal coordinates have known, were derived from CSD (Cambridge Structural Database System). The ligands were manually docked into the 3D models of the opioid receptors, then the structure of each ligand–receptor complex was optimized with energy minimization.

First, we docked the morphine molecule into the binding sites 1 and 2 of the μ opioid receptor. When morphine molecule was docked into the binding site 1, the sulfur atom of Cys 79 in TM I and the phenyl ring of Tyr325 in TM VII were considerably apart from morphine (7.1 and 5.8 Å, respectively). Moreover, no other amino acid residue that was participating in ligand binding was found. On the other hand, when the morphine molecule was docked into the binding site 2, the morphine molecule fitted the binding site well. The cationic nitrogen of the morphine could have electrostatic interaction with Asp 147 (2.7 Å) and the phenyl ring of the morphine could have aromatic interaction with Tyr148 and Tyr 299.[43]

Moreover the morphine molecule was stabilized by hydrogen bonding between the phenolic hydroxyl group of morphine and the phenolic hydroxyl group of Tyr148 and the amino group of side chain of Lys303, respectively (Fig. 9).

We also analyzed the docking of the morphine molecule into the binding sites of δ and κ opioid receptors. The results indicated that the morphine molecule could fit only the binding site 2 of the δ and κ opioid receptors. From these results, we estimated the binding site 2 as the binding sites of the morphine.

As mentioned above, Fukuda et al. expressed chimeric receptors between the rat μ- and δ- opioid receptors from cDNA and analysed their ligand binding properties; major determinant for binding of the m-selective morphine is demonstrated to exist in the region spanning of TM V-VII (like binding site 2).[10] For further analysis of the predicted binding site 2, we have analysed docking of the affinity labeling ligands **1** and **2** into the binding site 2 of the μ opioid receptor (Fig. 10). After optimization of the structure of each complex, the distances were measured between the amino acid residues in the predicted binding sites and each ligand. Although the distance between Asp147 and the cationic nitrogen of each ligand (2.7–2.8 Å) and the distance between the phenyl ring of Tyr299 in TM VI and the phenyl ring of each ligand (3.3–3.5 Å) were almost equal, only the distance between the thiol group of Cys321 and the activated thiol group of compounds **1** and **2** were considerably different (3.4 and 6.5 Å, respectively). This observation may result in the difference of the irreversible binding abilities between compounds **1** and **2**. The present results may account for our results of affinity labeling experiments.

In conclusion, we constructed three-dimensional molecular models of the multiple opioid receptor subtypes by homology modeling method using the bacteriorhodopsin as a template. We designed the ligand possessing an activated sulfhydryl group in a suitable position and utilized as the specific probe for the cysteine group in the ligand binding pocket. The affinity labeling experiment using such ligands could define the relative position of cysteine residue toward the ligand binding in the receptor. This made it

possible to predict the ligand binding sites in the receptor models and the manner of the ligand binding. Finally, the predicted binding site was confirmed by docking the morphine derivatives into the receptor model. All the ligands tested were proved to fit the predicted binding sites reasonably, and the binding site could also explain the mode of the binding of the opioid agonists and antagonists. There are some GPCRs possessing cysteine residues in the ligand binding pockets,[23–25] and they are expected to be characterized in the same manner.

The present models of the multiple opioid receptor subtypes are preliminary, and further modification is necessary for quantitative analysis of the receptor–ligand interaction. Especially additional data of site-directed mutagenesis and affinity labeling experiments would improve the reliability of our model considerably. It is, however, at least qualitatively useful for evaluation of our ligand design. In fact, a potent κ agonist was developed in our laboratory based on the structure of the putative binding sites.[44]

Because the elucidation of the three-dimensional structures of most of the membrane spanning receptors are now difficult, the construction of more accurate receptor's models would be essential for the study of functions of these membrane receptors and for the drug design.

Experimental

Synthesis

Starting materials and reagents purchased from commercial suppliers were generally used without further purification. Solvents were dried by distillation from the appropriate drying agent immediately prior to use. Tetrahydrofuran was distilled from sodium and benzophenone under argon atmosphere. Dichloromethane was distilled from calcium hydride under argon. All solvents used for routine isolation of products and chromatography were reagent grade and glass distilled. Air- and moisture-sensitive reactions were performed under an argon atmosphere. Residual

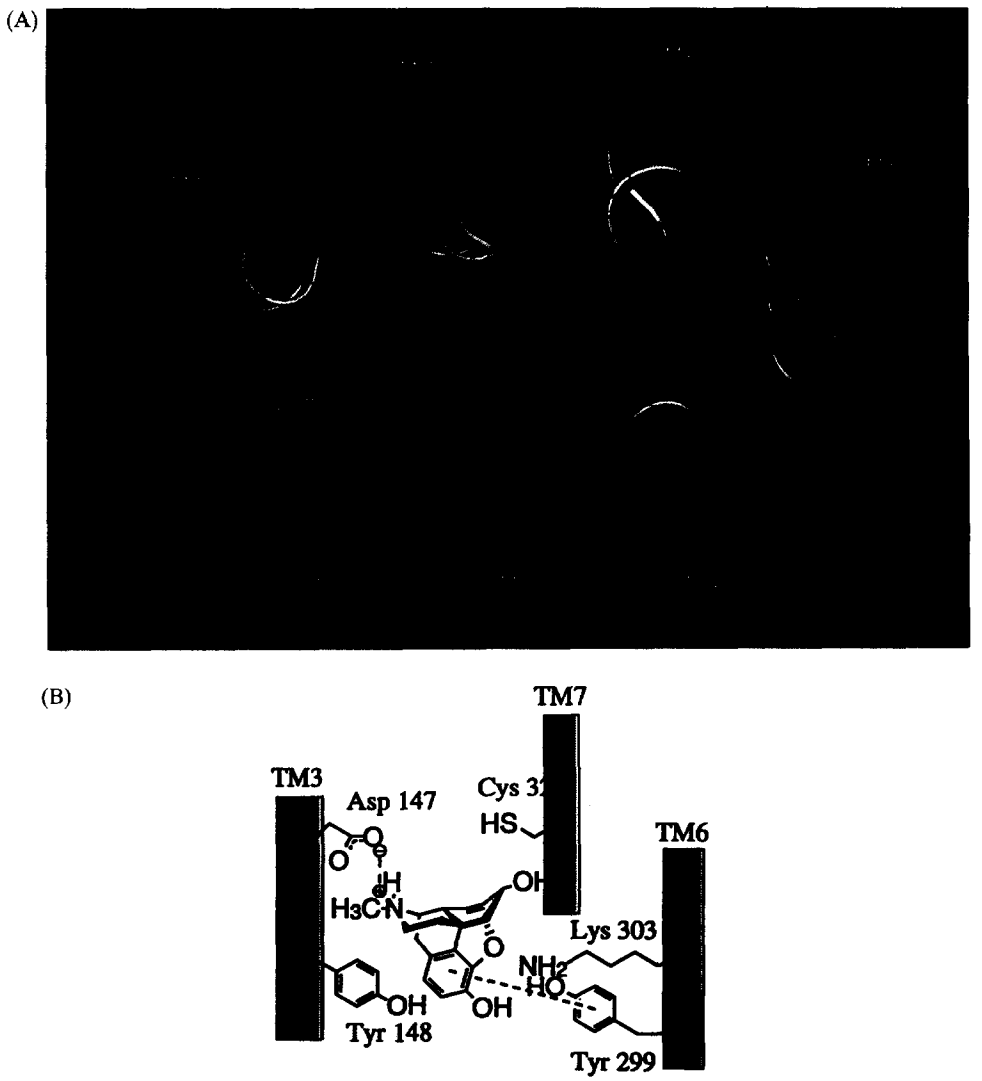


Figure 9. (A) The morphine molecule docked into the predicted binding site 2 of the μ opioid receptor (extracellular view). (B) Schematic representation of the interaction between morphine and the predicted binding site 2 of the μ opioid receptor.

solvent was removed under high vacuum at less than 1 Torr. Analytical thin-layer chromatography was performed using precoated aluminum TLC plates (0.2 mm layer thickness of silica gel 60 F-254). Column chromatography was carried out using silica gel 60 (230–400 mesh). Melting points were measured on Yanaco micro-melting point apparatus and are uncorrected. Optical rotations were measured with a Jasco DIP 360 polarimeter at ambient temperature using a 1 dm cell of 1 mL capacity. Infrared spectra were recorded on a Jasco IR A-100 infrared spectrophotometer. Carbon and proton nuclear magnetic resonance spectra were recorded on a Jeol GX-270 spectrometer. Chemical shifts are reported in parts per million down field from tetramethylsilane on the δ scale. ^{1}H NMR data are reported in the order of chemical shift, number of protons, multiplicity and coupling constant in hertz (Hz). Fast atom bombardment (FAB) mass spectra were measured on Jeol DX-300. Elemental analyses were performed on a Yanaco MT2 CHN recorder.

3-Acetoxy-6β-acetylthio-dihydromorphine (4). To a soln of triphenylphosphine (4.8 g, 18.28 mmol) in anhydrous tetrahydrofuran (120 ml) was added diisopropylazodicarboxylate (3.6 g, 18.28 mmol) at 0 °C. The mixture was stirred at 0 °C for 0.5 h. To the mixture were added **3** (1.00 g, 3.05 mmol) in tetrahydrofuran (50 ml) and thioacetic acid (1.31 ml, 18.28 mmol). The mixture was stirred overnight. After the solvent was evapd the residue was dissolved in ethyl acetate (50 ml) and was extracted with aq 10% HCl. To the extracts was added sodium bicarbonate carefully until the pH of the solution above 8. Then the mixture was extracted with chloroform (3 × 50 ml). The combined extracts were dried (sodium sulfate), and the solvent evapd. The residual oil was purified by chromatography on silica gel, using 2% methanol in chloroform, to give 0.90 g (76%) of **4** as a yellow oil. Compound **4**: $[\alpha]^{20}_D$ −0.99° (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 2.27 (s, 3H), 2.32 (s, 3H), 2.40 (s, 3H), 4.52 (d, *J* = 8.91 Hz, 1H), 6.70 (d, *J* = 8.91 Hz, 1H), 6.84 (d, *J* = 8.25 Hz, 1H), 7.27 (s, 2H); IR ($CHCl_3$; cm^{-1}) 1100, 1340, 2940; HRMS *m/z* 387.4995 (calcd for $C_{21}H_{25}NO_4S$ 387.4998).

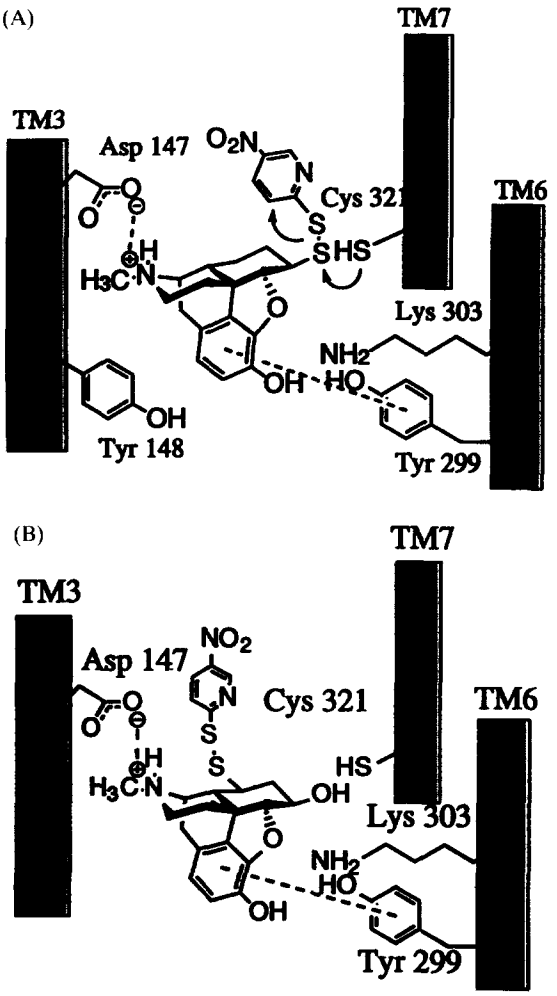


Figure 10. (A) Schematic representation of the interaction between ligand **1** and the predicted binding site 2 of the μ opioid receptor. (B) Schematic representation of the interaction between ligand **2** and the predicted binding site 2 of the μ opioid receptor.

6β-[5-Nitro-2-pyridinesulfenyl]thio-dihydromorphine (1). To a soln of **4** (400 mg, 1.03 mmol) in ethanol (50 ml) was added sodium borohydride (221 mg, 5.84 mmol) at 0 °C. The mixture was stirred at room temperature. After 1 h, aq ammonium chloride (20 ml) was added to the mixture. The resulting mixture was extracted with chloroform (3 × 20 ml). The combined extracts were dried (sodium sulfate), and the solvent evaporated. The residual oil (531 mg) in dichloromethane (50 ml) was added potassium carbonate (147 mg, 1.06 mmol) and 5-nitro-2-pyridinesulfenylchloride (458 mg, 2.4 mmol) at 0 °C. The mixture was stirred at room temperature for 10 min. To this solution was added water (20 ml), the layers were separated and the aqueous phase was extracted with chloroform (3 × 20 ml). The combined extracts were dried (sodium sulfate), and the solvent was evaporated. The residual oil was purified by chromatography on silica gel, using 10% methanol in chloroform, to give 397 mg (84% from **3**) of **1** as a yellow oil. Compound **1** was converted to its HCl salt. Compound **1**: $[\alpha]^{20}_D$ −5.98° (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 2.41(s, 3H), 3.00 (d, *J* = 18.48 Hz, 1H), 3.17 (bs, 1H), 4.40 (d, *J* = 8.58 Hz, 1H), 6.58 (d, *J* = 7.92 Hz, 1H), 6.67 (d, *J* = 7.92 Hz, 1H), 7.27 (s, 2H), 8.12 (d, *J* = 8.58 Hz, 1H), 8.48 (dd, *J* = 8.91 Hz, 3.00 Hz, 1H); IR ($CHCl_3$; cm^{-1}) 2950, 3250; MS *m/z* 457 [M^+]; Anal. calcd for $C_{22}H_{24}N_3O_4S_2Cl \cdot 0.5H_2O$: C, 52.53; H, 5.01; N, 8.35. Found: C, 52.40; H, 5.21; N, 8.37.

3-Acetoxy-morphinone (7). To a stirred soln of dimethylsulfoxide (0.66 ml, 9.40 mmol) in dichloromethane (15 ml) was added trifluoroacetic anhydride (0.98 ml, 6.91 mmol) in dichloromethane (10 ml) at −60 °C. After 10 min, **6** (1.34 g, 1.03 mmol) in dichloromethane (50 ml) was added to the mixture. After 2 h, triethylamine (1.66 ml) was added to the mixture and the mixture was allowed to warm to room temperature with stirring. To this solution was added triethylamine (8.0 ml), the layers were sepd and the aq

phase extracted with chloroform (3 × 20 ml). The combined extracts were dried (sodium sulfate), and the solvent evapd. The residual oil was purified by chromatography on silica gel, using 10% methanol in chloroform, to give 1.13 g (85%) of **7** as a colorless oil. Compound **7**: $[\alpha]^{23}_{D}$ −188.7° (*c* 1.0, $CHCl_3$);^{1}H NMR ($CDCl_3$): δ 2.27 (s, 3H), 2.61 (s, 3H), 4.74 (s, 1H), 6.09 (dd, *J* = 10.23, 2.97 Hz, 1H), 6.62–6.66 (m, 1H), 6.66 (d, *J* = 8.25 Hz, 1H), 6.81 (d, *J* = 8.25 Hz, 1H); IR ($CHCl_3$; cm^{-1}) 1750; HRMS *m/z* 3326.3719 (calcd for $C_{19}H_{20}NO_4$ 326.3721).

3-Acetoxy-8b-acetylthio-dihydromorphinone (8). To a soln of **7** (325 mg, 1.00 mmol) in benzene (50 ml) were added 2,6-lutidine (230 ml, 2.00 mmol) and thioacetic acid (130 ml, 2.00 mmol) at 0 °C. The mixture was stirred at 0 °C for 1 h. To this soln was added water (30 ml), the layers were sepd and the aq phase extracted with chloroform (3 × 20 ml). The combined extracts were dried (sodium sulfate), and the solvent evapd. The residual oil was purified by chromatography on silica gel, using 10% methanol in chloroform, to give 320 mg (79%) of **8** as a colorless oil. **8**: $[\alpha]^{20}_{D}$ −38.3° (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.85 (m, 1H), 2.05 (dt, *J* = 12.2 Hz, 4.8 Hz, 1H), 2.21 (dt, *J* = 12.2 Hz, 3.7 Hz, 1H), 2.32 (s, 6H), 2.38 (dd, *J* = 19.3 Hz, 6.3 Hz, 1H), 2.43 (s, 3H), 2.56 (m, 1H), 2.60 (dd, *J* = 14.2 Hz, 12.6 Hz, 1H), 2.63 (dd, *J* = 12.5 Hz, 3.2 Hz, 1H), 2.69 (dd, *J* = 13.9 Hz, 4.0 Hz, 1H), 3.01 (d, *J* = 19.1 Hz, 1H), 3.30 (dt, *J* = 12.5 Hz, 4.2 Hz, 1H), 3.46 (m, 1H), 4.70 (s, 1H), 6.73 (d, *J* = 8.3 Hz, 1H), 6.88 (d, *J* = 8.3 Hz, 1H);^{13}C-NMR(270 MHz, $CDCl_3$) d : 203.45 (s), 193.64 (s), 168.43 (s), 147.76 (s), 131.88 (s), 126.81 (s), 123.02 (d), 120.22 (d), 91.05 (d), 57.06 (d), 47.23 (t), 46.89(t), 46.07(d), 42.84 (q), 39.81 (d), 35.63 (t), 30.73 (q), 20.76 (q), 20.01 (t); IR ($CHCl_3$; cm^{-1}) 750, 1180, 1760, 2920; HRMS *m/z* 402.4920 ($C_{21}H_{24}NO_5S$ 402.4913).

8β-[5-Nitro-2-pyridinesulfenyl]thio-dihydromorphine (2). To a soln of **8** (146 mg, 0.364 mmol) in ethanol (50 ml) was added sodium borohydride (68.9 mg, 1.82 mmol) at 0 °C. The mixture was stirred at room temperature. After 1 h, aq ammonium chloride (20 ml) was added to the mixture. The resulting mixture was extracted with chloroform (3 × 20 ml). The combined extracts were dried (sodium sulfate), and the solvent evapd. To the residual oil (121 mg) in dichloromethane (20 ml) were added potassium carbonate (104.9 mg, 0.758 mmol) and 5-nitro-2-pyridinesulfenylchloride (132 mg, 0.69 mmol) at 0 °C. The mixture was stirred at room temperature for 10 min. To this soln was added water (20 ml), the layers were sepd and the aq phase extracted with chloroform (3 × 20 ml). The combined extracts were dried (sodium sulfate), and the solvent evapd. The residual oil was purified by chromatography on silica gel, using 10% methanol in chloroform, to give 103 mg (60% from **8**) of **2** as a yellow oil. Compound **2** was converted to its HCl salt. Compound **2**: $[\alpha]^{20}_{D}$ −153.25° (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 2.44 (s, 3H), 3.06 (d, *J* = 18.48 Hz, 1H), 3.65 (bs, 1H), 4.10 (m, 1H), 4.58(d, *J* = 4.95 Hz, 1H), 6.55 (d, *J* = 8.25 Hz, 1H), 6.64 (d, *J* = 7.92 Hz, 1H), 7.26 (s, 2H), 7.78 (d, *J* = 8.58 Hz, 1H), 8.33 (dd, *J* = 8.91, 2.64 Hz, 1H); IR ($CHCl_3$; cm^{-1}) 760, 1180, 2450, 3090, 3420; MS *m/z* 474 [M^+]; Anal. calcd for $C_{22}H_{25}N_3O_5S_2Cl \cdot 0.5H_2O$: C, 50.81; H, 5.04; N, 8.08. Found: C, 50.97; H, 5.11; N, 8.15.

Mechanical Response

Male guinea pigs weighing 250–300 g were fasting overnight prior to the day of the experiment and were killed by a blow on the neck. After the ileum was excised, strips of longitudinal muscle with attached myentric plexus were prepared using the method of Kosterlitz.[45] Tissues were mounted in a 20 ml organ bath containing Krebs solution (NaCl 118, KCl 4.75, $CaCl_2 \cdot 2H_2O$ 2.54, $NaHCO_3$ 25.0, KH_2PO_4 1.19, $MgSO_4$ 1.20 and glucose 11.0 mM) and gassed with a mixture of 95% O_2 and 5% CO_2 at 37 °C. Two platinum electrodes (2 × 35 mm) were placed at an interval of 5 mm and field stimulation of the ileum was carried out by passing a rectangular pulse of 0.1 msec duration, supramaximal voltage and a frequency of 0.1 Hz between the two electrodes. The ileal strips responded to a single pulse. The twitch response to the electrical stimulation was recorded isometrically with an initial tension of 0.5 g.

Receptor binding studies materials

6b-[5-Nitro-2-pyridinesulfenyl]thio-dihydromorphine (**1**) and 8β-[5-nitro-2-pyridinesulfenyl]thio-dihydromorphine (**2**) were synthesized from morphine in our laboratory.[31] Bacitracin was purchased from Sigma (St. Louis, MO, U.S.A.), bovine serum albumin from Janssen (Olen, Belgium). The other reagents, of analytical grade, were purchased from Nakarai Tesque (Kyoto, Japan). [^{3}H]Naloxone, [^{3}H][D-Ser^2, D-Leu^5]enkephalin ([^{3}H] DSLET) and [^{3}H]U-69593 were purchased from New England Nuclear (Boston, MA, U.S.A.).

Receptor binding assay

Radioligand receptor binding assays using guinea pig brain membrane preparations were carried out essentially as described previously.[46] [^{3}H]naloxone (1.5 TBq/mmol), [^{3}H]DSLET ([^{3}H][D-Ser^2,Leu^5]-enkephalyl-Thr^6) (1.5 TBq/mmol) and [^{3}H]U-69593 (1.5 TBq/mmol) were used as tracers specific for μ, δ and κ opioid receptors, respectively, at the final concentration of 0.25 nM. Incubations were carried out at 25 °C for 60 min in 50 mM Tris–HCl buffer (pH 7.5) containing 0.1% bovine serum albumin. Bacitracin (100 mg/ml) was added to the buffer as an enzyme inhibitor. Dose–response curves were constructed utilizing 7–10 doses. The results were analysed by the computer program ALLFIT[47] and the data were used to construct the least-squares estimates of the logistic curves relating the binding of labeled ligand [^{3}H]naloxone, [^{3}H]DSLET and [^{3}H]U-69593 to concentrations of unlabeled ligands.

Affinity labeling of opioid receptors

Guinea pig brain membranes in 10 mM Tris–HCl buffer (pH 7.5) were incubated with *S*-activated ligands (0.2 nM–20 mM) or without ligands (control) in the presence of bacitracin (100 mg/ml) at 25 °C for 30 min. Membranes were then centrifuged (40,000 ***g***, 15 min) and suspended in the same volume of buffer to homogenize with Polytoron homogenizer, then incubated at 25 °C for 15 min. These washing operations were repeated to remove completely the ligands bound reversibly to membranes, and at least five times washings were required (data not shown). Washed membranes were finally assayed for radio-ligand receptor binding assays as described above.

Molecular modeling of multiple opioid receptors and ligand docking analysis

Receptor modeling including energy minimization were carried out using InsightII/Discover.[48] The construction of the model for opioid receptors was done by using a strategy which was already described.[30] The coordinate of bacteriorhodopsin was from the Protein Data Bank (Brookhaven National Laboratory). Energy minimizations for the model were carried out using molecular mechanics calculations with CVFF force field in Discover. The parameters were the distance-dependent dielectric constant of 2 and the non-bonded cut off of 15, and no solvent molecules were included in the calculation. The models were energy minimized for 500 steps with steepest descent minimizer and subsequently until rms energy gradient was less than 0.1 kcal/mol Å with the conjugate gradient minimizer.

The model for the opioid receptors was constructed from the amino acid sequences according to the following procedures: (1) the hydrophobic moment[49] of each helix of bacteriorhodopsin was calculated, and the amplitude and direction of the moment were indicated by a vector. (2) The average hydrophobic moment of the three opioid receptors was obtained from the aligned amino acid sequences. (3) The seven transmembrane helices in each opioid receptor were constructed by taking the backbone (φ, ψ) angles as ($-59°$, $-44°$)[50] and the preferable side-chain rotamer structures, as determined by Ponder and Richards.[51] (4) Each helix was rotated around the helical axis so that the direction of the hydrophobic moment agreed with that of bacteriorhodopsin. Here, the second, sixth and seventh helices were further rotated so that the side-chains of aspartate, cysteine and the aromatic residues would be oriented to the interior of the helices. (5) Each helix was moved in the direction normal to the membrane on the graphics screen. (6) The whole structural energy of each transmembrane domain in the three opioid receptors was minimized to get rid of bad contacts in each final model structure.

An initial model for the opioid ligand–receptor complex was obtained by docking ligand into receptor model by manual adjustment. The complex was optimized with the procedure mentioned above. The global energy minimization was not used for the final model, because global energy minimization moved protein structure away from its well determined crystal structure.[21]

References and Notes

1. Martin, W. R.; Eades, C. G.; Thompson, J. A.; Huppler, R. E.; Gilbert, P. E. *J. Pharmcol. Exp. Ther.* **1976**, *197*, 517.

2. Lord, J. A.; Waterfield, A. A.; Hughes, J.; Kosterlitz, H. W. *Nature* **1977**, *267*, 495.

3. Uhl, G. R.; Childers, S.; Pasternak, G. *TINS* **1994**, *17*, 89.

4. Kong, H.; Raynor, K.; Yasuda, K.; Moe, S. T.; Portoghese, P. S. *J. Biol. Chem.* **1993**, *268*, 23055.

5. Surratt, C. K.; Johnson, P. S.; Moriwaki, A.; Seidleck, B. K.; Blaschak, C. J.; Wang, J. B.; Uhl, G. R. *J. Biol. Chem.* **1994**, *269*, 20548.

6. Kong, H.; Raynor, K.; Yano, H.; Takeda, J.; Bell, G. I.; Reisine, T. *Proc. Natl Acad. Sci. U.S.A.* **1994**, *91*, 8042.

7. Xue, J. C.; Chen, C.; Zhu, J.; Kunapuli, S.; DeRiel, J. K.; Yu, L.; Liu-Chen, L. Y. *J. Biol. Chem.* **1994**, *269*, 30195.

8. Onogi, T.; Minami, M.; Katao, Y.; Nakagawa, T.; Aoki, Y.; Toya, T.; Katsumata, S.; Satoh, M. *FEBS Letters* **1995**, *357*, 93.

9. Hjorth, S. A.; Thirstruo, K.; Grandy, D. K.; Schwartz, T. W. *Mol. Pharmacol.* **1995**, *47*, 1089.

10. Fukuda. K.; Kato, S.; Mori, K. *J. Biol. Chem.* **1995**, *270*, 6702.

11. Meng, F.; Hoversten, M. T.; Thompson, R. C.; Taylor, L.; Waston, S. J.; Akil, H. *J. Biol. Chem.* **1995**, *270*, 12730.

12. Xue, J. C.; Chen, C.; Zhu, J.; Kunapuli, S. P.; DeRiel, J. K.; Yu, L.; Liu-Chen, L. Y. *J. Biol. Chem.* **1995**, *270*, 12977.

13. Findlay, J.; Eliopoulos, E. *Trends Pharmacol. Sci.* **1990**, *11*, 492.

14. Dahl, S. F.; Edvardsen, O.; Sylte, I. *Proc. Natl Acad. Sci. U.S.A.* **1991**, *88*, 8111.

15. Grötzinger, J.; Engels, M.; Jacoby, E.; Wollmer, A.; Straussburger, W. *Protein. Eng.* **1991**, *4*, 767.

16. Hibert, M. F.; Trumpp-Kallmeyer, S.; Bruinvels, A.; Hoflack, J. *Mol. Pharmacol.* **1991**, *40*, 8.

17. Livingstone, C. D.; Strange, P. G.; Naylor, L. H. *Biochem. J.* **1992**, *287*, 277.

18. Trumpp-Kallmeyer, S.; Hoflack, J.; Bruinvels, A.; Hibert, M. *J. Med. Chem.* **1992**, *35*, 3448.

19. Zhang, D.; Weinstein, H. *J. Med. Chem.* **1993**, *230*, 292.

20. Yamamoto, Y.; Kamiya, K.; Terao, S. *J. Med. Chem.* **1994**, *36*, 820.

21. Teeter, M. M.; Froimowitz, M.; Stec, B.; DuRand, C. J. *J. Med. Chem.* **1994**, *37*, 2874.

22. Hedberg, M. H.; Johansson, A. M., Nordvall, G.; Yliniemela, A.; Li, H. B.; Martin, A. R.; Hjorth, S.; Unelius, L.; Sundell, S.; Hacksell, U. *J. Med. Chem.* **1995**, *38*, 647.

23. Karlin, A.; Bartles, E. *Biochim. Biophys. Acta* **1966**, *126*, 525.

24. Mukherjee, C.; Lefkowitz. R. J. *Mol. Pharmacol.* **1977**, *13*, 291.

25. Stadel, J. M.; Lefkowitz. R. J. *Mol. Pharmacol.* **1979**, *16*, 709.

26. Abood, M. E.; Law, P. Y.; Loh, H. H. *Biochem. Biophys. Res. Commun.* **1985**, *127*, 477.

27. Smith, J.; Simon, E. J. *Proc. Natl Acad. Sci. U.S.A.* **1980**, *77*, 281.

28. Niwa, H.; Nozaki, M.; Fujimura, H. *Life Sci.* **1983**, *33*, 211.

29. Larsen, N. E.; Kilpatrick, D. M.; Blume, A. J. *Mol. Pharmacol.* **1981**, *20*, 255.

30. Fujii, I.; Nakamura, H.; Sagara, T.; Kanematsu, K. *Med. Chem. Res.* **1994**, *4*, 424.

31. Affinity labeling of μ and δ opioid receptors were reported in Sagara, T.; Okamura, M.; Shimohigashi, Y.; Ohno, M.; Kanematsu, K. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1609.

32. Fukuda, K., Kato, S.; Mori, K.; Nishi, M.; Takeshima, H. *FEBS Lett.* **1993**, *327*, 311.

33. Yasuda, K.; Raynor, K.; Kong, H., Breder, C. D.; Takeda, J.; Reisine, T.; Bell, G. I. *Proc. Natl Acad. Sci. U.S.A.* **1993**, *90*, 6736.

34. Mimami, M., Toya, T., Katao, Y., Maekawa, K., Nakamura, S., Onogi, T., Kaneko, S., Satoh, M. *FEBS Lett.* **1993**, *329*, 291.

35. Kyte, J., Doolittle, R. F. *J. Mol. Biol.* **1982**, *157*, 105.

36. Henderson, R., Baldwin, J. M.; Ceska, T. A.; Zemlin, F.; Beckmann, E.; Downing, K. H. *J. Mol. Biol.* **1990**, *213*, 899.

37. Lean, A. D.; Munson, P.J.; Rodbard, D. *Am. J. Physiol.* **1978**, *235*, E97.

38. Kanematsu, K.; Naito, R.; Shimohigashi, Y.; Ohno, M.; Ogasawara, T.; Kurono, M.; Yagi, K. *Chem. Pharm. Bull.* **1990**, *38*, 1438.

39. Gacel, G.; Fournie-Zaluski, M. C.; Roques, B. P. *FEBS Lett.* **1980**, *118*, 245.

40. Lord, J. A. H.; Waterfield, A. A.; Hughes, J.; Kosterlitz, H. W. *Nature (London)* **1977**, *267*, 495.

41. Goldstein, A.; Naidu, A. *Mol. Pharmacol.* **1989**, *36*, 265.

42. Yasunaga, T.; Shimohigashi, Y.; Kodama, H.; Miyazaki, M.; Nagaishi, M.; Ohno, M.; Kondo, M. *Bull. Chem. Soc. Jpn* **1994**, *67*, 296.

43. A referee suggested that the CH/π interaction in comparison with the π/π interaction plays an important role in biopolymer. However, the CH/π interaction is still far from complete.

44. Sagara, T.; Ozawa, S.; Kushiyama, E.; Koike, K.; Takayanagi, I.; Kanematsu, K. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1505.

45. Kosterlitz, H. K.; Waterfield, A. A. *A. Rev. Pharmac.* **1975**, *15*, 29.

46. Shimohigashi, Y.; English, M. L., Stammer; C. H., Costa, T. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 583.

47. Lean, A. D.; Munson, P.J.; Rodbard, D. *Am. J. Physiol.* **1978**, *235*, E97.

48. Discover user guide Version 3.1, Biosym Technologies, San Diego **1993**.

49. Eisenberg, D.; Weiss, R.M.; Termilliger, T. C. *Proc. Natl Acad. Sci. U.S.A.* **1984**, *81*, 140.

50. Blundell, T.; Barlow, D.; Borlakoti, N.; Thornton, J. *Nature* **1983**, *306*, 281.

51. Ponder, J. M.; Richards, F. M. *J. Mol. Biol.* **1987**, *193*, 775.

(*Received in Japan* 6 *June* 1996; *accepted* 13 *August* 1996)