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DAMGO recognizes four residues in the third extracellular loop to discriminate between μ- and κ-opioid receptors

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Abstract

Previously, we reported that replacement of the region from the fifth transmembrane domain to the C-terminus of κ-opioid receptor with the corresponding region of μ-opioid receptor gives high affinity for [D-Ala^2, *N*-MePhe^4, Gly-ol^5]enkephalin (DAMGO), a μ-opioid receptor-selective ligand, to the resultant chimeric receptor, suggesting that the difference in the amino acid sequence within this region is critical for the discrimination between μ- and κ-opioid receptors by DAMGO. In the present study, we constructed further six μ/κ-chimeric receptors and revealed that at least two separate regions around the third extracellular loop are critical for the discrimination between μ- and κ-opioid receptors by DAMGO. Furthermore, we constructed several mutant receptors by a site-directed mutagenesis technique and found that the difference between Glu^{297} of κ-opioid receptor and Lys^{303} of μ-opioid receptor in one region, and the difference between Ser^{310}, Tyr^{312} and Tyr^{313} of κ-opioid receptor and Val^{316}, Trp^{318} and His^{319} of μ-opioid receptor in the other region, are critical for the discrimination between these receptors by DAMGO. The mutant receptor, κ (E297K + Y313H + Y312W + S310V), in which the Glu^{297}, Ser^{310}, Tyr^{312} and Tyr^{313} of κ-opioid receptor were changed to Lys, Val, Trp and His, respectively, bound to DAMGO with high affinity ($K_d = 8.7 \pm 1.2$ nM) and efficiently mediated the inhibitory effect of DAMGO on intracellular cAMP accumulation. The present results showed that these four amino acid residues act as determinants for the discrimination between μ- and κ-opioid receptors by DAMGO.

Keywords: DAMGO ([D-Ala^2, *N*-MePhe^4, Gly-ol^5]enkephalin); Opioid receptor; Chimeric receptor; Site-directed mutagenesis

1. Introduction

Endogenous opioid peptides and opiate drugs like morphine act on specific receptors to produce various physiological and pharmacological effects, such as analgesia, respiratory effect, euphoria and modulation of neuroendocrine. The opioid receptors have been classified into at least three types, μ-, δ- and κ-types (Pasternak, 1988). Recently, we and others have cloned cDNAs for these three types of opioid receptors (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Thompson et al., 1993; Wang et al., 1993; Fukuda et al., 1993; Minami et al., 1994, 1993; Yasuda et al., 1993; Meng et al., 1993). Hydropathy analyses of the deduced amino acid sequences of these clones suggest that these receptors have seven putative transmembrane helices characteristic of G protein-coupled receptors. Comparison of the amino acid sequences across the μ-, δ- and κ-opioid receptors revealed about 60% identity among these three receptors. In spite of such high homology, the types can be discriminated by the ligands selective to each. The molecular basis of such discrimination is unknown and of keen interest.

Extensive studies using chimeric and mutant opioid receptors have elucidated the regions and/or amino acid residues in these receptors which are important for discrimination of receptor types by type-selective opioid ligands (Onogi et al., 1995; Minami et al., 1995, 1996; Xue et al., 1995; Wang et al., 1995; Meng et al., 1996; Hjorth et al., 1995; Valiquette et al., 1996; Pepin et al., 1997). Previously, we reported that [D-Ala^2, *N*-MePhe^4, Gly-ol^5]enkephalin (DAMGO), a μ-opioid receptor-selective ligand, discriminates between μ- and δ-opioid receptors by recognition of a difference in only one amino acid residue located in the first extracellular loop, that is, Lys^{108} in δ-opioid receptor and Asn^{127} in μ-opioid receptor

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(Minami et al., 1996). Furthermore, our investigation with μ/κ chimeric receptors revealed that DAMGO discriminates between μ- and κ-opioid receptors at the region around the third extracellular loop (Minami et al., 1995), different from the case of the discrimination between μ- and δ-opioid receptors. In the present study, by construction of further μ/κ chimeric receptors, we showed that at least two distinct regions around the third extracellular loop are necessary for the μ-opioid receptor-selective binding of DAMGO. Site-directed mutagenesis in κ-opioid receptor, followed by expression in COS-7 (CV-1 Origin, SV40) cells and binding experiments, revealed that four amino acid residues located in the third extracellular loop are the determinants for discrimination between μ- and κ-opioid receptors by DAMGO.

2. Materials and methods

2.1. Materials

The rat μ- and κ-opioid receptor cDNAs were cloned as previously described (Minami et al., 1994, 1993). [Tyrosyl-3,5-^{3}H(N)]DAMGO (50.5 Ci/mmol), [phenyl-3,4-^{3}H]U69,593 ((+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl] benzeneacetamide) (47.5 Ci/mmol) and (−)-[9-^{3}H(N)]bremazocine (29.8 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, MA, USA). DAMGO, dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) and CTOP (D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2) were purchased from Peninsula Laboratories (Belmont, CA, USA). Met-enkephalin was from Peptide Institute (Minoh, Japan). Morphine hydrochloride was from Takeda Chemical Industries (Osaka, Japan). Naloxone hydrochloride was from Sigma (St. Louis, MO, USA).

2.2. Construction of chimeric receptors and site-directed mutagenesis

The chimeric receptors between μ- and κ-opioid receptors were constructed by using the intrinsic (*Ban*II site in μ- and κ-opioid receptor cDNAs, *Pvu*II-1 site in κ-opioid receptor cDNA, and *Pvu*II-2 site in μ-opioid receptor cDNA) and introduced (*Pvu*II-1 site in μ-opioid receptor cDNA, and *Pvu*II-2 site in κ-opioid receptor) restriction enzyme recognition sites which exist at the corresponding positions of both receptor cDNAs (Fig. 1). The appropriate restriction enzyme fragments of μ- and κ-opioid receptor cDNAs were ligated and cloned into the multiple cloning site of the pcDNA3 expression vector (Invitrogen, Carlsbad, CA, USA). In vitro site-directed mutagenesis was carried out using a Transformer™ Site-Directed Mutagenesis Kit (2nd version) (CLONTECH Laboratories, Palo Alto, CA, USA) as described (Minami et al., 1996). For the site-directed mutagenesis, the coding region of rat κ-opioid receptor cDNA was subcloned into the pBluescript II (Stratagene, San Diego, CA, USA). The fragment containing the full-length coding region of each mutant κ-opioid receptor cDNA was subcloned into the pcDNA3 vector. The sequence of each construct was confirmed by sequencing analysis using ABI PRISM dye terminator cycle-sequencing ready-reaction kit (Perkin Elmer, Foster City, CA, USA).

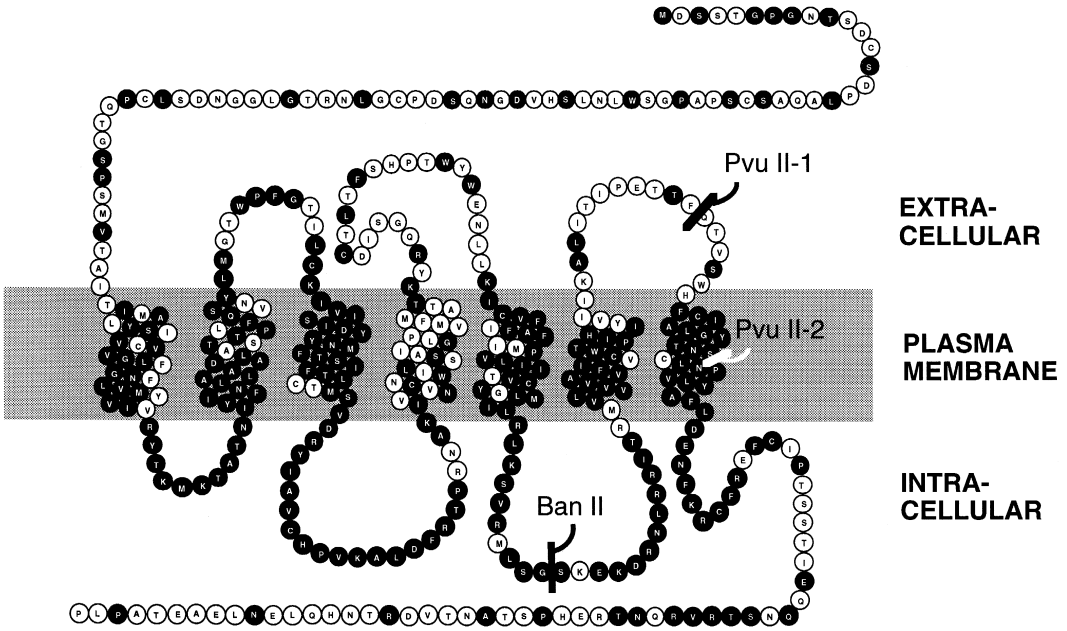

Fig. 1. Proposed transmembrane topology of the rat μ-opioid receptor. Solid circles indicate the amino acid residues conserved between the μ- and κ-opioid receptors. Noted are restriction enzyme sites used to construct chimeric receptors.

2.3. Expression of wild type, chimeric and mutant receptors and radioligand binding assay

The COS-7 cells were grown in Dulbecco's modified Eagle Medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37°C. For transient expression of the wild type, chimeric and mutant receptors, each plasmid cDNA (2–5 μg/ml) was transfected to COS-7 cells by the DEAE–dextran method (Selden, 1987). After cultivation for 65 h, the cells were harvested and homogenized in 50 mM Tris (pH 7.4) containing 10 mM $MgCl_2$ and 1 mM EDTA. After centrifugation for 20 min at $30\,000 \times g$, the pellet was resuspended in the same buffer and used as membrane preparations in the radioligand binding assay. Saturation binding experiments were performed with [^{3}H]DAMGO, a μ-opioid receptor-selective ligand, and [^{3}H]bremazocine, a non-selective opioid ligand. The membrane preparations were incubated with various concentrations of [^{3}H]DAMGO or [^{3}H]bremazocine at 25°C for 1 h. Non-specific binding was determined in the presence of 10 μM unlabelled DAMGO or bremazocine, respectively. The incubations were terminated by the addition of ice-cold buffer, immediately followed by rapid filtration over Whatman GF/C glass fiber filters which were pretreated with 0.3% polyethyleneimine, and the radioactivity on each filter was measured by liquid scintillation counting. K_d values of [^{3}H]DAMGO and [^{3}H]bremazocine for each receptor were obtained by Scatchard analyses of the data from saturation-binding experiments.

The Chinese hamster ovary (CHO) cells were grown in F-12 medium, supplemented with 10% fetal calf serum in 5% CO_2 at 37°C. The cells were transfected by the lipofectin method, with the plasmid containing the wild type μ- or κ-opioid receptor cDNA or a mutant receptor cDNA. A single clone expressing each receptor was selected by cultivation in the presence of 500 μg/ml G418 (GIBCO BRL, Gaithersburg, NY, USA) followed by a binding assay with the tritiated ligand. The expression of the mRNA for each receptor was confirmed by Northern blot analysis. The affinities of various μ-opioid ligands for the wild type μ- and κ-opioid receptors, and for a mutant receptor, were examined using the CHO cells stably expressing each of these receptors. Preparation of cell membranes and radioligand binding experiments were carried out, as well, as in the experiments with COS-7 cells. Saturation binding experiments for Scatchard analyses were performed with various concentrations of [^{3}H]DAMGO or [^{3}H]U69,593, a κ-opioid receptor-selective ligand. For competitive binding assay, the cell membranes were incubated with [^{3}H]DAMGO or [^{3}H]U69,593 and various concentrations of unlabelled μ-opioid receptor ligands. 1 nM [^{3}H]DAMGO and 2 nM [^{3}H]U69,593 were used for wild type μ- and κ-opioid receptors, respectively, and 6 nM [^{3}H]DAMGO and 4 nM [^{3}H]U69,593 for the mutant receptor. K_d values of the radiolabelled ligands were obtained by Scatchard analyses. K_i values were calculated from the IC_{50} values obtained from the competitive binding assay, in accordance with the equation, $K_i = IC_{50}/(1 + [\text{radiolabelled ligand}]/K_d)$ (Cheng and Prusoff, 1973), where IC_{50} is the concentration of unlabelled ligand producing a 50% inhibition of the specific binding of radiolabelled ligand. The results of binding assays are presented as the mean ± S.E. of three to five separate experiments.

2.4. cAMP assay

For cAMP assay, 1×10^5 CHO cells expressing the wild type μ- or κ-opioid receptor or the mutant receptor were seeded in each well of a 24-well plate. After cultivation for 24 h, the cells were washed with HEPES-buffered saline (140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose and 15 mM HEPES, pH 7.4) and incubated in 0.45 ml HEPES-buffered saline containing 1 mM 3-isobutyl-1-methylxanthine for 10 min at 37°C. Stimulation was started by addition of 50 μl of HEPES-buffered saline containing 100 μM forskolin (final concentration = 10 μM) and 1 mM 3-isobutyl-1-methylxanthine in the presence of various concentrations of DAMGO. After incubation for 10 min, the stimulation was terminated by the addition of 0.5 ml of ice-cold 10% trichloroacetic acid to each well. Then, the plate was chilled on ice for 15 min. After being frozen and thawed, the trichloroacetic acid solution was transferred to a microtube and centrifuged at 2000 rpm for 5 min at 4°C. 0.5 ml of the supernatant was transferred to another tube, and then added with 1 ml of chilled water-saturated diethyl ether and voltexed for 20 s. After aspiration of the ether phase, the same procedure for ether extraction was repeated once more. The sample was incubated for 20 min at 40°C to evaporate residual ether, and then 5 μl of each sample was taken for cAMP assay. The concentration of cAMP was measured using a radioimmunoassay kit (Amersham, Buckinghamshire, UK). IC_{50} values were determined as the concentration of DAMGO, producing 50% of the maximal inhibition in cAMP accumulation. The results of cAMP assays are presented as the mean ± S.E.M. of three separate experiments.

3. Results

3.1. Determination of the regions involved in discrimination between μ- and κ-opioid receptors by DAMGO

The affinities of the wild type μ- and κ-opioid receptors and chimeric receptors expressed in COS-7 cells for DAMGO were estimated by saturation binding assay using [^{3}H]DAMGO as a radiolabelled ligand (Table 1). Although the μ-opioid receptor bound [^{3}H]DAMGO with high affinity ($K_d = 3.5 \pm 0.8$ nM), the κ-opioid receptor poorly bound [^{3}H]DAMGO, and the K_d value could not be determined. The chimeric receptor κ(*Ban*II)μ, in which

Table 1
K_d values of [^{3}H]DAMGO and [^{3}H]bremazocine for the wild type μ- and κ-opioid receptors and μ/κ chimeric receptors

	μ	κ	μ(Ban II)κ	κ(Ban II)μ
[^{3}H]DAMGO	3.5 ± 0.8	N.D.	N.D.	4.4 ± 1.2
[^{3}H]bremazocine	2.9 ± 0.2	3.1 ± 0.4	3.9 ± 0.5	7.6 ± 0.4

	μ(Pvu II-2)κ	κ(Pvu II-2)μ	μ(Pvu II-1)κ	κ(Pvu II-1)μ
[^{3}H]DAMGO	1.1 ± 0.2	N.D.	N.D.	N.D.
[^{3}H]bremazocine	7.9 ± 0.6	6.9 ± 0.9	3.4 ± 0.5	9.9 ± 1.1

K_d (nM) values of [^{3}H]DAMGO and [^{3}H]bremazocine were determined by Scatchard analyses of the data from saturation binding experiments. Values are the mean ± S.E. of three to five experiments. ND means impossible to determine K_d values due to the very low affinity for [^{3}H]DAMGO.

the region from the *Ban*II site in the third intracellular loop (Fig. 1) to the carboxyl-terminus of κ-opioid receptor was replaced with the corresponding region of μ-opioid receptor, exhibited high affinity for DAMGO ($K_d = 4.4 \pm 1.2$ nM), equivalent to that of the wild type μ-opioid receptor, while the reciprocal chimeric receptor μ(*Ban*II)κ poorly bound [^{3}H]DAMGO and the K_d value could not be determined as in the case of the wild type κ-opioid receptor. The chimeric receptor μ(*Pvu*II-2)κ, in which the region from the *Pvu*II site in the seventh transmembrane domain (*Pvu*II-2 site, Fig. 1) to the carboxyl-terminus of μ-opioid receptor was replaced with the corresponding region of κ-opioid receptor, exhibited high affinity for DAMGO ($K_d = 1.1 \pm 0.15$ nM) equivalent to the wild type μ-opioid receptor. On the other hand, the reciprocal chimeric receptor κ(*Pvu*II-2)μ did not show any specific binding to [^{3}H]DAMGO. Because all chimeric receptors were verified to bind [^{3}H]bremazocine, a non-selective opioid ligand with high affinity, the lack of [^{3}H]DAMGO binding to μ(*Ban*II)κ and κ(*Pvu*II-2)μ was not due to the low expression of these chimeric receptors nor the overall alteration in tertiary structures of the receptors. To better identify the region for the discrimination by DAMGO, we constructed further two chimeric receptors, μ(*Pvu*II-1)κ and κ(*Pvu*II-1)μ, using another *Pvu*II site in the third extracellular loop (*Pvu*II-1 site, Fig. 1). These chimeric receptors did not bind [^{3}H]DAMGO, although both the receptors showed high affinity for [^{3}H]bremazocine (Table 1).

3.2. Identification of amino acid residue(s) involved in discrimination between μ- and κ-opioid receptors by DAMGO in the region between BanII and PvuII-1 sites

To identify the amino acid residue(s) involved in discrimination between μ- and κ-opioid receptors by DAMGO in the region between *Ban*II and *Pvu*II-1 sites, the amino acid residue in the region of the chimeric receptor κ(*Pvu*II-1)μ was mutated to the amino acid found at the corresponding position of μ-opioid receptor. Glu^{297} of κ(*Pvu*II-1)μ, which is located in the boundary region between the sixth transmembrane domain and third extracellular loop (Fig. 2), was replaced with Lys. The resultant mutant receptor κ(*Pvu*II-1)μ(E297K) (Fig. 3, top), different from the parent chimeric receptor κ(*Pvu*II-1)μ, showed high affinity for DAMGO ($K_d = 11 \pm 2.7$ nM; Fig. 3, middle).

3.3. Identification of amino acid residue(s) involved in discrimination between μ- and κ-opioid receptors by DAMGO in the region between PvuII-1 and PvuII- 2 sites

In the region between *Pvu*II-1 and *Pvu*II-2 sites, there are only five amino acid residues that differ between μ-

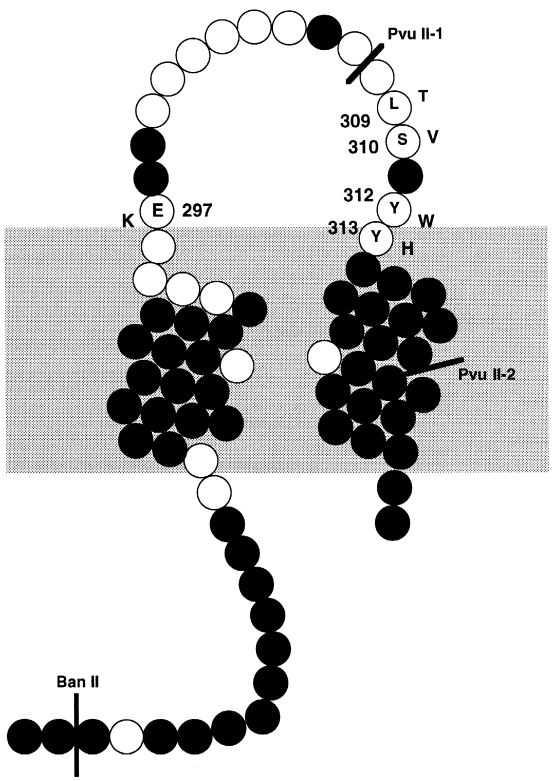

Fig. 2. Structure of the region between *Ban*II and *Pvu*II-2 sites of the κ-opioid receptor. (●) Residues conserved between the μ- and κ-opioid receptors. (○) Residues specific for the κ-opioid receptor. The targets of site-directed mutagenesis are noted by indicating the sort of amino acid by a one-letter symbol with each residue number, and the amino acids found at the corresponding position of the μ-opioid receptor are indicated outside the circle.

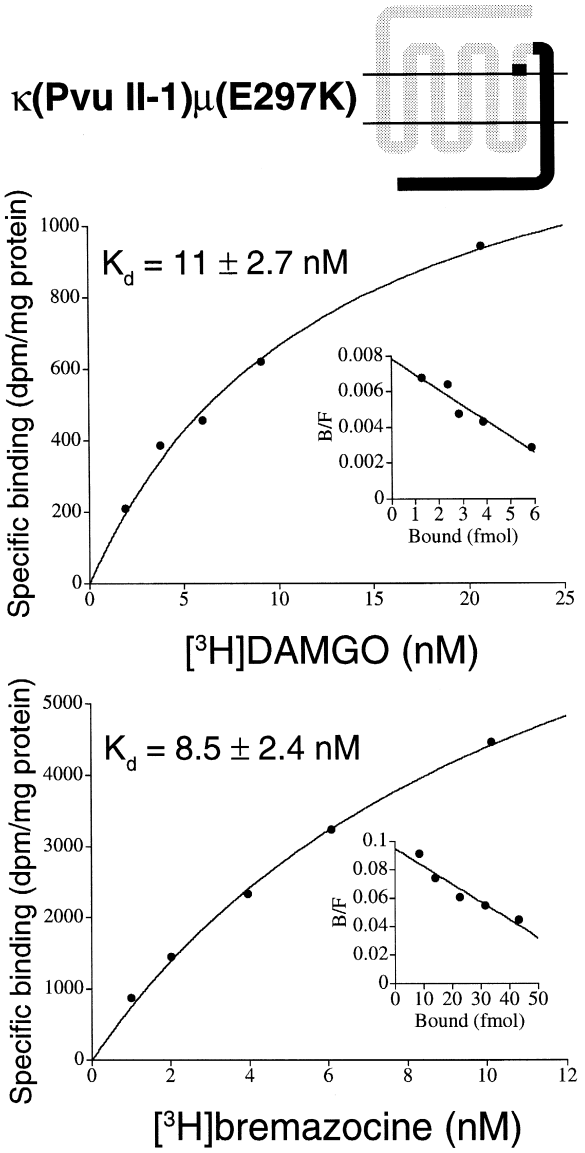

Fig. 3. Top: a schema of the mutant receptor $\kappa(Pvu\text{II-}1)\mu(\text{E297K})$, in which the gray and black regions are derived from κ- and μ-opioid receptors, respectively. Middle and bottom: saturation binding of [^{3}H]DAMGO and [^{3}H]bremazocine to the membrane preparation of COS-7 cells expressing the mutant receptor $\kappa(Pvu\text{II-}1)\mu(\text{E297K})$. Insets show the Scatchard analyses of [^{3}H]DAMGO and [^{3}H]bremazocine bindings.

and κ-opioid receptors (Fig. 2). To determine the amino acid residue(s) involved in discrimination between μ- and κ-opioid receptors by DAMGO in this region, these amino acid residues of the κ-opioid receptor were replaced with the amino acids found at the corresponding position of μ-opioid receptor, in addition to the replacement of Glu^{297} with Lys. The mutant receptor κ(E297K), in which Glu^{297} of the wild type κ-opioid receptor mutated to Lys, did not bind [^{3}H]DAMGO (Table 2). Firstly, the amino acid residues between *Pvu*II-1 and *Pvu*II-2 sites of κ(E297K) were accumulatively mutated to the amino acids found at the corresponding position of μ-opioid receptor, starting from Tyr^{313}, which was located at the boundary region between the third extracellular loop and seventh transmembrane domain, in the direction of the amino-terminus (Table 2, left column). The mutant receptors κ(E297K + Y313H) and κ(E297K + Y313H + Y312W) did not bind [^{3}H]DAMGO. Different from these receptors, the mutant receptor κ(E297K + Y313H + Y312W + S310V) bound [^{3}H]DAMGO with high affinity ($K_d = 8.7 \pm 1.2$ nM). The mutant receptor κ(E297K + Y313H + Y312W + S310V + L309T) also bound [^{3}H]DAMGO with high affinity ($K_d = 17 \pm 1.7$ nM), but the affinity did not increase as compared with that of κ(E297K + Y313H + Y312W + S310V).

Secondly, to examine whether the mutations at all of these three amino acid residues, that is, Y313H, Y312W and S310V, are indispensable for high-affinity binding of DAMGO, we constructed further four mutant receptors, κ(E297K + S310V), κ(E297K + Y312W), κ(E297K + Y312W + S310V) and κ(E297K + Y313H + S310V) (Table 2, right column). Among these receptors, κ(E297K + S310V), κ(E297K + Y312W) and κ(E297K + Y312W + S310V) did not bind [^{3}H]DAMGO. Although κ(E297K + Y313H + S310V) bound to [^{3}H]DAMGO with relatively high affinity ($K_d = 45 \pm 9.5$ nM), this affinity was about five times lower than that of κ(E297K + Y313H + Y312W + S310V).

3.4. Coupling of mutant κ-opioid receptor to adenylate cyclase

To evaluate the agonistic activity of DAMGO in the mutant receptor, the effects of DAMGO on forskolin-induced cyclic AMP accumulation via the wild type μ- and κ-opioid receptors and the mutant receptor κ(E297K + Y313H + Y312W + S310V) expressed in CHO cells were examined (Table 3). In the wild type μ-opioid receptor, DAMGO inhibited the cAMP accumulation and the IC_{50} value was 3.0 ± 0.6 nM. On the other hand, in the wild type κ-opioid receptor, the potency of DAMGO was very weak and the IC_{50} value was > 3000 nM. DAMGO efficiently inhibited the cAMP accumulation via the mutant receptor κ(E297K + Y313H + Y312W + S310V) and the IC_{50} value was 36 ± 11 nM.

3.5. Affinity of various μ-opioid receptor ligands for κ(E297K + Y313H + Y312W + S310V) mutant receptor

The affinities of various μ-opioid receptor ligands for wild type μ- and κ-opioid receptors and κ(E297K + Y313H + Y312W + S310V) mutant receptor were examined by competitive binding assay (Table 4). [^{3}H]DAMGO and [^{3}H]U69,593 were used as radiolabelled ligands for the μ- and κ-opioid receptors, respectively, and both the tritiated ligands for the mutant receptor. The mutant receptor κ(E297K + Y313H + Y312W + S310V) expressed in CHO cells, as well as that in COS-7 cells, bound

Table 2
K_d values of [^{3}H]DAMGO and [^{3}H]bremazocine for the mutant κ-opioid receptors

		K_d (nM)	
		[^{3}H]DAMGO	[^{3}H]bremazocine
κ(E297K)		N.D.	3.9 ± 0.3
κ(E297K+Y313H)		N.D.	8.9 ± 1.5
κ(E297K+Y313H +Y312W)		N.D.	2.1 ± 0.9
κ(E297K+Y313H +Y312W+S310V)		8.7 ± 1.2	6.3 ± 0.5
κ(E297K+Y313H +Y312W+S310V +L309T)		17 ± 1.7	5.0 ± 1.7
κ(E297K+S310V)		N.D.	5.0 ± 0.8
κ(E297K+Y312W)		N.D.	2.6 ± 0.4
κ(E297K+Y312W +S310V)		N.D.	5.6 ± 0.4
κ(E297K+Y313H +S310V)		45 ± 9.5	12 ± 0.8

K_d values of [^{3}H]DAMGO and [^{3}H]bremazocine were determined by Scatchard analyses of the data from saturation binding experiments. Values are the mean ± S.E. of three to five experiments. ND means impossible to determine K_d values due to the very low affinity for [^{3}H]DAMGO. The structure around the third extracellular loop is illustrated. Black circles indicate the amino acid residues conserved between the μ- and κ-opioid receptors. Gray circles indicate the amino acid residues mutated from κ- to μ-type. Black bar indicates *Pvu*II-1 site.

[^{3}H]DAMGO with high affinity ($K_d = 8.9 \pm 1.5$ nM). The mutant receptor also bound [^{3}H]U69,593 with high affinity ($K_d = 7.5 \pm 0.7$ nM). We used two radiolabelled ligands, [^{3}H]DAMGO and [^{3}H]U69,593, for the mutant receptor, because Hjorth et al. (1996) have reported the case that there are considerable discrepancies between the K_i values determined by the competitive binding assays with two different radiolabelled ligands in the mutant κ-opioid receptors. However, each μ-opioid receptor ligand examined in the present study displaced the binding of [^{3}H]DAMGO and [^{3}H]U69,593 to the mutant receptor with similar K_i values.

Table 3
Agonistic activity of DAMGO in the wid-type μ- and κ-opioid receptors and the mutant receptor

	IC_{50} (nM)		
	μ	κ(E297K + Y313H + Y312W + S310V)	κ
DAMGO	3.0 ± 0.6	36 ± 11	> 3000

Effects of DAMGO of forskolin-stimulated cAMP accumulation in the CHO cells expressing the wild-type μ- and κ-opioid receptors and the mutant receptor were evaluated by IC_{50} values. Data are expressed as the mean ± S.E.M. of three experiments.

The peptidic μ-opioid receptor-selective agonist dermorphin, peptidic μ-opioid receptor-selective antagonist, CTOP, and endogenous opioid peptide, Met-enkephalin, bound to the μ-opioid receptor with high affinity ($K_i = 1.5 \pm 0.3$, 40 ± 8 and 3.0 ± 0.3 nM, respectively), but not to the κ-opioid receptor ($K_i = > 3000$, > 3000 and 750 ± 220 nM, respectively). The replacement of Glu^{297}, Tyr^{313}, Tyr^{312} and Ser^{310} of the κ-opioid receptor to Lys, His, Trp and Val, respectively, did not increase the affinity for dermorphin, CTOP and Met-enkephalin ($K_i = > 3000$, > 3000 and 340 ± 70 nM, respectively), in contrast with the case of DAMGO. Narcotic analgesics, such as morphine, methadone and fentanyl, and an opioid antagonist, naloxone, preferentially bound to the μ-opioid receptor ($K_i = 1.9 \pm 0.5$, 13 ± 1, 1.9 ± 0.6 and 4.1 ± 0.3 nM, re-

Table 4
Affinity of μ-opioid ligands in the wild type μ- and κ-opioid receptors and the κ(E297K + Y313H + Y312W + S310V)mutant receptor

	K_i (nM)			
	μ	κ(E297K + Y313H + Y312W + S310V)		κ
	[^{3}H]DAMGO; $K_d = 1.1 \pm 0.2$	[^{3}H]DAMGO; $K_d = 8.9 \pm 1.5$	[^{3}H]U69,593; $K_d = 7.5 \pm 0.7$	[^{3}H]U69,593; $K_d = 1.9 \pm 0.1$
Peptidic ligands				
DAMGO	0.87 ± 0.01	8.1 ± 2.6	16 ± 7	460 ± 100
Dermorphin	1.5 ± 0.3	> 3000	> 3000	> 3000
CTOP	40 ± 8	> 3000	> 3000	> 3000
Met-enkephalin	3.0 ± 0.3	340 ± 70	490 ± 120	750 ± 220
Nonpeptidic ligands				
Morphine	1.9 ± 0.5	190 ± 40	150 ± 30	130 ± 10
DL-Methadone	13 ± 1	1300 ± 300	> 3000	> 3000
Fentanyl	1.9 ± 0.6	330 ± 120	350 ± 110	270 ± 50
Naloxone	4.1 ± 0.3	31 ± 8	41 ± 15	19 ± 3

K_i values were determined by displacement of [^{3}H]DAMGO or [^{3}H]U69,593 binding with unlabelled μ-opioid ligands, followed by calculation in accordance with $K_i = IC_{50}/(1 + [\text{radiolabelled ligand}]/K_d)$. Data are expressed as the mean ± S.E. of three to five experiments.

spectively) compared with the κ-opioid receptor ($K_i = 130 \pm 10$, > 3000, 270 ± 50 and 19 ± 3 nM, respectively). The replacement of the four amino acid residues of κ-opioid receptor with the μ-type amino acids failed to increase the affinities for these nonpeptidic ligands ($K_i = 190 \pm 40$, 1300 ± 300, 330 ± 120 and 31 ± 8 nM, respectively).

4. Discussion

In the previous study, we constructed four types of μ/κ chimeric receptors and revealed that DAMGO discriminated between μ- and κ-opioid receptors at the region around the third extracellular loop (Minami et al., 1995). Similar results were also reported by Xue et al. (1995). In the present study, we first constructed four types of chimeric receptors. The chimeric receptors $\kappa(BanII)\mu$ and $\mu(PvuII\text{-}2)\kappa$ exhibited high affinities for DAMGO which were equivalent to that of the wild type μ-opioid receptor, while the chimeric receptors $\mu(BanII)\kappa$ and $\kappa(PvuII\text{-}2)\mu$ poorly bound [^{3}H]DAMGO, and the K_d values could not be determined as in the case of the wild type κ-opioid receptor. These results suggest that the region between *Ban*II site and *Pvu*II-2 site is responsible for discrimination between μ- and κ-opioid receptors by DAMGO. To identify the more restricted region for the discrimination, we constructed further two chimeric receptors $\mu(PvuII\text{-}1)\kappa$ and $\kappa(PvuII\text{-}1)\mu$. Attempts to examine the [^{3}H]DAMGO binding to these chimeric receptors met the unexpected results that neither $\mu(PvuII\text{-}1)\kappa$ nor $\kappa(PvuII\text{-}1)\mu$ bound [^{3}H]DAMGO, although both the receptors bound [^{3}H]bremazocine with high affinity. These results indicate that both regions around the third extracellular loop, that is, the regions between *Ban*II and *Pvu*II-1 sites and between *Pvu*II-1 and *Pvu*II-2 sites, are critical for the discrimination between μ- and κ-opioid receptors by DAMGO.

To identify the amino acid residue(s) critical for discrimination between μ- and κ-opioid receptors by DAMGO in the region between *Ban*II and *Pvu*II-1 sites, we introduced site-directed mutation to this region of the chimeric receptor $\kappa(PvuII\text{-}1)\mu$, in which another critical region between *Pvu*II-1 and *Pvu*II-2 sites was derived from the μ-opioid receptor. In the region between *Ban*II and *Pvu*II-1 sites, 16 amino acid residues differ between μ-and κ-opioid receptors. Among these residues, we first mutated Glu^{297} to Lys to create the mutant receptor $\kappa(PvuII\text{-}1)\mu$(E297K). We focused on this residue located in the boundary region between the sixth transmembrane domain and third extracellular loop because of the reports that the amino acid residues located at this position of κ- and δ-opioid receptors are important for the type-selective binding of norbinaltorphimine, a κ-opioid receptor-selective antagonist, and [D-Pen^2, D-Pen^5]enkephalin (DPDPE), a δ-opioid receptor-selective agonist, respectively (Hjorth et al., 1995; Valiquette et al., 1996; Pepin et al., 1997). The $\kappa(PvuII\text{-}1)\mu$(E297K) bound [^{3}H]DAMGO with high affinity, suggesting that, in the region between *Ban*II and *Pvu*II-1 sites, the difference between Glu^{297} of κ-opioid receptor and Lys^{303} of μ-opioid receptor is critical for the discrimination by DAMGO between these receptors. We cannot exclude the possibility that a difference in other amino acid residues in this region contributes to the discrimination by DAMGO. However, any such contribution is thought to be of minor importance, because the affinity of the mutant receptor $\kappa(PvuII\text{-}1)\mu$(E297K) for DAMGO was comparable to that of $\kappa(BanII)\mu$.

Next, we elucidated the amino acid residue(s) which was critical for the discrimination in the region between *Pvu*II-1 and *Pvu*II-2 sites, where only five amino acid

residues differ between μ- and κ-opioid receptors. In addition to the mutation of Glu^{297} to Lys, we mutated these amino acid residues of κ-opioid receptor to μ-type amino acids step by step, and demonstrated the possibility that the difference between Ser^{310}, Tyr^{312} and Tyr^{313} of κ-opioid receptor and Val^{316}, Trp^{318} and His^{319} of μ-opioid receptor are critical for the discrimination by DAMGO. Further study with four more types of mutant receptors revealed that replacement of any one or two of these three residues of the κ-opioid receptor to μ-type was not sufficient to increase the affinity for DAMGO to as high as that of $\kappa(PvuII\text{-}1)\mu$(E297K). Although the affinity of the mutant receptor κ(E297K + Y313H + S310V) was relatively high, it was about five times lower than that of κ(E297K + Y313H + Y312W + S310V). These results demonstrated that, in the region between *Pvu*II-1 and *Pvu*II-2 sites, the difference in all of these three amino acid residues, that is, the difference between Ser^{310}, Tyr^{312} and Tyr^{313} of κ-opioid receptor and Val^{316}, Trp^{318} and His^{319} of μ-opioid receptor, are involved in the discrimination between these receptors by DAMGO.

Consequently, DAMGO discriminated between μ- and κ-opioid receptors by recognition of the difference between Glu^{297}, Ser^{310}, Tyr^{312} and Tyr^{313} of κ-opioid receptor and Lys^{303}, Val^{316}, Trp^{318} and His^{319} of μ-opioid receptor. Replacement of these four amino acid residues of κ-opioid receptor to the μ-type remarkably increased not only the affinity for DAMGO but also the potency to transduce the agonistic activity of DAMGO. The IC_{50} value of DAMGO in the mutant receptor κ(E297K + Y313H + Y312W + S310V) was >80-fold smaller than that in the wild type κ-opioid receptor. We have previously reported that the difference between Lys^{108} of δ-opioid receptor and Asn^{127} of μ-opioid receptor, which is located in the first extracellular loop, plays an important role in discrimination between μ- and δ-opioid receptors by DAMGO (Minami et al., 1996). On the other hand, the present result shows that DAMGO discriminates between μ- and κ-opioid receptors at four amino acid residues located in the third extracellular loop. These findings suggest that for DAMGO, the mechanism for the discrimination between μ- and κ-opioid receptors is different from that between μ- and δ-opioid receptors. This idea is supported by the results from the competitive-binding experiments to examine the affinity of several μ-opioid receptor ligands for the mutant receptors. The replacement of Glu^{297}, Ser^{310}, Tyr^{312} and Tyr^{313} of κ-opioid receptor to μ-type amino acids markedly increased the affinity for DAMGO, but not for any other μ-opioid receptor ligand examined in the present study, that is, dermorphin, CTOP,

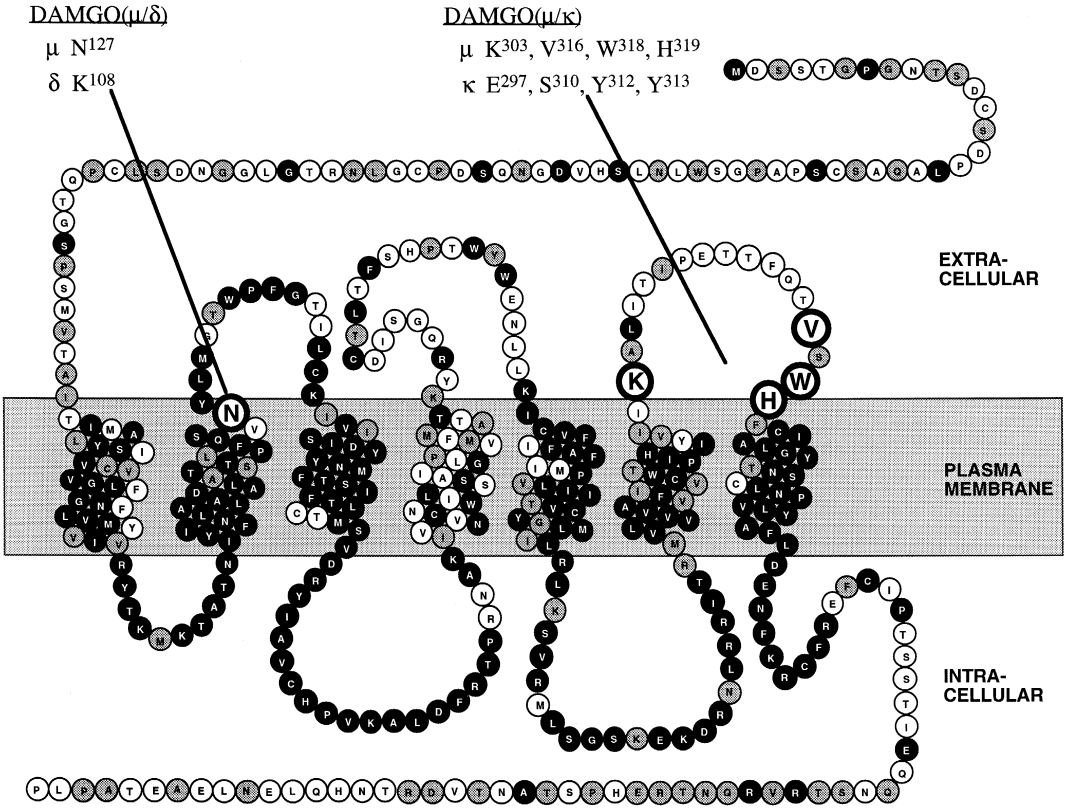

Fig. 4. Proposed transmembrane topology of the rat μ-opioid receptor. Amino acid residues of μ-opioid receptor conserved in both δ- and κ-opioid receptors, in either δ- or κ-opioid receptor, and in neither δ- nor κ-opioid receptor, are shown by black, gray and white circles, respectively. Larger circles with a solid face are the amino acid residues which are determinants for the discrimination between μ- and δ-opioid receptors or μ- and κ-opioid receptors by DAMGO. Such amino acid residues are found in the boundary region between the extracellular loops and transmembrane domains.

morphine, methadone, fentanyl and naloxone. These results indicate that the mechanism for the discrimination between μ- and κ-opioid receptors by DAMGO is specific for DAMGO. In contrast, the mechanism for the discrimination between μ- and δ-opioid receptors seems to be common to several μ-opioid receptor ligands. The replacement of Lys^{108} of δ-opioid receptor to Asn markedly increased the affinity for, not only DAMGO, but also other peptidic ligands such as dermorphin and CTOP, and slightly but significantly increased the affinity for nonpeptidic ligands such as morphine, methadone and naloxone (Minami et al., 1996).

It is absorbing interest and is now being elucidated in our laboratory how DAMGO interacts with these determinants; one is the determinant for the discrimination between μ- and κ-opioid receptors, which is located in the third extracellular loop, and the other is that for the discrimination between μ- and δ-opioid receptors, which is located in the first extracellular loop.

5. Conclusions

In the present study, we showed that four amino acid residues functioned as the determinants for discrimination between μ- and κ-opioid receptors by DAMGO; one residue is located in the boundary region between the sixth transmembrane domain and third extracellular loop, and the other three residues are located in the boundary region between the third extracellular loop and seventh transmembrane domain (Fig. 4). As we previously reported, the difference in only one amino acid residue, which is located in the boundary region between the second transmembrane domain and first extracellular loop, is critical for the discrimination between μ- and δ-opioid receptors by DAMGO (Minami et al., 1996). In addition, Hjorth et al. (1995) revealed that the difference between Glu^{297} of κ-opioid receptor and Lys^{303} of μ-opioid receptor is also critical for the distinction between these receptors by norbinaltorphimine. Furthermore, Valiquette et al. (1996) and Pepin et al. (1997) showed that Trp^{284}, Val^{296}, Val^{297} and Leu^{300}, which are located in the boundary region between the third extracellular loop and sixth and seventh transmembrane domains of δ-opioid receptor, are crucial for the δ-opioid receptor-selective binding of SNC-80 and DPDPE. Similar findings are also reported in other G protein-coupled receptor families, such as neurokinin (Gether et al., 1994), somatostatin (Kaupmann et al., 1995), α-adrenergic (Zhao et al., 1996) and endothelin (Krystek et al., 1994) receptor families. For instance, Glu^{193}, Lys^{194} and Tyr^{272}, which are located in the boundary region between the extracellular loops and transmembrane domains of the NK-1 neurokinin receptor, are critical for the binding of a nonpeptidic NK-1 selective antagonist, CP96,345 (Gether et al., 1994). These findings suggest that only one or few amino acid residue(s) located in the boundary regions between the extracellular loops and transmembrane domains play crucial roles in the discrimination of receptor subtypes by subtype-selective ligands in a considerable number of G protein-coupled receptor families. This notion may be useful in the design of novel subtype-selective ligands for the receptors belonging to G protein-coupled receptor families.

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