0026-895X/96/051413-10$3.00/0

MOLECULAR PHARMACOLOGY, 50:1413–1422 (1996).

A Single Residue, Lys108, of the δ-Opioid Receptor Prevents the μ-Opioid-Selective Ligand [D-Ala^2,*N*-MePhe^4,Gly-ol^5]Enkephalin from Binding to the δ-Opioid Receptor

MASABUMI MINAMI, TAKAYUKI NAKAGAWA, TAKAHIRO SEKI, TATSUHIRO ONOGI, YASUHIDE AOKI, YOSHIKAZU KATAO, SEISHI KATSUMATA, and MASAMICHI SATOH

Departments of Molecular Pharmacology (M.M., T.N., T.S., Y.A., Y.K., M.S.) and Pharmacology (T.O., S.K.), Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606–01, Japan

Received January 25, 1996; Accepted July 5, 1996

SUMMARY

Previously, we found that replacement of the region around the first extracellular loop of the δ-opioid receptor (OPR) with the corresponding region of the μ-OPR gives the high affinity for [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO), a μ-opioid-selective ligand, to the resultant chimeric receptor, DMDD, suggesting that the difference in the amino acid sequence within this region between the μ- and δ-OPRs is critical for the discrimination between these receptors by DAMGO. In the current study, we carried out systematic replacements of seven nonconserved residues in this region of the δ-OPR with the corresponding amino acid found in the μ-OPR. Among the seven mutant receptors, only one mutant receptor, δK108N, showed high affinity (K_i = 18.68 ± 5.27 nM) for DAMGO, which was comparable to that of the DMDD receptor (K_i = 23.77 ± 4.27 nM) and 75-fold higher than that of the wild-type δ-OPR (K_i = 1405 ± 161 nM). Lys108 in the δ-OPR was systematically replaced with 19 kinds of amino acids other than lysine. Among the resultant mutant receptors, 14 mutants bound DAMGO with K_i values comparable to those of the DMDD receptor, ranging from 4.20 to 43.38 nM. These findings suggest that Lys108 of the δ-OPR prevents DAMGO from binding to the δ-OPR rather than that the asparagine residue at the corresponding position in the μ-OPR is necessary for DAMGO binding. In addition, the replacement of Lys108 of the δ-OPR with asparagine dramatically increased the affinity for other peptidic μ receptor-selective ligands, such as dermorphin and D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2.

Endogenous opioid peptides and opiate drugs like morphine act on the specific receptors to produce various physiological and pharmacological effects, such as analgesia, respiratory depression, euphoria, and modulation of neuroendocrine. The presence of at least three types of OPRs in the nervous system has been established on the basis of differential pharmacological and binding properties (i.e., μ-, δ-, and κ-OPRs) (1). Although all three types of OPRs couple via pertussis toxin-sensitive G proteins to various effectors, including adenylate cyclase (2), Ca^{2+} channels, and K^+ channels (3), and many drugs act on all types, these types can be discriminated using the ligands selective to each type. The molecular basis for the discrimination action of these ligands among three types of OPRs is unknown and of great interest.

After the cloning of the δ-OPR (4, 5), we and several other groups (6–14) cloned the μ- and κ-OPRs. Hydropathy analyses of the deduced amino acid sequences of these clones suggest that these receptors have seven putative transmembrane helices characteristic to G protein-coupled receptors. Comparison across the amino acid sequences of the μ-, δ-, and κ-OPRs revealed ~60% identity among these three receptors. Higher identity was found in the transmembrane regions (73–76% identities) and intracellular regions (64–67% identities). Conversely, extracellular regions are considerably divergent (34–40% identities). It is possible that these divergent sequences are critical for the discrimination among these receptors by type-selective opioid ligands.

Construction of chimeric receptors between closely related receptors has been very useful to examine the receptor domains recognized by type-selective ligands, as it has been for epinephrine (15, 16), acetylcholine (17), dopamine (18), and tachykinin (19, 20) receptors. Recently, using chimeric μ/δ OPRs, we have shown that DAMGO, a μ-opioid-selective ligand, distinguishes between the μ- and δ-OPRs at the region around the first extracellular loop (21). There are only seven different amino acid residues between the μ- and δ-OPRs within this region (Fig. 2, *top*), and it is likely that

This study was supported by Grant-in-Aid from Ministry of Education, Science, Sports and Culture of Japan and by grants from the Japan Health Sciences Foundation and Uehara Memorial Foundation.

ABBREVIATIONS: DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin; DADLE, [D-Ala^2,D-Leu^5]enkephalin; OPR, opioid receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CTOP, D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2.

one or more of these amino acid residues are critical for discrimination between these receptors by DAMGO. In this study, by using a site-directed mutagenesis technique, we found that replacement of only a single amino acid residue, K108N, in the first extracellular loop of the δ-OPR markedly increased the affinity for DAMGO. In addition, the results from systematic replacements of Lys108 with other amino acids suggest that the lysine residue at position 108 in the δ-OPR prevents DAMGO from binding to the δ-OPR rather than that the asparagine residue at the corresponding position in the μ-OPR is necessary for DAMGO binding.

Experimental Procedures

Materials. The rat μ-OPR cDNA was cloned as described previously (10). The rat δ-OPR cDNA was a gift from Dr. K. Fukuda (Department of Anesthesia, Kyoto University Hospital, Kyoto, Japan) (9). The cDNA coding the DMDD chimeric receptor was constructed as described previously (21). Briefly, the region around the first extracellular loop (i.e., the region between the restriction enzyme *Bbs*I and *Afl*III) sites of the δ-OPR was replaced with the corresponding region of the μ-OPR. DADLE, DAMGO, dermorphin, and CTOP were purchased from Peninsula Laboratories (Belmont, CA). Met- and leu-enkephalins were from Peptide Institute (Minoh, Japan). Morphine hydrochloride was from Takeda Chemical Industries (Osaka, Japan). Naloxone hydrochloride was from Sigma Chemical (St. Louis, MO). [*tyrosyl*-3,5-^{3}H(N)]DADLE (33.5 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA).

Mutagenesis of δ-OPR. *In vitro* site-directed mutagenesis was carried out using a Transformer Site-Directed Mutagenesis Kit (version 2) (Clontech Laboratories, Palo Alto, CA). The coding region of rat δ-OPR cDNA was subcloned into the pBluescript II (Stratagene, San Diego, CA). Single-stranded DNA was prepared and simultaneously annealed by two kinds of oligonucleotide primers: one primer for the introduction of the desired mutation and the other primer for the mutation of a restriction site unique to the vector for the purpose of selection. After DNA elongation, ligation, and a primary selection by digestion with an adequate restriction enzyme, the mixture of mutated and unmutated plasmids was transformed into a *mut*S *Escherichia coli* strain defective in mismatch repair. Transformants were pooled, and plasmid DNA was prepared from the mixed bacterial population. The isolated DNA was then subjected to a second selective restriction enzyme digestion. Because the mutated DNA lacked the restriction enzyme recognition site, it was resistant to digestion. However, the parental DNA was sensitive to digestion and was linearized. Therefore, a final transformation using thoroughly digested DNA resulted in highly efficient recovery of the desired mutated plasmids. The sequence of each mutated receptor cDNA was confirmed through sequencing analysis using a Sequenase version 2 DNA sequencing kit (United States Biochemical, Cleveland, OH). Each fragment containing the full-length coding region of the mutated δ-OPR cDNA was subcloned into the *Hind*III/*Apa*I site of the pcDNA3 eukaryotic expression vector (InVitrogen, San Diego, CA).

Expression of wild-type, chimeric, and mutant receptors and binding assay. 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 (22). After cultivation for 65 hr, 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 × g, the pellet was resuspended in the same buffer and used in the radioligand binding assay. Saturation binding experiments for Scatchard analyses were performed with various concentrations of [^{3}H]DADLE. The membrane preparations with the B_{max} values within 200-2500 fmol/mg protein were used for the following competitive binding assay. For displacement studies, 1 nM [^{3}H]DADLE and various concentrations of unlabeled DAMGO were used. Nonspecific binding was determined in the presence of 10 μM unlabeled DADLE. Incubations of cell membranes with [^{3}H]DADLE in the presence or absence of competing opioid ligands were carried out at 25° for 1 hr and terminated by the addition of ice-cold buffer followed immediately by rapid filtration over Whatman GF/C glass-fiber filters that had been pretreated with 0.1% polyethyleneimine. The filters were washed with ice-cold buffer, and the radioactivity on each filter was measured by liquid scintillation counting. K_d and B_{max} values of [^{3}H]DADLE for the wild-type and mutant receptors were obtained by Scatchard analyses of the data from saturation binding experiments. K_i values of DAMGO were obtained from the data of displacement of [^{3}H]DADLE binding with unlabeled DAMGO through calculation in accordance with the equation $K_i = IC_{50}/(1 + [DADLE]/K_d)$ (23), where IC_{50} is the concentration of unlabeled DAMGO producing a 50% inhibition of the specific [^{3}H]DADLE binding.

Establishment of CHO cells expressing wild-type and mutant receptors and cAMP assay. CHO cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum in 5% CO_2 at 37°. The cells were transfected according to the Lipofectin method with the plasmid containing the wild-type μ- or δ-OPR cDNA or mutant receptor cDNA. A single clone expressing each receptor was selected by the cultivation in the presence of 500 μg/ml gentamycin (GIBCO BRL, Gaithersburg, MD) followed by the binding assay with the tritiated ligand. The expression of the mRNA for each receptor was confirmed by Northern blot analysis. For cAMP assay, 1×10^5 cells were seeded onto each well of a 24-well plate. After the cultivation for 24 hr, 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, 15 mM HEPES, pH 7.4) and incubated in 0.45 ml of HEPES-buffered saline containing 1 mM 3-isobutyl-1-methylxanthine for 10 min at 37°. Stimulation was started by the 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 or absence of various concentrations of DAMGO. After an incubation period of 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°. Next, 0.5 ml of the supernatant was transferred to another tube and then added to 1 ml of chilled water-saturated diethyl ether and vortex-mixed for 20 sec. After aspiration of the ether phase, the same procedure for ether extraction was repeated once. The sample was incubated for 20 min at 40° to evaporate residual ether, and then 5 μl of the aliquot 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.

Binding assay for various μ-opioid ligands in wild-type and mutant receptors. The affinities of various μ-opioid ligands for the wild-type μ- and δ-OPRs and for the δK108N mutant receptor were examined using the CHO cells stably expressing each of these receptors. Binding assay was carried out as well as in the case using COS-7 cells. Briefly, the cells were harvested and homogenized in 50 mM Tris containing 10 mM $MgCl_2$ and 1 mM EDTA. After centrifugation, the pellet was resuspended in the same buffer. Saturation binding experiments for Scatchard analyses were performed with various concentrations of [^{3}H]DADLE. In competitive binding assay, the cell membranes were incubated with 1 nM [^{3}H]DADLE and various concentrations of unlabeled μ-opioid ligands at 25° for 1 hr. Nonspecific binding was determined in the presence of 10 μM unlabeled DADLE. The incubations were terminated by the addition of ice-cold buffer immediately followed by rapid filtration over Whatman GF/C glass-fiber filters, and the radioactivity on each filter was measured by liquid scintillation counting. K_d and B_{max} values of [^{3}H]DADLE for the wild-type and mutant receptors were obtained from Scatchard analyses of the data of saturation binding experi-

ments. K_i values were calculated from the IC_{50} values obtained from the competitive binding assay in accordance with the equation $K_i = IC_{50}/(1 + [DADLE]/K_d)$.

Results

Determination of the amino acid residue involved in distinction between μ- and δ-OPRs by DAMGO. The affinity of the wild-type μ- and δ-OPRs and chimeric DMDD receptor for DAMGO was estimated by the competitive binding assay using [^{3}H]DADLE as a radiolabeled ligand. [^{3}H]DADLE bound the μ- and δ-OPRs and DMDD receptor with high affinity. Scatchard analyses of saturation binding experiments revealed that K_d values of [^{3}H]DADLE for the μ- and δ-OPRs and DMDD receptor were 8.67 ± 3.71, 1.91 ± 0.18, and 2.24 ± 0.25 nM, respectively (Table 1). In a competitive binding assay, DAMGO bound the μ-OPR and DMDD receptor with high affinity and displaced the binding of [^{3}H]DADLE with K_i values of 4.37 ± 0.24 and 23.77 ± 4.27 nM, respectively, whereas the affinity of the δ-OPR for DAMGO was very low (K_i = 1405 ± 161 nM) (Fig. 1 and Table 1).

In the region around the first extracellular loop, which was derived from the μ-OPR in the chimeric DMDD receptor, there were only seven different amino acid residues between the μ- and δ-OPRs (Fig. 2, *top*). To determine the amino acid residue(s) involved in the distinction between the μ- and δ-OPRs by DAMGO, these amino acid residues of the δ-OPR were individually replaced with the corresponding amino acid found in the μ-OPR. All of the seven mutant receptors showed high affinity for [^{3}H]DADLE (K_d = 0.83–4.06 nM) (Table 1), indicating that the entire structural integrity of these mutant receptors was not impaired. Among the seven mutant receptors, only one receptor, δK108N, showed high affinity for DAMGO (Fig. 2, *middle* and *bottom*). The K_i value of DAMGO for δK108N was 18.68 ± 5.27 nM, which was comparable to that for the DMDD receptor and 75-fold smaller than that for the wild-type δ-OPR (Table 1). The mutant receptors δA107V, δE112G, δE118T, δL119I, δA123I, and δL125I showed very low affinity for DAMGO. The K_i values of DAMGO for these six mutant receptors were 1198 ± 182, 1432 ± 199, 1077 ± 74, 708 ± 205, 1007 ± 94, and 1158 ± 193 nM, respectively, which were almost the same as those for the wild-type δ-OPR (Table 1).

TABLE 1

Ligand-binding properties of the mutant receptors

Data are expressed as mean ± standard error of three to five experiments. K_d and B_{max} values for DADLE were determined by Scatchard analysis using [^{3}H]DADLE. K_i values for DAMGO were determined by displacement of [^{3}H]DADLE binding with unlabeled DAMGO followed by calculation in accordance with $K_i = IC_{50}/(1 + [DADLE]/K_d)$.

	[^{3}H]DADLE		DAMGO
	B_{max}	K_d	K_i
	fmol/mg protein		*nM*
μ	1712 ± 221	8.67 ± 3.71	4.37 ± 0.24
δ	1319 ± 288	1.91 ± 0.18	1405 ± 161
DMDD	1884 ± 169	2.24 ± 0.25	23.77 ± 4.27[a]
δA107V	909 ± 92	1.12 ± 0.06	1198 ± 182
δK108N	1383 ± 225	4.06 ± 1.26	18.68 ± 5.27[a]
δE112G	1682 ± 220	3.57 ± 0.36	1432 ± 199
δE118T	1460 ± 242	3.33 ± 0.92	1077 ± 74
δL119I	1230 ± 362	2.96 ± 0.53	708 ± 205
δA123I	1576 ± 279	3.10 ± 0.85	1007 ± 94
δL125I	1323 ± 298	0.83 ± 0.11	1158 ± 193

[a] Significantly different from wild-type δ-OPR ($p < 0.01$, Student's t test).

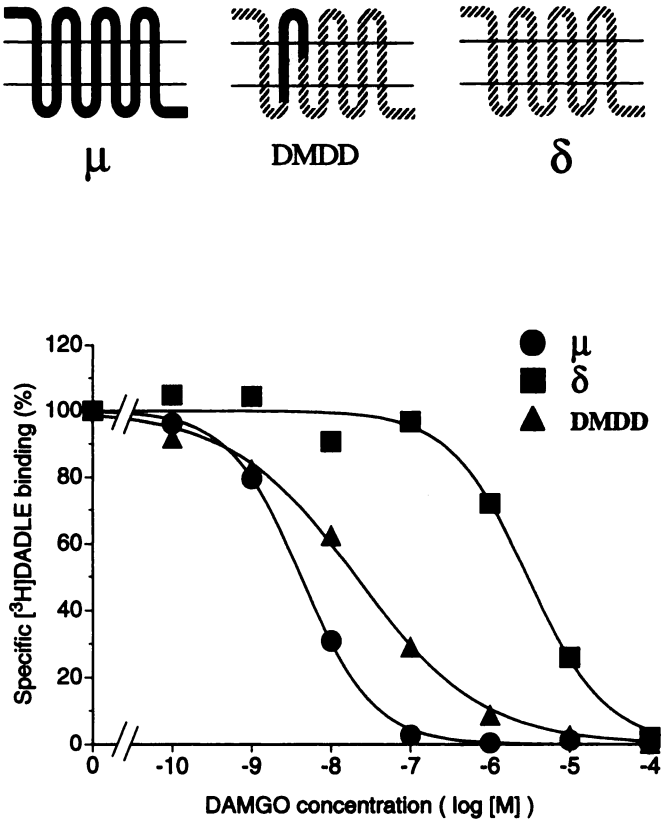

Fig. 1. Displacement of [^{3}H]DADLE binding with DAMGO in the wild-type μ- and δ-OPRs and DMDD chimeric receptor. *Top*, representation of the structures of the wild-type μ- and δ-OPRs and DMDD chimeric receptor. *Bottom*, displacement of the specific binding of [^{3}H]DADLE to the membrane of COS-7 cells expressing the μ- and δ-OPRs and DMDD receptor with unlabeled DAMGO. *Curves*, representative of three or four experiments.

Replacement of Lys108 of δ-OPR. Lys108 in the δ-OPR was systematically replaced with 19 kinds of amino acids other than lysine. All 19 mutant receptors bound [^{3}H]DADLE with K_d values comparable to those of the wild-type μ- and δ-OPRs, ranging from 1.65 to 8.23 nM (Table 2), indicating that the entire structural integrity of these mutant receptors was not impaired.

Replacement of Lys108 with arginine, another basic amino acid, slightly increased the affinity for DAMGO (Fig. 3, *top left*), but the affinity (K_i = 375 ± 72 nM) was 86 and 16-fold lower than those of the wild-type μ-OPR and chimeric DMDD receptor, respectively (Table 2). In the case of the replacement with acidic amino acids (Fig. 3, *middle left*), δK108D bound DAMGO with 65-fold higher affinity than the wild-type δ-OPR, and the K_i value (21.70 ± 3.01 nM) was comparable to that for the DMDD receptor. However, the affinity of δK108E for DAMGO was low, and the K_i value was 155 ± 45 nM. Changes in the free carboxyl group to amide slightly increased the affinity for DAMGO. The K_i values of δK108N and δK108Q for DAMGO were 18.68 ± 5.27 and 74.00 ± 10.64 nM, respectively.

For the amino acids with a polar group (i.e., hydroxyl or sulfhydryl group) in their side chain, the affinities of δK108S, δK108T, and δK108C for DAMGO were comparable to those of the DMDD receptor and 44–47-fold higher than those of

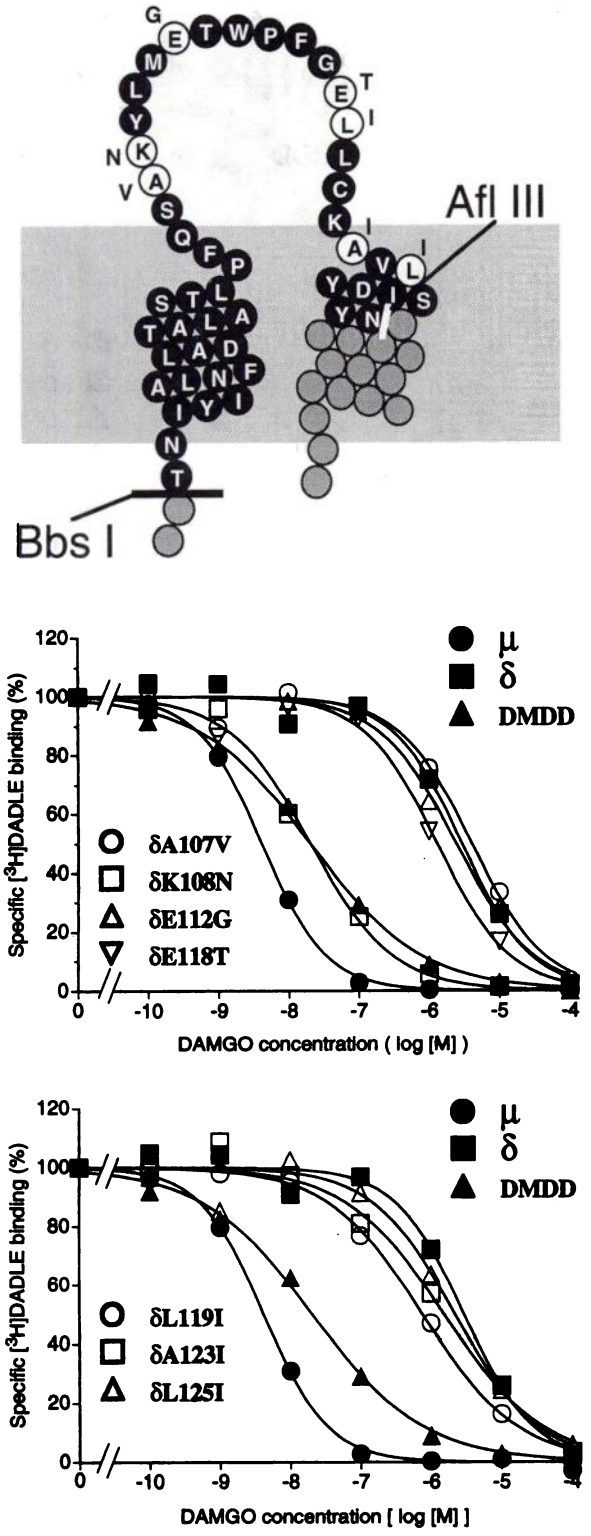

Fig. 2. Identification of amino acid residue(s) of the δ-OPR involved in distinction between the μ- and δ-OPRs by DAMGO. *Top*, structure of the region around the first extracellular loop of the δ-OPR. ●, Residues conserved between the μ- and δ-OPRs. ○, Residues specific for the δ-OPR. The corresponding residues found in the μ-OPR are indicated. The unique restriction enzyme *Bbs*I and *Afl*III sites were used to construct the DMDD chimeric receptor. *Middle* and *bottom*, displacement of the specific binding of [^{3}H]DADLE with DAMGO to the membrane of COS-7 cells expressing the wild-type μ- and δ-OPRs, DMDD receptor, and mutant receptors. *Curves*, representative of three to five experiments.

TABLE 2

Ligand-binding properties of the mutant receptors

Data are expressed as mean ± standard error of three to five experiments. K_d and B_{max} values for DADLE were determined by Scatchard analysis using [^{3}H]DADLE. K_i values for DAMGO were determined by displacement of [^{3}H]DADLE binding with unlabeled DAMGO followed by calculation in accordance with $K_i = IC_{50}/(1 + [DADLE]/K_d)$.

	[^{3}H]DADLE		DAMGO
	B_{max}	K_d	K_i
	fmol/mg protein		*nM*
μ	1712 ± 221	8.67 ± 3.71	4.37 ± 0.24
δ (δK108K)	1319 ± 288	1.91 ± 0.18	1405 ± 161
DMDD	1884 ± 169	2.24 ± 0.25	23.77 ± 4.27[a]
δK108R	501 ± 279	3.22 ± 0.32	375 ± 72[a]
δK108D	474 ± 37	6.27 ± 0.70	21.70 ± 3.01[a]
δK108E	246 ± 163	4.75 ± 0.75	155 ± 45[a]
δK108N	1383 ± 225	4.06 ± 1.26	18.68 ± 5.27[a]
δK108Q	1394 ± 489	4.57 ± 1.73	74.00 ± 10.64[a]
δK108S	577 ± 132	3.68 ± 0.71	30.31 ± 8.45[a]
δK108T	1713 ± 339	6.94 ± 0.60	31.65 ± 3.03[a]
δK108C	469 ± 75	5.09 ± 0.99	29.79 ± 6.59[a]
δK108M	943 ± 129	1.65 ± 0.19	31.91 ± 5.55[a]
δK108A	2144 ± 181	7.06 ± 0.81	43.38 ± 14.53[a]
δK108V	1230 ± 111	8.23 ± 0.28	32.57 ± 6.79[a]
δK108L	486 ± 33	3.12 ± 0.51	7.24 ± 1.88[a]
δK108I	473 ± 92	5.22 ± 1.07	12.25 ± 2.54[a]
δK108P	622 ± 118	2.34 ± 0.51	4.44 ± 0.58[a]
δK108G	554 ± 21	3.31 ± 0.85	146 ± 42[a]
δK108F	915 ± 45	2.36 ± 0.12	4.20 ± 1.23[a]
δK108Y	1093 ± 132	2.08 ± 0.14	11.54 ± 1.27[a]
δK108H	901 ± 372	3.93 ± 1.21	32.30 ± 12.09[a]
δK108W	469 ± 115	6.10 ± 0.83	136 ± 57[a]
μN127K	425 ± 69	4.40 ± 1.00	56.27 ± 12.41[b]

[a] Significantly different from wild-type δ-OPR ($p < 0.01$, Student's t test).
[b] Significantly different from wild-type μ-OPR ($p < 0.05$, Student's t test).

the wild-type δ-OPR (Fig. 3, *bottom left*). The K_i values were 30.31 ± 8.45, 31.65 ± 3.03, and 29.79 ± 6.59 nM, respectively (Table 2). The mutant receptor, which possessed another sulfur-containing amino acid, methionine, at position 108, showed an affinity (K_i = 31.91 ± 5.55 nM) similar to that of δK108C.

Replacements of Lys108 with the amino acids with aliphatic side chains increased the affinity for DAMGO by 32–194-fold (Fig. 3, *top right*). The K_i values of DAMGO for δK108A, δK108V, δK108L, and δK108I were 43.38 ± 14.53, 32.57 ± 6.79, 7.24 ± 1.88, and 12.25 ± 2.54 nM, respectively (Table 2). Among the amino acids with aliphatic side chains, proline requires special mention because its side chain forms a cyclic structure by taking an α-amino group. Replacement of Lys108 with proline markedly increased the affinity for DAMGO (Fig. 3, *middle right*). The K_i value of DAMGO for δK108P (4.44 ± 0.58 nM) was 316-fold smaller than that for the wild-type δ-OPR and comparable to that for the wild-type μ-OPR (Table 2). Glycine has no side chain (or only a single hydrogen atom as its side chain). Replacement of Lys108 with glycine increased the affinity for DAMGO (Fig. 3, *middle right*), but the K_i value of DAMGO for δK108G (146 ± 42 nM) was 6-fold larger than that for the DMDD receptor (Table 2).

For the amino acids with aromatic rings, the affinity of δK108F for DAMGO was comparable to that of the wild-type μ-OPR and was 335-fold higher than that of the wild-type δ-OPR (Fig. 3, *bottom right*). The K_i value was 4.20 ± 1.23 nM (Table 2). The addition of a hydroxyl group to the aromatic ring decreased the affinity for DAMGO by ~3-fold, and the K_i

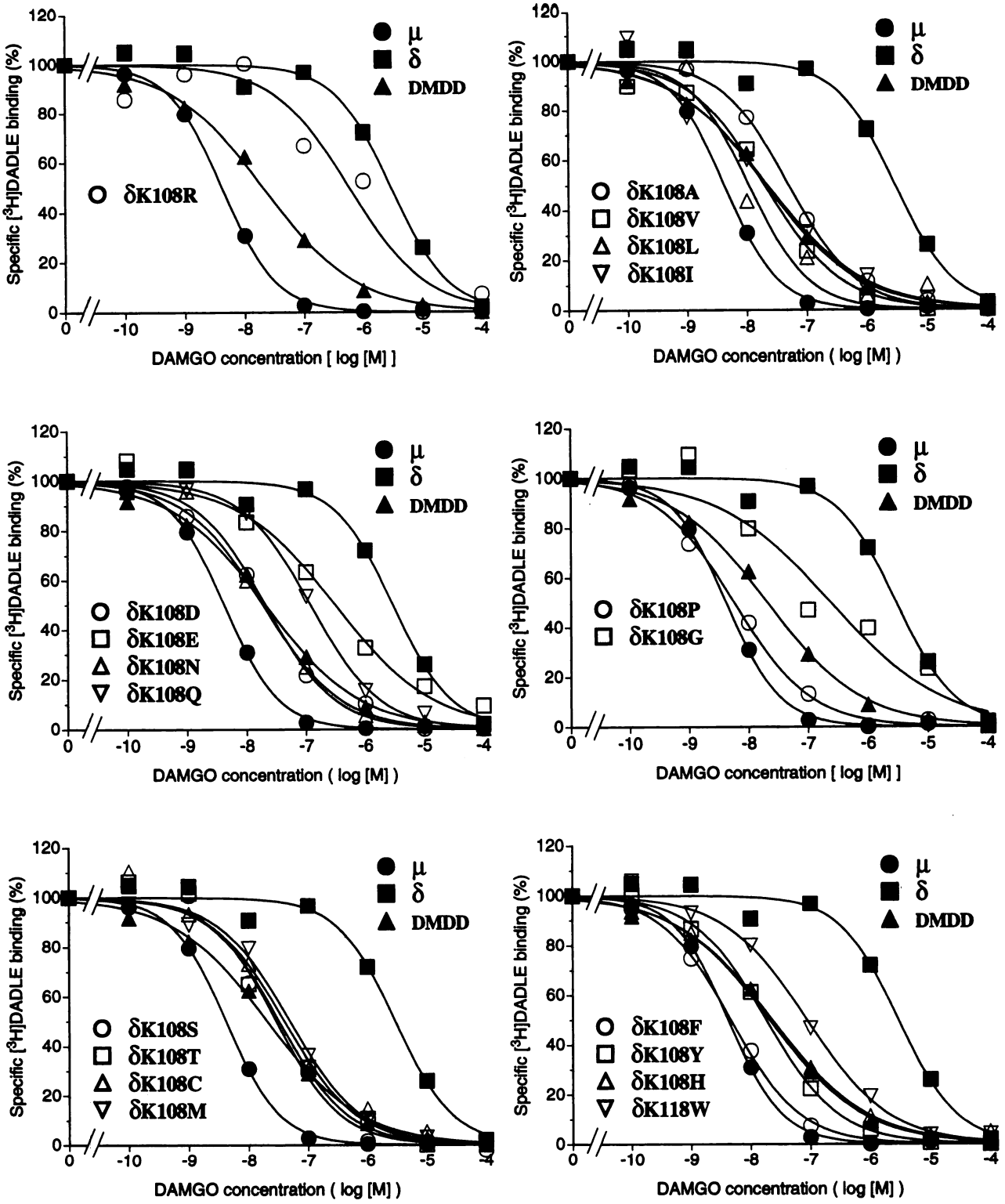

Fig. 3. Displacement of [^{3}H]DADLE binding with DAMGO in the mutant receptors. Displacement of the specific binding of [^{3}H]DADLE with DAMGO to the membrane of COS-7 cells expressing the wild-type μ- and δ-OPRs, DMDD receptor, and mutant receptors. *Curves*, representative of three to five experiments.

value of DAMGO for δK108Y was 11.54 ± 1.27 nM. Replacement of Lys108 with histidine increased the affinity for DAMGO by 43-fold compared with the wild-type δ-OPR, but the K_i value of DAMGO for δK108H (32.30 ± 12.09 nM) was larger than those for δK108F and δK108Y. The mutant receptor δK108W, which possessed tryptophan at position 108, showed a low affinity for DAMGO (K_i = 136 ± 57 nM).

Replacement of Asn127 of μ-OPR with lysine. Asn127 of the μ-OPR, which was the amino acid residue at the corresponding position to Lys108 of the δ-OPR, was replaced

with lysine. The resultant mutant receptor, μN127K, bound [^{3}H]DADLE with a K_d value (4.40 ± 1.00 nM) comparable to those of the wild-type μ- and δ-OPRs (Table 2), indicating that the entire structural integrity of this mutant receptor was not impaired. Replacement of Asn127 with lysine decreased the affinity for DAMGO by 13-fold (Fig. 4). The K_i value of DAMGO for μN127K was 56.27 ± 12.41 nM (Table 2).

Coupling of mutant δ-OPR to adenylate cyclase. To evaluate the agonistic activity of DAMGO in the mutant receptors, the effects of DAMGO on forskolin-induced cAMP accumulation via the wild-type μ- and δ-OPRs and mutant receptors δK108N and δK108A were examined (Fig. 5). In the wild-type μ-OPR, DAMGO maximally inhibited the cAMP accumulation to <10% of the control level, and the IC_{50} value was 3.13 ± 0.75 nM. In the wild-type δ-OPR, the IC_{50} value of DAMGO was 618 ± 50 nM. In the mutant receptors δK108N and δK108A, DAMGO maximally inhibited the accumulation of cAMP to an extent similar to that in the wild-type μ-OPR, and the IC_{50} values were 1.67 ± 0.06 and 6.47 ± 0.72 nM, respectively.

Affinity of various μ-opioid ligands for the δK108N mutant receptor. δK108N mutant receptor expressed on CHO cells, as well as that expressed on COS-7 cells, bound DAMGO with high affinity (K_i = 22.32 ± 3.60 nM) (Table 3). The affinity was 53-fold higher than that for the wild-type δ-OPR. Another peptidic μ-selective agonist, dermorphin, bound the wild-type μ- and δ-OPRs with K_i values of 7.37 ± 3.16 and 712 ± 98 nM, respectively. Replacement of Lys108 of the δ-OPR with asparagine increased the affinity of dermorphin by 32-fold. The K_i value of dermorphin for the δK108N receptor was 21.91 ± 3.61 nM. Replacement of Lys108 of the δ-OPR with asparagine also increased the affinity of CTOP, a peptidic μ-selective antagonist, by >100-fold. The K_i value of CTOP for the δK108N receptor (K_i = 31.66 ± 7.55 nM) was comparable to that for the wild-type μ-OPR (K_i = 33.56 ± 10.49 nM). The endogenous opioid peptides met- and leu-enkephalins bound both the μ- and δ-OPRs with high affinities. These peptides also showed high affinities for the δK108N mutant receptor. The K_i values of met- and leu-enkephalins for the δK108N were 5.23 ± 1.06 and 3.20 ± 0.71 nM, respectively. Narcotic analgesics, such as morphine and methadone, and an opioid antagonist, naloxone, preferentially bound to the μ-OPR compared with the δ-OPR. The affinities of morphine, methadone, and naloxone for the μ-OPR were 56-, 65-, and 8-fold higher than those for the δ-OPR. Replacement of Lys108 of the δ-OPR with asparagine increased the affinities of these nonpeptidic ligands by 3–6-fold. The K_i values of morphine, methadone, and naloxone for δK108N were 266 ± 50, 423 ± 55, and 23.09 ± 3.96 nM, respectively.

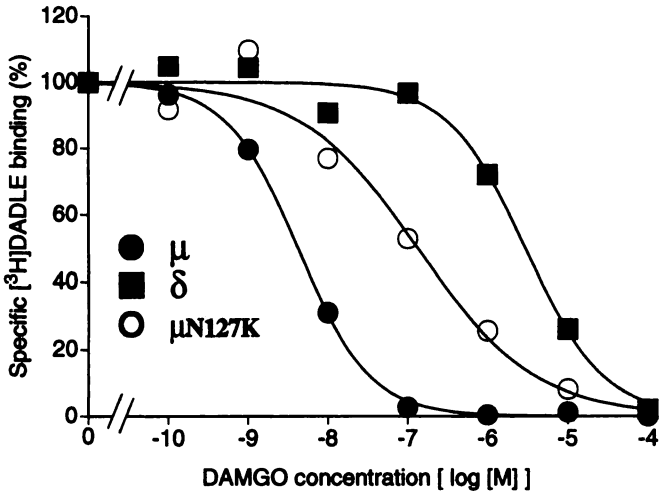

Fig. 4. Displacement of [^{3}H]DADLE binding with DAMGO in the wild-type μ- and δ-OPRs and mutant receptor μN127K. Displacement of the specific binding of [^{3}H]DADLE with DAMGO to the membrane of COS-7 cells expressing the wild-type μ- and δ-OPRs and mutant receptor μN127K. *Curves*, representative of three experiments.

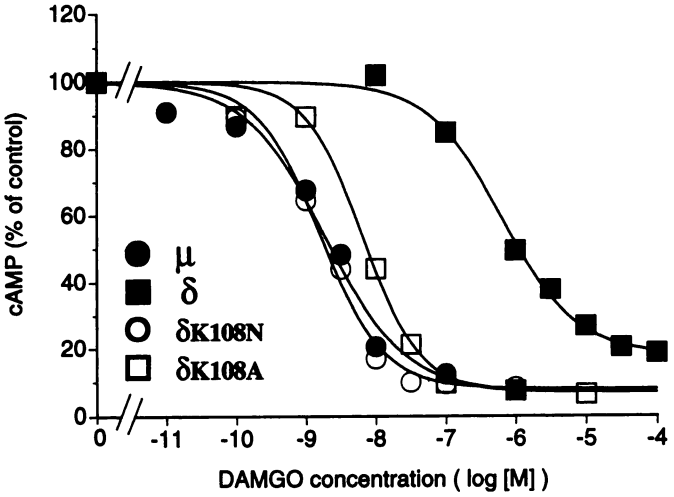

Fig. 5. Agonistic activity of DAMGO in the wild-type μ- and δ-OPRs and mutant receptors. Effects of DAMGO on forskolin-stimulated cAMP accumulation in the CHO cells expressing the wild-type μ- and δ-OPRs and mutant receptors δK108N and δK108A. *Curves*, representative of three experiments.

Discussion

Previously, we reported that replacement of the region around the first extracellular loop of the δ-OPR with the corresponding region of the μ-OPR gave a high affinity for DAMGO to the resultant chimeric receptor, DMDD (21). We demonstrated the high affinity of DMDD receptor for DAMGO through the use of a saturation binding experiment followed by Scatchard analysis using [^{3}H]DAMGO as a radiolabeled ligand. In the current study, we used [^{3}H]DADLE as a radiolabeled ligand because it was known to bind with high affinity to both the μ- and δ-OPRs and was expected to bind to the chimeric DMDD receptor and all of the mutant receptors in which parts of the δ-OPR were replaced with the homologues of the μ-OPR. As expected, the results revealed that the DMDD receptor and mutant receptors, as well as the μ- and δ-OPRs, bound [^{3}H]DADLE with high affinity. In the competitive binding assay using [^{3}H]DADLE as a radiolabeled ligand, DAMGO bound the μ-OPR and DMDD receptor with high affinity and displaced the binding of [^{3}H]DADLE with K_i values of 4.37 and 23.77 nM, respectively, whereas the affinity of the δ-OPR for DAMGO was very low (K_i = 1405 nM). These findings are consistent with our previous report that showed high affinity of the DMDD receptor for DAMGO through the use of saturation binding experiments using [^{3}H]DAMGO as a radiolabeled ligand, confirming that the region around the first extracellular loop is critical for the distinction between the μ- and δ-OPRs by DAMGO.

TABLE 3

Affinity of μ-opioid ligands in the δK108N mutant receptor

Data are expressed as mean ± standard error of three to five experiments. K_i values were determined by displacement of [^{3}H]DADLE binding with unlabeled μ-opioid ligands followed by calculation in accordance with $K_i = IC_{50}/(1 + [DADLE]/K_d)$.

	K_i		
	μOPR	δK108N	δOPR
		nM	
Peptidic ligands			
DAMGO	5.48 ± 0.43	22.32 ± 3.60[a]	1188 ± 84
Dermorphin	7.37 ± 3.16	21.91 ± 3.61[a]	712 ± 98
CTOP	33.56 ± 10.49	31.66 ± 7.55[a]	>3000
Met-enkephalin	4.35 ± 1.27	5.23 ± 1.06	3.21 ± 0.99
Leu-enkephalin	12.54 ± 4.22	3.20 ± 0.71	4.54 ± 0.39
Nonpeptidic ligands			
Morphine	27.10 ± 4.53	266 ± 50[b]	1508 ± 342
dl-Methadone	41.28 ± 5.70	423 ± 55[a]	2685 ± 388
Naloxone	7.86 ± 1.08	23.09 ± 3.96[a]	65.77 ± 10.16

[a] Significantly different from wild-type δ-OPR ($p < 0.01$, Student's t test).
[b] Significantly different from wild-type δ-OPR ($p < 0.05$, Student's t test).

In the region around the first extracellular loop, there are only seven different amino acid residues between the μ- and δ-OPRs. To determine the amino acid residue(s) involved in the discrimination between these receptors by DAMGO, these amino acid residues of the δ-OPR were individually replaced with the corresponding amino acids found in the μ-OPR. Among the seven mutant receptors, only one receptor, δK108N, showed high affinity for DAMGO. The K_i value of DAMGO for this mutant receptor was 18.68 nM which was comparable to that for the DMDD chimeric receptor (K_i = 23.77 nM). The K_i values of DAMGO for the mutant receptors δA107V, δE112G, δE118T, δL119I, δA123I, and δL125I were similar to those for the wild-type δ-OPR. These findings indicate that the replacement of Lys108 with asparagine is entirely responsible for the high affinity of the DMDD receptor for DAMGO. The affinities of the δK108N and DMDD receptors for DAMGO were 4–6-fold lower than that of the wild-type μ-OPR, suggesting that another region or regions other than that around the first extracellular loop also contribute to the high affinity of the μ-OPR for DAMGO. Our previous results from experiments with various chimeric receptors showed that the region posterior to the third transmembrane domain had some involvement in the high affinity binding of μ-OPR for DAMGO (21). Further studies using chimeric and mutant receptors are necessary to fully clarify the molecular basis for the high affinity binding of DAMGO to the μ-OPR. Nevertheless, the current results revealed that the difference in the region around the first extracellular loop, especially the difference between Lys108 in the δ-OPR and Asn127 in the μ-OPR, is principally critical for distinction between the μ- and δ-OPRs by DAMGO.

To examine the role of the residue at position 108 of the δ-OPR in the distinction between μ- and δ-OPRs by DAMGO, Lys108 of the δ-OPR was systematically replaced with other amino acids. Valine is found at the corresponding position of the κ-OPR. The mutant receptor δK108V showed the high affinity for DAMGO. This finding is consistent with our previous report that the region around the first extracellular loop is not involved in the distinction between μ- and κ-OPRs by DAMGO (24). In that report, we used chimeric μ/κ receptors to reveal that the region from the fifth transmembrane domain to the carboxyl terminus is important for the distinction between μ- and κ-OPRs by DAMGO. A similar finding was reported by Xue *et al.* (25). An endogenous opioid peptide, enkephalin, which is a prototype for DAMGO, is well known to exhibit a high affinity for both the μ- and δ-OPRs but not for the κ-OPR. Also in the current study, met- and leu-enkephalins showed high affinities for μ- and δ-OPRs and δK108N mutant receptor. The ability to distinguish between the μ- (or δ-) and κ-OPRs is intrinsically possessed by enkephalin, and the artificial modification gives DAMGO additional ability to distinguish between the μ- and δ-OPRs, so it is likely that the mechanism for the distinction by DAMGO between the μ- and δ-OPRs is different from that for the distinction between the μ- and κ-OPRs. Also, for the replacements of Lys108 with other amino acids with aliphatic side chains, the affinity of the resultant mutants for DAMGO increased by 32–194-fold compared with the wild-type δ-OPR. Furthermore, the addition of a hydroxyl or sulfhydryl group to the aliphatic chain did not affect the affinity for DAMGO because the K_i values of DAMGO for δK108S, δK108T, and δK108C were comparable to those for δK108A and δK108V. These findings suggest that it is not necessary for the amino acid residue at position 108 in the mutant δ-OPR to be an asparagine for high affinity binding to DAMGO to occur.

Replacement of Lys108 with arginine improved the affinity for DAMGO by very little. Both lysine and arginine possess positively charged side chains. Furthermore, although the replacement of Lys108 with glutamic acid with a negatively charged side chain slightly improved the affinity for DAMGO, the affinity of δK108E for DAMGO was ~10-fold lower than those of δK108N and DMDD receptors. These findings suggest that either positively or negatively charged side chain possessed by the amino acid residue at position 108 prevents DAMGO binding. It is possible that the charged side chain forms an obstacle by interacting with the neighboring oppositely charged group and prevents DAMGO from binding to or approaching the binding pocket of the receptor. This idea is supported by the finding that the change in the free carboxyl group in the δK108E to amide (δK108Q) increased the affinity for DAMGO. The replacement of Lys108 with aspartic acid improved the affinity for DAMGO to a level comparable to that of the DMDD receptor. Aspartic acid as well as glutamic acid possesses negatively charged side chains, but the side chain of the former is shorter than that

of the latter. Furthermore, lysine and arginine, which have longer side chains than aspartic acid and glutamic acid, have lower affinity for DAMGO when they exist at the position 108. These findings suggest that the longer side chain is more advantageous to interact with the neighboring oppositely charged group; the replacement of Lys108 with the amino acids with aromatic rings is in agreement. The affinity of δK108F for DAMGO was very high and comparable to that of the wild-type μ-OPR. The addition of the phenolic hydroxyl group, which is weakly ionized, to the aromatic ring (δK108Y) decreased the affinity for DAMGO by 3-fold. The δK108H with the histidine, which contains the weakly ionized imidazole group, at position 108 showed ~8-fold lower affinity for DAMGO than δK108F. The replacement of Lys108 with tryptophan, which has a longer side chain than phenylalanine, tyrosine, and histidine, made the resultant receptor δK108W exhibit the 32-, 12-, and 4-fold lower affinity for DAMGO compared with δK108F, δK108Y, and δK108H, respectively. Although the side chain of tryptophan contains no charged group, it could interact with the neighboring aromatic ring through π/π interaction to form an obstacle to DAMGO binding. The idea that Lys108 of the δ-OPR prevents DAMGO from binding to the δ-OPR is also supported by the findings obtained in the binding experiments using another mutant receptor, μN127K. Asn127 of the μ-OPR is the amino acid residue in the corresponding position to Lys108 of the δ-OPR, and the replacement of Asn127 with lysine markedly decreased the affinity for DAMGO. The binding experiments using mutant receptors revealed that the existence of the amino acid residues with the charged and relatively long side chains at position 108 in the mutant receptors caused low affinity for DAMGO. However, one exception was found in the mutant receptor δK108G. Although glycine has only a single hydrogen atom as its side chain, which is not charged and very small, replacement of Lys108 with glycine scarcely improved the affinity for DAMGO, suggesting that carbon atom at the β position is necessary for DAMGO binding.

The current findings suggest that Lys108 of the δ-OPR obstructs the binding of DAMGO to the δ-OPR rather than that the asparagine residue at the corresponding position in the μ-OPR is necessary for DAMGO binding. This implies that the receptor domain or domains responsible for the affinity for DAMGO exist in the region or regions other than those relevant to the distinction between the μ- and δ-OPRs by DAMGO, and such a domain is conserved between these receptors. In the mutant receptor δK108N, DAMGO maximally inhibited the production of cAMP to an extent similar to that in the wild-type μ-OPR. The IC_{50} value of DAMGO in the δK108N receptor (IC_{50} = 1.67 ± 0.06 nM) was comparable to that in the wild-type μ-OPR (IC_{50} = 3.13 ± 0.75 nM), whereas the K_i value of DAMGO in the δK108N receptor was 4-fold larger than that in the wild-type μ-OPR. Because δK108N has the same primary structure as the δ-OPR except for one amino acid residue, the efficacy for coupling to the G proteins to inhibit cAMP production might differ between the δK108N and μ-OPR. Also, replacement of Lys108 in the δ-OPR with alanine, which has a methyl group as its side chain, improved the agonistic activity, as well as the binding affinity, of DAMGO. Although the potency of DAMGO in the δK108A (IC_{50} = 6.48 ± 0.72 nM) was ~4-fold lower than that in the δK108N, the intrinsic activities of DAMGO on these receptors are considered to be similar when it is taken into account that the affinity of δK108A for DAMGO was ~2-fold lower than that of the δK108N. These findings indicate that the receptor domain or domains responsible for the expression of agonistic activity of DAMGO are also conserved between the μ- and δ-OPRs.

The results from the systematic replacements of Lys108 of the δ-OPR suggest that the lysine residue at position 108 of the δ-OPR prevents DAMGO from binding to the δ-OPR rather than that the asparagine residue at the corresponding position of the μ-OPR is necessary for DAMGO binding. Recently, similar results were reported by Fukuda *et al.* (26). However, they have, at least in part, seem to have misunderstood the role of the residue at position 108 of the δ-OPR because they replaced Lys108 of the δ-OPR with only four amino acids (δK108N, δK108R, δK108D, and δK108A). They concluded that a positively charged amino acid, but not a noncharged or negatively charged amino acid, at position 108 of the δ-OPR prevents DAMGO binding to the δ-OPR, but we showed that tryptophan, a noncharged amino acid, and glutamic acid, a negatively charged amino acid, at position 108 could prevent DAMGO binding. In addition, Fukuda *et al.* did not conduct an experiment to examine whether the mutant receptors can couple to the second messenger system. We examined the coupling of mutant receptors δK108N and δK108A to the inhibitory system of cAMP production and clarified that the receptor domain or domains responsible for the signal transduction exist in the region or regions other than those relevant to the distinction between the μ- and δ-OPRs by DAMGO.

In the current study, we demonstrated that the difference in a specific residue (i.e., the difference between Lys108 of the δ-OPR and Asn127 of the μ-OPR) located just outside the second transmembrane domain is critical for the distinction between these receptors by DAMGO. Recently, Hjorth *et al.* (27) revealed that the difference in a specific residue (i.e., the difference between Glu297 of the κ-OPR and Lys303 of the μ-OPR) located just outside the sixth transmembrane domain is critical for the distinction between these receptors by norbinaltorphimine, a κ-selective antagonist. Taking into account the fact that the positive charge at the N17 position of norbinaltorphimine is critical for its κ-selective binding (28), Glu297 of the κ-OPR interacts with this positive charge to increase the affinity between the κ-OPR and norbinaltorphimine. Prevention of DAMGO binding by Lys108 of the δ-OPR is important for the distinction between the μ- and δ-OPRs by DAMGO, whereas additional binding affinity produced by the interaction between Glu297 of the κ-OPR and norbinaltorphimine is thought to be important for the distinction between the μ- and κ-OPRs by norbinaltorphimine. These findings indicate that the amino acid residues just outside the transmembrane domains of OPRs play critical roles as determinants of the recognition by type-selective opioid ligands. Such an example is also reported in the neurokinin receptors (29). Lys193, Glu194, and Tyr272, located just outside the transmembrane domains of the NK-1 neurokinin receptor, are critical for the binding of the nonpeptidic NK-1-selective antagonist CP96,345. On the other hand, the current findings suggest that the receptor domain or domains involved in the expression of agonistic activity of DAMGO are conserved between the μ- and δ-OPRs and exist in the region or regions not relevant for the distinction between the μ- and

δ-OPRs by DAMGO. Taking it into account that several opiate compounds, such as etorphine, which are relatively small and lipophilic compared with peptidic opioid ligands, bind to all of the μ-, δ-, and κ-OPRs and produce agonistic activity, the region or regions that bind opioid agonists and are responsible for signal transduction process probably exist in the transmembrane domains, which are highly conserved across these three receptors. Several specific residues that exist in the transmembrane regions and are conserved across the μ-, δ-, and κ-OPRs have been shown to be critical for both the binding and the activity of agonists (30, 31)

Fig. 6 shows the hypothetical model illustrating the affinity and selectivity of the opioid ligands for the μ- and δ-OPRs. Both DAMGO and enkephalin possess the specific region to bind the "binding pocket" (as indicated) of the receptors and to exert the signal transduction process. The structure of the binding pocket is thought to be considerably conserved between the μ- and δ-OPRs and exist in the transmembrane region. On the other hand, at least one of the "selectivity domains" is located just outside the transmembrane domain. For the distinction between the μ- and δ-OPRs by DAMGO, a single specific residue Lys108 in the selectivity domain located just outside the second transmembrane domain of the δ-OPR prevents the binding of DAMGO to the δ-OPR. This residue, Lys108, is also critical for the discrimination between the μ- and δ-OPRs by other peptidic μ-selective ligands, such as dermorphin and CTOP. On the other hand, replacement of Lys108 of the δ-OPR with asparagine increased the affinities of nonpeptidic μ-opioid ligands, such as morphine and methadone, by 6-fold. However, the increase in the affinity for these nonpeptidic ligands was not remarkable compared with peptidic μ-selective ligands, in which the replacement of the amino acid residue increased the affinities by >30-fold. These findings indicate that the most critical determinant or determinants for discrimination between μ- and δ-OPRs by nonpeptidic ligands, such as morphine, exist at a different region than that around the first extracellular loop. Fukuda *et al.* (32) showed the importance of the region from the fifth to seventh transmembrane domains for the distinction between the μ- and δ-OPRs by morphine. It is likely that relatively small and lipophilic ligands such as morphine stay almost entirely within the transmembrane region when binding to the binding pocket, so the most critical selectivity domain for the type-selectivity of such ligands possibly exists in the transmembrane regions (Fig. 6). Further investigations are necessary to fully elucidate the structures involved in the selectivity of μ-opioid-selective ligands other than DAMGO.

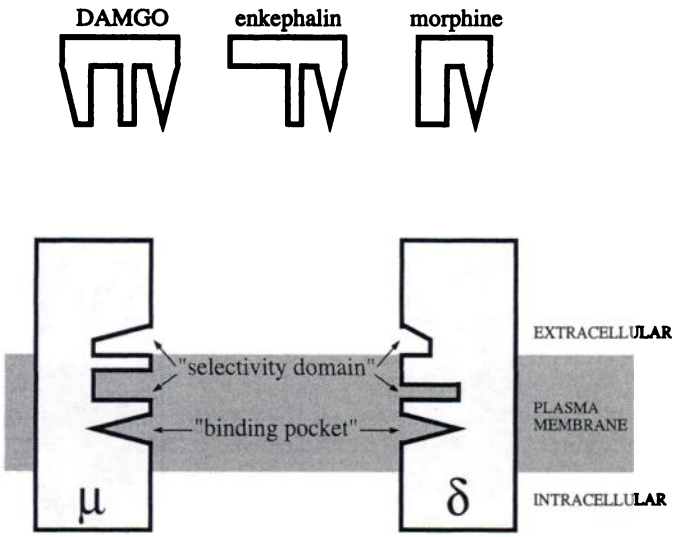

Fig. 6. Hypothetical model of the binding selectivity and agonistic activity of μ-opioid agonists. Details are described in Discussion.

Acknowledgments

We thank Dr. Kazuhiko Fukuda (Department of Anesthesia, Kyoto University Hospital, Kyoto, Japan) for providing the rat δ-opioid receptor cDNA.

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Send reprint requests to: Masamichi Satoh, Ph.D., Department of Molecular Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606–01, Japan. E-mail: msatoh@pharm.kyoto-u.ac.jp