Identification of the amino acid residues involved in selective agonist binding in the first extracellular loop of the δ- and μ-opioid receptors

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Received 14 August 1995

Abstract Effects of amino acid substitutions in the first extracellular loop region of the δ- and μ-opioid receptors were examined. Substitution of lysine-108 of the δ-receptor (δK108) with asparagine improved affinity to [D-Ala^2,MePhe^4,Gly-ol^5]enk ephalin (DAGO), a μ-selective peptide agonist, to be comparable with that of the μ-receptor. On the other hand, replacement of mN127 with lysine decreased the affinity to DAGO by ~15-fold. These results suggest that dK108 and mN127, which correspond to each other in the aligned amino acid sequences, mainly determine the difference in DAGO binding affinity between the δ- and μ-receptors.

Key words: Opioid receptor; Site-directed mutagenesis; cDNA expression; Agonist-binding affinity

1. Introduction

Opioid analgesics interact specifically with the opioid receptor present in the nervous system to produce various cellular responses through the action of the guanine nucleotide-binding regulatory proteins (G-proteins). Pharmacologically, the opioid receptor has been classified into at least three types (δ, μ and κ), on the basis of the difference in binding affinities to opioid ligands [1,2]. The δ-receptor is characterized by high-affinity binding of a peptide agonist [D-penicillamine^2,D-penicillamine^5]enkephalin (DPDPE), while the μ-receptor has been shown to bind a peptide agonist [D-Ala^2,MePhe^4,Gly-ol^5]enk ephalin (DAGO) and an alkaloid agonist morphine with high affinity [3].

Recently, the cDNAs encoding three types of the opioid receptor (δ, μ and κ) have been cloned. Analysis of the primary structures deduced from the nucleotide sequences of the cDNAs revealed that the three opioid receptors possess characteristic structural features of the G-protein-coupled receptor family, including the presence of seven putative transmembrane segments, and have high amino acid sequence identities (~60%) to each other [4]. Furthermore, attempts have been made to understand the structural basis of the ligand selectivity of the opioid receptors, by means of site-directed mutagenesis and cDNA expression [5–13]. Previously, we have reported that the region extending from the intracellular loop region (ICL-I to the amino-terminal half of the transmembrane segment (TM)-III of the μ-opioid receptor contains the major determinant for the high-affinity binding of the μ-selective peptide agonist DAGO [10]. In contrast, the major binding determinant for the δ-selective peptide DPDPE and that for the μ-selective alkaloid morphine were shown to exist within the region spanning TM V-VII.

In this investigation, to identify the amino acid residues involved in determination of the difference in DAGO binding affinity between the δ- and the μ-opioid receptors, we examined agonist binding properties of the receptors in which the amino acid residues divergent between the δ- and the μ-receptors around the extracellular loop region (ECL)-I were mutated. The results obtained indicate that the difference in DAGO binding affinity is mainly determined by the difference of one amino acid residue in ECL-I in the aligned amino acid sequences (δK108 and μN127; amino acid residues followed by residue numbers). Furthermore, μG131 was suggested to be located near the agonist binding site of the μ-receptor.

2. Materials and methods

2.1. Construction of expression plasmids for mutant receptors

cDNAs encoding the rat δ- and μ-opioid receptors [14] were cloned into bacteriophage M13mp18. Mutations were introduced by oligonucleotide-directed mutagenesis (Amersham), and confirmed by sequencing the single-stranded DNAs. The entire protein-coding sequences of the mutant cDNAs were excised from the double-stranded DNAs, and inserted into the *Hin*dIII site of an expression vector pKCRH2 [15] in the same orientation with respect to SV40 early promoter. Expression plasmids for the wild-type rat δ- and μ-opioid receptors were described previously [10].

2.2. Transfection and ligand-binding assay

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum at 37°C in a humidified atmosphere of 95% air/5% CO_2 and transfected with expression plasmids for the wild-type or mutant receptors by the calcium phosphate method [16].

After 72-h incubation at 37°C, cells were washed with phosphate-buffered saline, harvested and stored at −80°C before use. Frozen cells were thawed and homogenized with a Dounce homogenizer in 50 mM Tris-HCl, pH 7.5. The homogenate was centrifuged at 1,000 × *g* for 10 min and the precipitate suspended in the same buffer, homogenized and centrifuged at 1,000 × *g* for 10 min. The two supernatants were combined and centrifuged at 20,000 × *g* for 30 min. The pellet was suspended in the same buffer and used for binding assays. [^{3}H]Ethylketocyclazocine (EKC) or [^{3}H]DAGO binding reaction was performed with membrane preparations (20–50 μg of protein) in 0.2 ml of 50 mM Tris-HCl, pH 7.5, at 37°C for 1 h. After incubation, the samples were collected on GF/B filters (Whatman) and washed with 10 ml of 50 mM Tris-HCl, pH 7.5. The filters were then counted for radioactivity. K_i values for DAGO, DPDPE and morphine were obtained by displacement of [^{3}H]EKC binding by unlabeled ligands and calculation by the equation $K_i = IC_{50}/(1 + L/K_d^*)$ [17], where IC_{50} is concentration of the unlabeled ligand producing a 50% reduction in the [^{3}H]EKC binding, L is concentration of [^{3}H]EKC, and K_d^* is the apparent dissociation

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Abbreviations: G-proteins, guanine nucleotide-binding regulatory proteins; DPDPE, [D-penicillamine^2,D-penicillamine^5]enkephalin; DAGO, [D-Ala^2,MePhe^4,Gly-ol^5]enk ephalin; ICL, intracellular loop; TM, transmembrane segment; ECL, extracellular loop; EKC, ethylketocyclazocine.

SSDI 0014-5793(95)01034-3

constant for [^{3}H]EKC estimated by Scatchard analysis. K_d values for DAGO were determined by Scatchard analysis using [^{3}H]DAGO. All determinations were performed in duplicate.

3. Results

Previously, we have demonstrated that the difference in DAGO binding affinity between the δ- and the μ-receptors is mainly determined by the amino acid sequences of the region spanning from ICL-I to the amino-terminal half of TM-III [10] (Fig. 1). The amino acid sequence of this region is well conserved between the δ- and the μ-receptors [14]. In fact, amino acid residues are identical at 48 of 56 positions in this region of these receptors, and 7 of 8 non-identical residues are clustered around ECL-I (Fig. 1B). Since it is unlikely that amino acid residues in the cytoplasmic region directly contributes to ligand binding, we expected the non-identical amino acid residues around ECL-I to be chiefly involved in determining the difference in DAGO binding affinity of these receptors. To find out the amino acid residues involved in determining DAGO binding affinity, mutant receptors, in which the amino acid residues around ECL-I divergent between the δ- and μ-receptors were changed, were expressed transiently in COS-7 cells and tested for their agonist binding properties using crude membrane preparations. All mutant receptors bound EKC with K_d values comparable with those of the wild-type δ- and μ-receptors, ranging from 1.7 to 12.0 nM (Table 1), suggesting that global structural deterioration was not induced by the mutations. Binding affinities to DAGO, DPDPE and morphine were estimated by displacement of [^{3}H]EKC binding.

Substitutions of six of the seven non-conserved residues around ECL-I of the δ-receptor with the corresponding residues of the μ-receptor (δA107V, δE112G, δE118T, δL119I, δA123I and δL125I; amino acid residues in the wild-type and the mutant preceding and following the number of the altered residue, respectively) (Fig. 1B) affected little the binding affinities to DAGO, DPDPE and morphine (Table 1). In contrast, replacement of δK108 with the corresponding residue of the μ-receptor (δK108N) caused drastic change in DAGO binding affinitiy (Table 1; Fig. 2A). The K_i value for DAGO of δK108N was 41 ± 4 nM (mean ± S.E.M.; n = 5), comparable with that of the wild-type μ-receptor and more than 30-fold lower than that of the δ-receptor. The binding affinities to DPDPE and morphine were also affected by this replacement (Table 1; Fig. 2B,C), but the changes in K_i values were much smaller than in the case of DAGO. To assess the role of amino acid residue-108 of the δ-receptor in defining agonist binding property, mutant receptors in which δK108 was replaced with arginine, aspartic acid or alanine were tested (Table 1; Fig. 2). The affinity to DAGO of δK108R was comparable with that of the wild-type δ-receptor, whereas the K_i values for DAGO of δK108D and δK108A were comparable with that of δK108N and 15–20-fold lower than that of the wild-type δ-receptor. These mutations caused small changes in the affinities to DPDPE and morphine.

Next, we analyzed effects of mutations around ECL-I of the μ-receptor. Replacement of five of the seven divergent amino acid residues of the μ-receptor with corresponding residues of the δ-receptor (μV126A, μT137E, μI138L, μI142A and μI144L) (Fig. 1B) did not significantly affect binding affinities to DAGO, DPDPE and morphine (Table 1). The mutation μN127K, that is the reciprocal mutation of dK108N (see above), increased the K_i value for DAGO ~15-fold (Table 1; Fig. 2A). The K_i value for morphine was ~2.5-fold increased, and that for DPDPE was ~3-fold decreased by this substitution (Table 1; Fig. 2B,C). Replacement of μG131 with the corresponding amino acid residue (μG131E) dramatically changed the agonist

Table 1
Agonist-binding properties of the mutant receptors

Receptor	K_d (nM) EKC		K_i (nM) DAGO		DPDPE		Morphine	
δ	8.6 ± 1.6	(4)	1,300 ± 300	(4)	32 ± 6	(4)	330 ± 60	(4)
μ	4.6 ± 0.3	(4)	17 ± 5	(3)	10,420 ± 2,030	(6)	32 ± 6	(4)
δA107V	6.3 ± 1.7	(3)	990 ± 130	(3)	19 ± 6	(3)	350 ± 10	(3)
δK108N	3.2 ± 0.6	(3)	41 ± 4	(5)	80 ± 3	(3)	140 ± 10	(4)
δK108R	5.2 ± 0.3	(3)	810 ± 110	(3)	153 ± 10	(3)	470 ± 30	(3)
δK108D	3.1 ± 0.3	(3)	60 ± 13	(3)	102 ± 4	(3)	110 ± 5	(3)
δK108A	3.4 ± 0.1	(3)	90 ± 6	(3)	137 ± 5	(3)	100 ± 7	(3)
δE112G	8.7 ± 0.8	(3)	1,330 ± 220	(4)	32 ± 2	(3)	440 ± 10	(4)
δE118T	7.5 ± 1.1	(3)	1,890 ± 420	(4)	33 ± 5	(3)	450 ± 10	(4)
δL119I	6.3 ± 0.6	(3)	1,050 ± 80	(3)	15 ± 2	(3)	320 ± 10	(3)
δA123I	6.8 ± 1.1	(3)	1,280 ± 0	(3)	25 ± 1	(3)	400 ± 20	(3)
δL125I	8.9 ± 1.9	(3)	1,140 ± 40	(3)	14 ± 5	(3)	550 ± 30	(3)
μV126A	4.8 ± 0.9	(3)	23 ± 6	(4)	14,020 ± 1,110	(3)	37 ± 2	(3)
μN127K	8.3 ± 0.7	(3)	270 ± 50	(5)	3,660 ± 270	(6)	81 ± 14	(5)
μG131E	12.0 ± 1.0	(3)	2,380 ± 410	(5)	160 ± 30	(6)	1,220 ± 380	(5)
μG131A	3.8 ± 0.2	(3)	24 ± 1	(3)	8,080 ± 700	(4)	45 ± 2	(3)
μT137E	4.1 ± 0.6	(3)	35 ± 8	(5)	6,540 ± 1,370	(3)	43 ± 6	(5)
μI138L	3.8 ± 0.4	(3)	15 ± 2	(4)	8,390 ± 320	(3)	25 ± 2	(3)
μI142A	3.1 ± 0.2	(3)	17 ± 3	(4)	8,440 ± 560	(3)	19 ± 4	(3)
μI144L	1.7 ± 0.1	(3)	24 ± 3	(4)	13,120 ± 1,420	(3)	19 ± 2	(3)

Data are given as means ± S.E.M.; numbers in parentheses refer to the number of experiments. K_d for EKC was estimated by Scatchard analysis using [^{3}H]EKC. K_i for DAGO, DPDPE and morphine were determined by measuring displacement of [^{3}H]EKC binding by unlabeled ligands (see Figs. 2, 3).

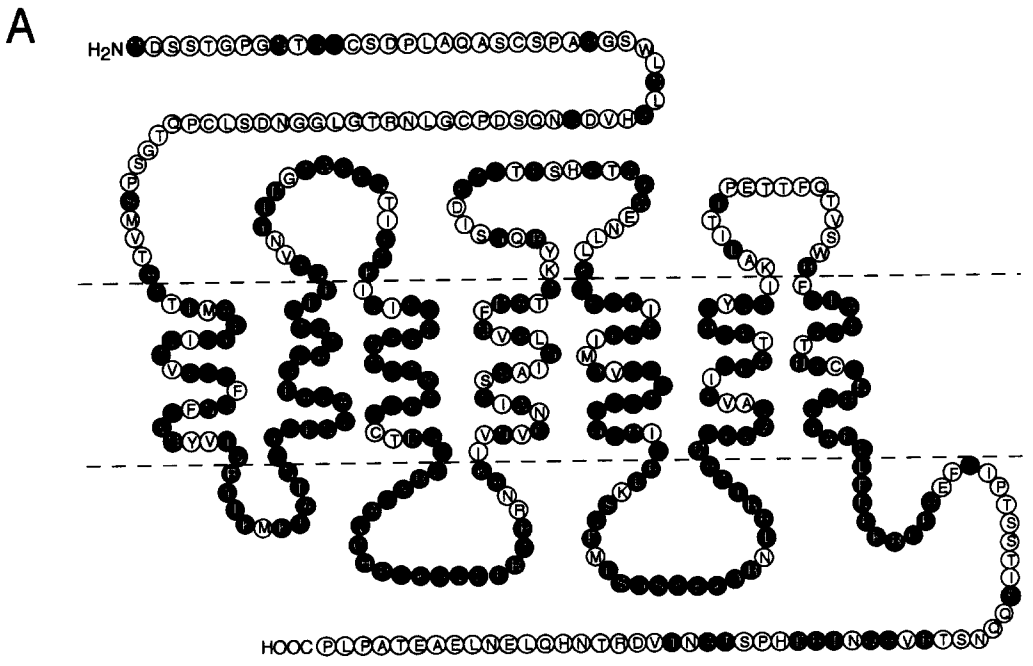

Fig. 1. Topographical model and sequence alignment of the opioid receptor. (A) Proposed two-dimensional structure and amino acid sequence of the rat μ-opioid receptor. The model is based on the amino acid sequence of the rat μ-receptor deduced from the cDNA sequence [14]. The amino acid residues (one-letter code) conserved between the aligned sequences of the δ- and μ-receptors are indicated by closed circles, and divergent ones by open circles. (B) Alignment of the amino acid sequences of the rat δ- and μ-opioid receptors in the region spanning from ICL-I to the amino-terminal half of TM-III, which were previously shown to contain the major determinant for the difference in DAGO binding affinity [10]. Amino acid residues (one-letter code) divergent between the δ- and μ-receptors are boxed and numbered [14]. The positions of the predicted transmembrane segments (TM-II and -III) are indicated by bars.

binding property, in contrast with the reciprocal mutation δE112G (Table 1; Fig. 3). The K_i values for DAGO and morphine were increased ~140-fold and ~40-fold, respectively, to be even higher than those of the wild-type δ-receptor. The K_i value for DPDPE was decreased more than 60-fold, to be only ~5-fold higher than that of the wild-type δ-receptor. However, the mutation μG131A did not significantly change the binding affinities to agonists (Table 1; Fig. 3).

Scatchard analysis with [^{3}H]DAGO demonstrated that the K_d value for DAGO of dK108N was 2.7 ± 0.2 nM (n = 3), which was comparable with that of the wild-type μ-receptor (1.7 ± 0.2 nM; n = 3) (Fig. 4). The K_d values of the wild-type δ-receptor, δE112G, μN127K and μG131E could not be obtained, because they poorly bound [^{3}H]DAGO, although all of them were shown to bind [^{3}H]EKC with high affinities (Table 1). These results are consistent with the data obtained by displacement of [^{3}H]EKC binding, indicating that δK108 and μN127 are important in determining the difference in DAGO binding affinity, and that the DAGO binding affinity is impaired by the mutation μG131E but not significantly changed by the reciprocal mutation δE112G.

4. Discussion

In this investigation, we have attempted to identify amino acid residues involved in selective agonist binding around ECL-I of the δ- and μ-opioid receptor molecules. The results obtained indicate that δK108 and μN127, corresponding to each other in the aligned amino acid sequences, are important in defining the difference in DAGO binding affinity between the δ- and the μ-receptors.

Replacement of δK108, which is positively charged, with a positively charged amino acid (δK108R) did not significantly affect the binding affinity to DAGO. On the other hand, replacement with a non-charged or negatively charged amino acid (δK108N, δK108A or δK108D) improved the DAGO binding affinity to be comparable with that of the wild-type μ-receptor. This result suggests that a positive charge at amino acid residue-108 in the δ-receptor determines the low DAGO binding affinity of the δ-receptor probably by hindering interaction of DAGO with the agonist binding site, rather than that μN127, corresponding to δK108, determines the high affinity to DAGO of the μ-receptor. The DAGO binding affinity of the

κ-receptor is as low as that of the δ-receptor [18], although the amino acid residue-108 in the amino acid sequence of the κ-receptor, corresponding to δK108 and μN127, is valine, which does not have a positive charge. This may suggest that the amino acid sequence of the κ-receptor other than ECL-I plays a major role in determining the low DAGO binding affinity of the κ-receptor. This notion is supported by the recent report that DAGO distinguishes between the μ- and κ-receptors at the region around ECL-III, different from the case of distinction between the δ- and μ-receptors [12].

Replacement of μG131 with the corresponding residue in the δ-receptor (μG131E) resulted in the agonist binding profile

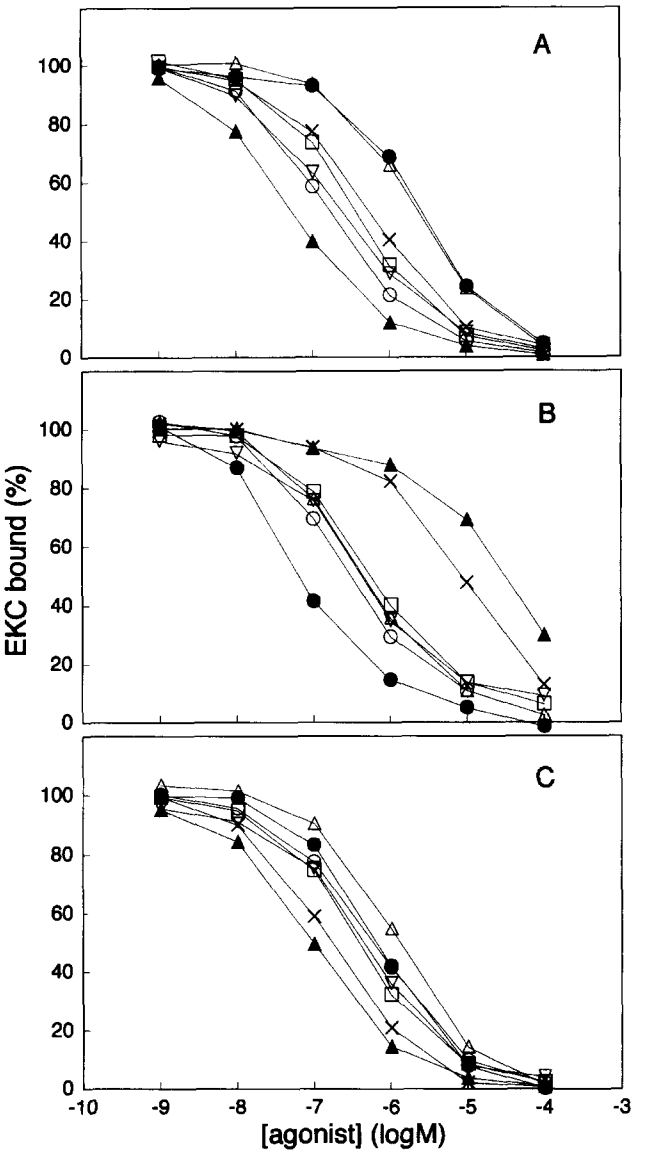

Fig. 2. Displacement of [^{3}H]EKC binding to the wild-type and mutant opioid receptors expressed in COS-7 cells. Membrane was prepared from COS-7 cells expressing the wild-type δ- (●) and μ- (▲) receptors, δK108N (○), δK108R (△), δK108D (▽), δK108A (□) and μN127K (×). Competitive ligand binding assay in the presence of 10 nM [^{3}H]EKC and increasing concentrations of unlabeled DAGO (A), DPDPE (B) and morphine (C) was performed. Each point represents mean from 3–6 experiments. Values for 100% and 0% were determined by measurements in the absence of unlabeled ligands and in the presence of 1 mM naloxone, respectively.

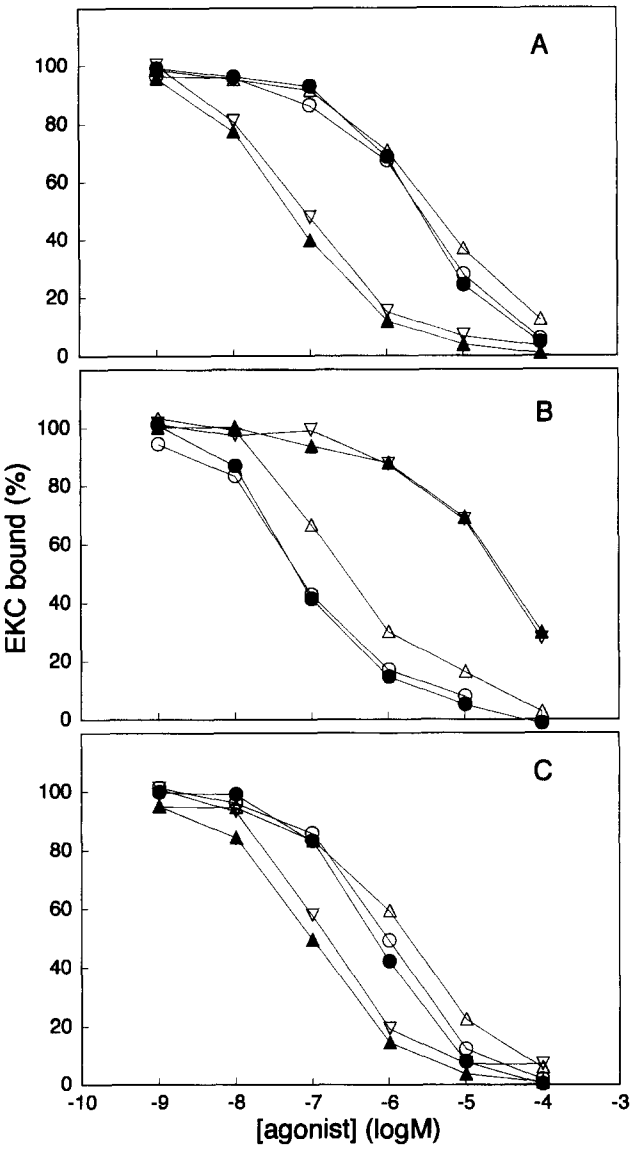

Fig. 3. Competition binding experiments in the wild-type and mutant opioid receptors. Binding affinities to DAGO (A), DPDPE (B) and morphine (C) of the wild-type δ- (●) and μ- (▲) receptors, δE112G (○), μG131E (△) and μG131A (▽) were estimated as described in the legend to Fig. 2. Each point represents mean from 3–6 experiments.

resembling that of the δ-receptor. However, δE112G, the reciprocal mutation of μG131E, and μG131A did not significantly change the agonist binding property of the δ-receptor and that of the μ-receptor, respectively. These results together may suggest that μG131 does not directly interact with agonists but is located near the agonist binding site. It might be possible that replacement of μG131 with glutamic acid, a negatively charged amino acid, indirectly changes the local conformation of the agonist binding site of the μ-receptor to be similar as that of the δ-receptor.

In our previous study on the chimeric receptors [10], the major determinants for the difference in binding affinity to DPDPE and morphine between the δ- and the μ-receptors were demonstrated to exist in the region spanning TM-V–VII. On the other hand, in the present study amino acid replacements

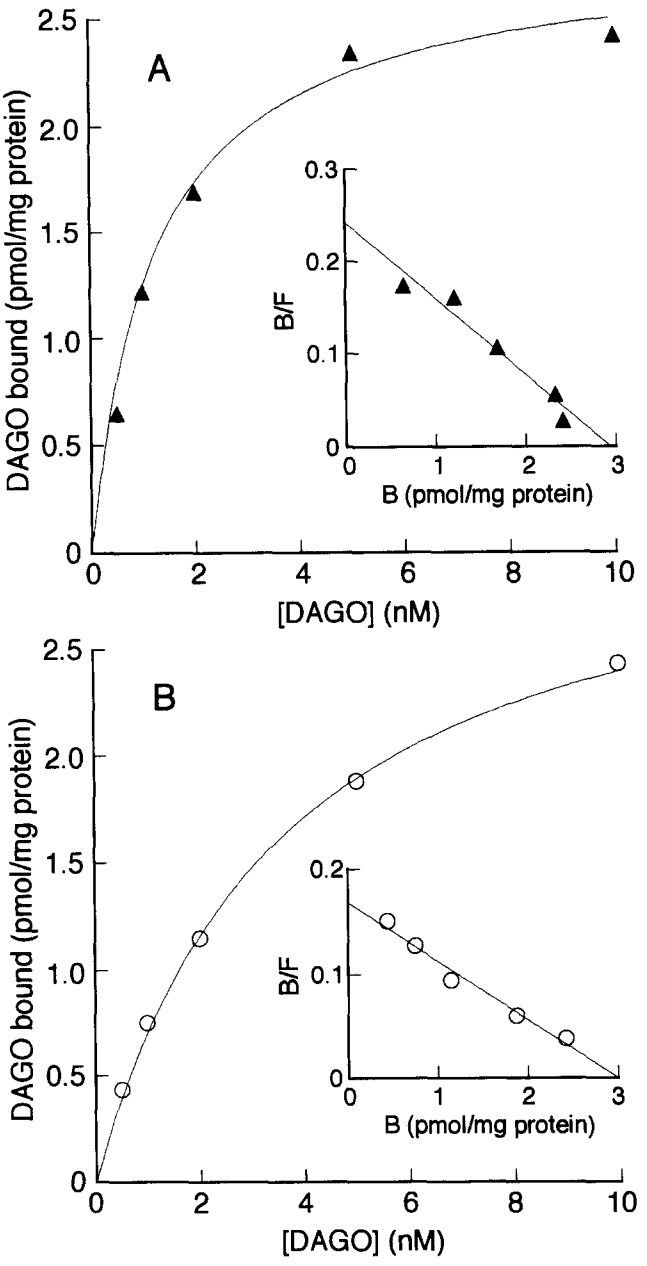

Fig. 4. [^{3}H]DAGO binding to the wild-type μ-receptor and δK108N. Membrane preparations from COS-7 cells expressing the wild-type μ-receptor (A) and δK108N (B) were subjected to saturation analysis with [^{3}H]DAGO. The non-specific binding was measured in the presence of 10 μM unlabeled DAGO. Results of representative experiments are shown. The insets show Scatchard plots of the data.

in the ECL-I of the δ- and μ-receptors produced small changes in binding affinities to DPDPE and morphine, suggesting that ECL-I of the opioid receptor also partly contributes to forming the binding sites for DPDPE and morphine. These observations together may imply that the agonist binding site of the opioid receptor is constituted from multiple parts of the receptor polypeptide. In conjunction with this idea, it has been suggested that amino acid residues involved in binding with peptide agonists are scattered throughout the receptor structure of the neurokinin receptor [19,20] and the angiotensin II receptor [21].

In conclusion, our results indicate that the one amino acid difference in ECL-I in the aligned amino acid sequences (δK108 and μN127) mainly determines the difference in DAGO binding affinity between the δ- and the μ-receptors, which is possibly due to the hindrance of interaction of DAGO with the agonist binding site by a positive charge at amino acid residue-108 of the δ-receptor, and partly contributes to determining the difference in binding affinities to DPDPE and morphine. It was further suggested that μG131 in ECL-I is located near the agonist binding site of the μ-receptor. These results would provide important information to elucidate the structural basis of the ligand selectivity of the opioid receptor, and would facilitate development of highly selective opioid analgesics, which might have side effects less than opioids currently used and might be useful to clarify the physiological roles of individual opioid receptor types.

Acknowledgements: We thank Keiko Komatsu and Natsumi Kikkawa for technical assistance. This investigation was supported by a research grant from the Ministry of Education, Science and Culture of Japan.

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