DAMGO, a μ-opioid receptor selective ligand, distinguishes between μ-and κ-opioid receptors at a different region from that for the distinction between μ- and δ-opioid receptors

Masabumi Minami[a], Tatsuhiro Onogi[b], Takayuki Nakagawa[a], Yoshikazu Katao[a], Yasuhide Aoki[a], Seishi Katsumata[b], Masamichi Satoh[a,*]

[a]*Department of Molecular Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606-01, Japan*
[b]*Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606-01, Japan*

Received 14 March 1995

Abstract The structural basis of opioid receptors (OPRs) for the subtype-selective binding of DAMGO, a μ-opioid receptor selective ligand, was investigated using chimeric μ/κ-OPRs. Replacement of the region from the middle of the fifth transmembrane domain to the C-terminal of μ-OPR with the corresponding region of μ-OPR remarkably decreased the binding affinity to DAMGO, while the reciprocal chimera revealed the high affinity to DAMGO. These results indicate that DAMGO distinguishes between μ- and μ-OPRs at the region around the third extracellular loop, different from the case of the distinction between μ-and δ-OPRs in which the region around the first extracellular loop is important. Furthermore, displacement studies revealed that the region around the third extracellular loop is involved in the discrimination between μ- and κ-OPRs not only by peptidic μ- selective ligands but also by non-peptidic ligands, such as morphine and naloxone.

Key words: Opioid receptor; Chimeric receptor; Ligand binding; μ-Type; κ-Type; [D-Ala^2,MePhe^4,Gly(ol)^5]Enkephalin

1. Introduction

Endogenous opioid peptides and opiate drugs like morphine act on the specific receptors expressing on cell surface to exert various physiological and pharmacological effects, such as analgesia, sedation, respiratory depression, euphoria and modulation of neuroendocrine. The presence of at least three subtypes of opioid receptors (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 of the three subtypes of OPRs couple via pertussis toxin-sensitive G proteins to various effectors including adenylate cyclase [2] and Ca^{2+} and K^+ channels [3] and many drugs act on the all subtypes, these subtypes can be discriminated using the ligands selective to each subtype. The structural basis for how those ligands discriminate among the three subtypes of OPRs is yet unknown and of great interest.

Following the cloning of δ-OPR cDNA [4,5], we as well as several other groups cloned the cDNAs for μ- and κ-OPRs [6–8]. 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 of the amino acid sequences of μ-, δ- and κ-OPRs reveals that these receptors show an overall about 60% identity to one another. Higher identities are found in the transmembrane regions (73–76% of identities) and intracellular regions (63–66% of identities), while the extracellular regions are considerably divergent (34–40% of identities). It is likely that these divergent sequences are responsible for the discrimination among these subtypes by the subtype-selective opioid ligands.

Cloning of the cDNAs for these OPRs has allowed to investigate the structural basis for subtype specificity of the OPRs in their ligand bindings using the various molecular biological techniques. The construction of chimeric receptors is thought to be one of the very powerful approaches to the issue, as it has been demonstrated in the cases of adrenaline [9,10], acetylcholine [11], tachykinin [12,13] and endothelin [14] receptors. Recently, using chimeric μ/δ-OPRs, we have shown that DAMGO, a μ-OPR selective ligand, distinguishes between μ- and δ-OPRs at the region around the first extracellular loop and that this region is partly involved in the discrimination between μ- and δ-OPRs also by other several peptidic μ-selective ligands, such as dermorphin, morphiceptin and CTOP, but not by non-peptidic ligands, such as morphine and naloxone [15]. In the present study, we constructed chimeric μ/κ-OPRs and examined their binding characteristics to DAMGO and other several μ-selective opioid ligands. We will show here that DAMGO distinguishes between μ- and κ-OPRs at the different region from that for the distinction between μ- and δ-OPRs, and that the region around the third extracellular loop is involved in the discrimination between μ- and κ-OPRs not only by peptidic μ-selective ligands but also by non-peptidic ligands, such as morphine and naloxone.

2. Materials and methods

2.1. Materials

[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-pyrroli dinyl)-1-oxaspiro[4,5]dec-8-yl]benzeneacetamide) (52.9 Ci/mmol) and (−)-[9-^{3}H(N)]bremazocine (29.8 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, USA). DAMGO was purchased from Cambridge Research Biochemicals Ltd. (Cheshire, UK). Morphine hydrochloride was from Takeda Chemical Industries Ltd. (Osaka, Japan). Naloxone hydrochloride was from Sigma Chemical Co. (St. Louis, USA). Dermorphin, morphiceptin and CTOP were from Peninsula Laboratories Inc. (Belmont, USA).

*Corresponding author. Fax: (81) (75) 753-4586.

Abbreviations: CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; DAMGO, [D-Ala^2,MePhe^4,Gly(ol)^5]en kephalin; G protein, GTP binding protein; OPR, opioid receptor.

SSDI 0014-5793(95)00340-1

2.2. Construction of chimeric receptors

The chimeric receptors between μ- and κ-OPRs were constructed by using the intrinsic (*Afl*III sites in μ- and κ-OPR cDNAs and *Bgl*II site in κ-OPR cDNA) and introduced (*Bgl*II site in μ-OPR cDNA) restriction enzyme sites which exist at the corresponding positions of both receptor cDNAs (Fig. 1). *Bgl*II site was introduced into μ-OPR cDNA by in vitro site-directed mutagenesis using a Transformer Site-Directed Mutagenesis Kit (2nd ver.) (Clontech Laboratories Inc., Palo Alto, CA, USA). The appropriate restriction enzyme fragments of the μ- and κ-OPRs were ligated and cloned into the *Hin*dIII-*Apa*I site of the pcDNA3 eukaryotic expression vector (Invitrogen, San Diego, CA, USA). The sequence of each construct was confirmed by sequencing analysis using Sequenase ver.2 DNA sequencing kit (United States Biochemical, Cleveland, USA). Each constructed chimeric receptor was given a name on the basis of the origins of its four extracellular domains.

2.3. Expression of wild type and chimeric receptors and binding assay

For transient expression of the wild type and chimeric OPRs, the plasmid containing each receptor cDNA was transfected to COS-7 cells by the DEAE-dextran method [16]. 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 × *g*, the pellet was resuspended in the same buffer and used in the radioligand binding assay. Binding experiments for Scatchard analyses were performed with various concentrations of [^{3}H]DAMGO or [^{3}H]bremazocine. For displacement studies, 1 nM [^{3}H]DAMGO or 1 nM [^{3}H]U69,593 and various concentrations of unlabeled opioid ligands were used. Non-specific binding was determined in the presence of 10 mM unlabeled DAMGO, bremazocine or U69,593. Incubations of cell membranes with [^{3}H]DAMGO, [^{3}H]bremazocine or [^{3}H]U69,593 in the presence or absence of competing opioid ligands were carried out at 25°C for 1 h and terminated by the addition of ice-cold buffer and rapid filtration over Whatman GF/C glass fiber filters which were 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.

3. Results and discussion

To determine which the first or latter half of the receptor is involved in the discrimination between μ- and κ-OPRs by DAMGO, we constructed two chimeric receptors using restriction enzyme *Afl*III. The chimeric receptor KKMM, in which the amino-terminal half of the μ-OPR was replaced with the corresponding region of κ-OPR, exhibited an equivalent affinity (K_d = 4.38 ± 0.90 nM; n = 3) to DAMGO compared with the wild type μ-OPR (K_d = 3.46 ± 0.84 nM; n = 3), while the reciprocal chimeric receptor MMKK poorly bound [^{3}H]DAMGO and the K_d value could not be determined as in the case of the wild type κ-OPR (Fig. 2). These results suggest that the latter half of the receptor structure is critical for DAMGO to distinguish between μ- and κ-OPRs. Interestingly, this is quite the reverse of the case that the first half of the receptor is important to the discrimination between μ- and δ-OPRs by DAMGO [15]. For further clarification of the region responsible for subtype-selective binding of DAMGO, we constructed two chimeric receptors using the restriction enzyme *Bgl*II. The chimeric receptor KKKM, in which the region from amino-terminal to the middle of the fifth transmembrane domain of μ-OPR was replaced with the corresponding region of κ-OPR, exhibited an equivalent affinity to DAMGO (K_d = 3.56 ± 0.92 nM; n = 3) compared with the wild type μ-OPR. On the contrary, the chimeric receptor MMMK did not display any specific binding to [^{3}H]DAMGO. Because all of the four chimeric receptors were verified to bind [^{3}H]bremazocine, a non-selective opioid ligand, with high affinity (data not shown), the lack of [^{3}H]DAMGO binding to chimeric receptors MMKK and MMMK was not due to the low expression of these chimeric receptors nor due to the overall alteration in tertiary structures of the receptors. These results indicate that the region around the third extracellular loop is critical for DAMGO to distinguish between μ- and κ-OPRs. This is very different from the case of the discrimination between μ- and δ-OPRs by DAMGO in which the region around the first extracellular loop is important.

Next, to examine the importance of the region around the third extracellular loop to the discrimination between μ- and κ-OPRs by other μ-selective opioid ligands, we carried out the displacement studies using μ- and κ-OPRs and the chimeric

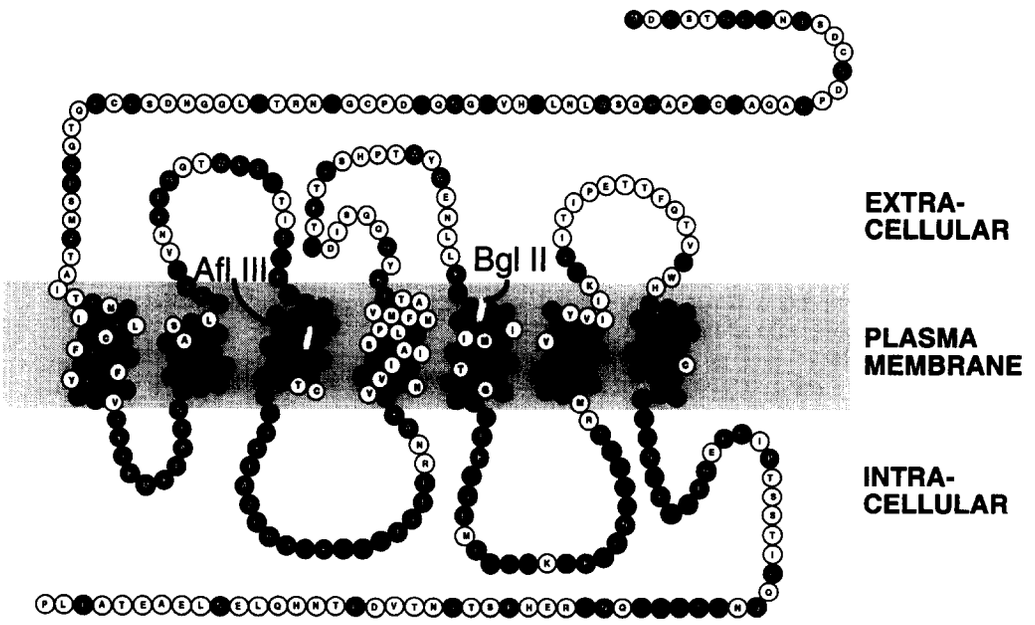

Fig. 1. Proposed model for membrane topography of the rat μ-opioid receptor. Solid circles indicate amino acid residues conserved between μ- and κ-opioid receptors. Noted are the unique restriction enzyme sites used to construct chimeric receptors.

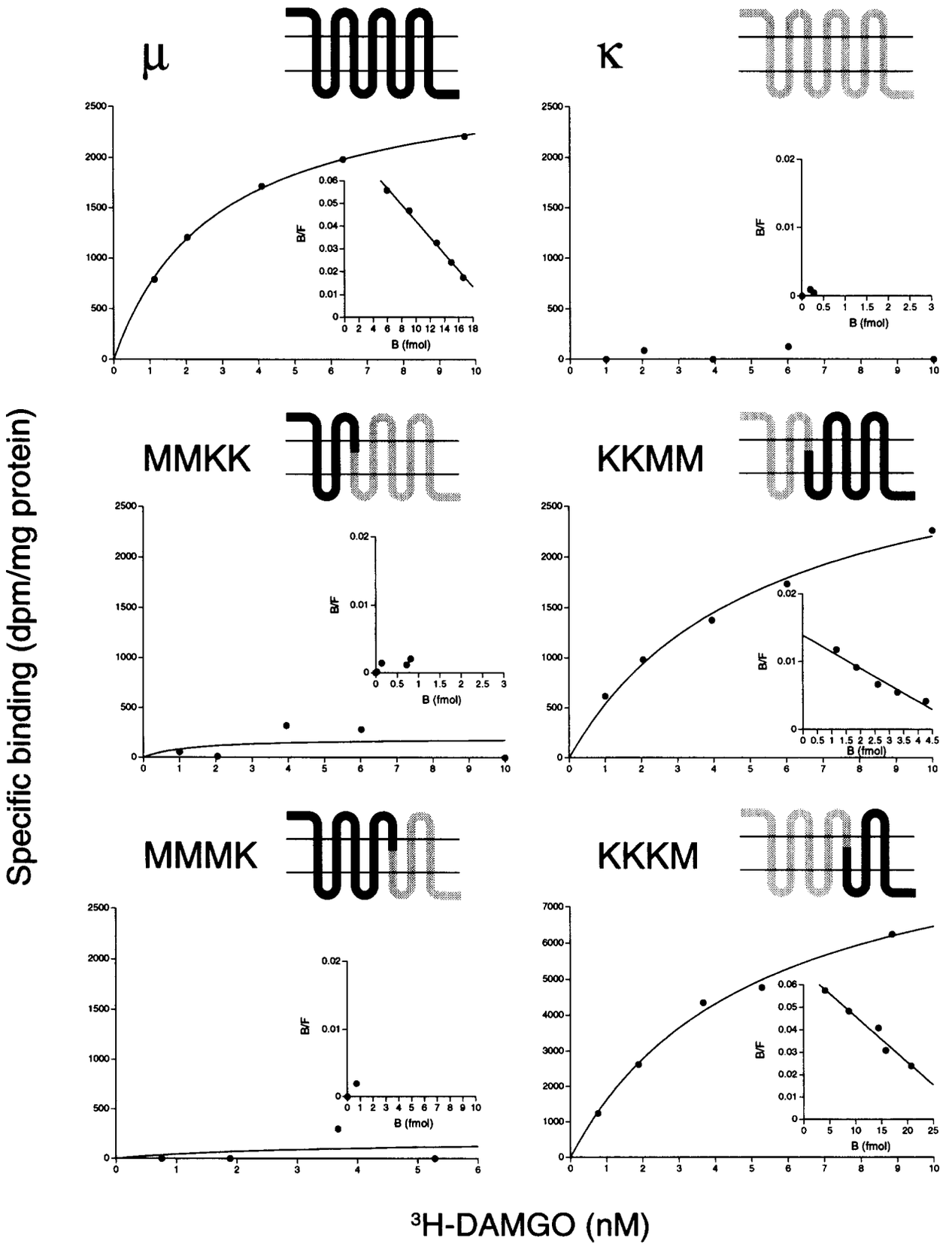

Fig. 2. Saturation binding of [^{3}H]DAMGO to the membrane of COS-7 cells which expressed wild type μ- or κ-opioid receptor or chimeric MMKK, KKMM, MMMK or KKKM receptor. Insets show the scatchard analyses of [^{3}H]DAMGO binding.

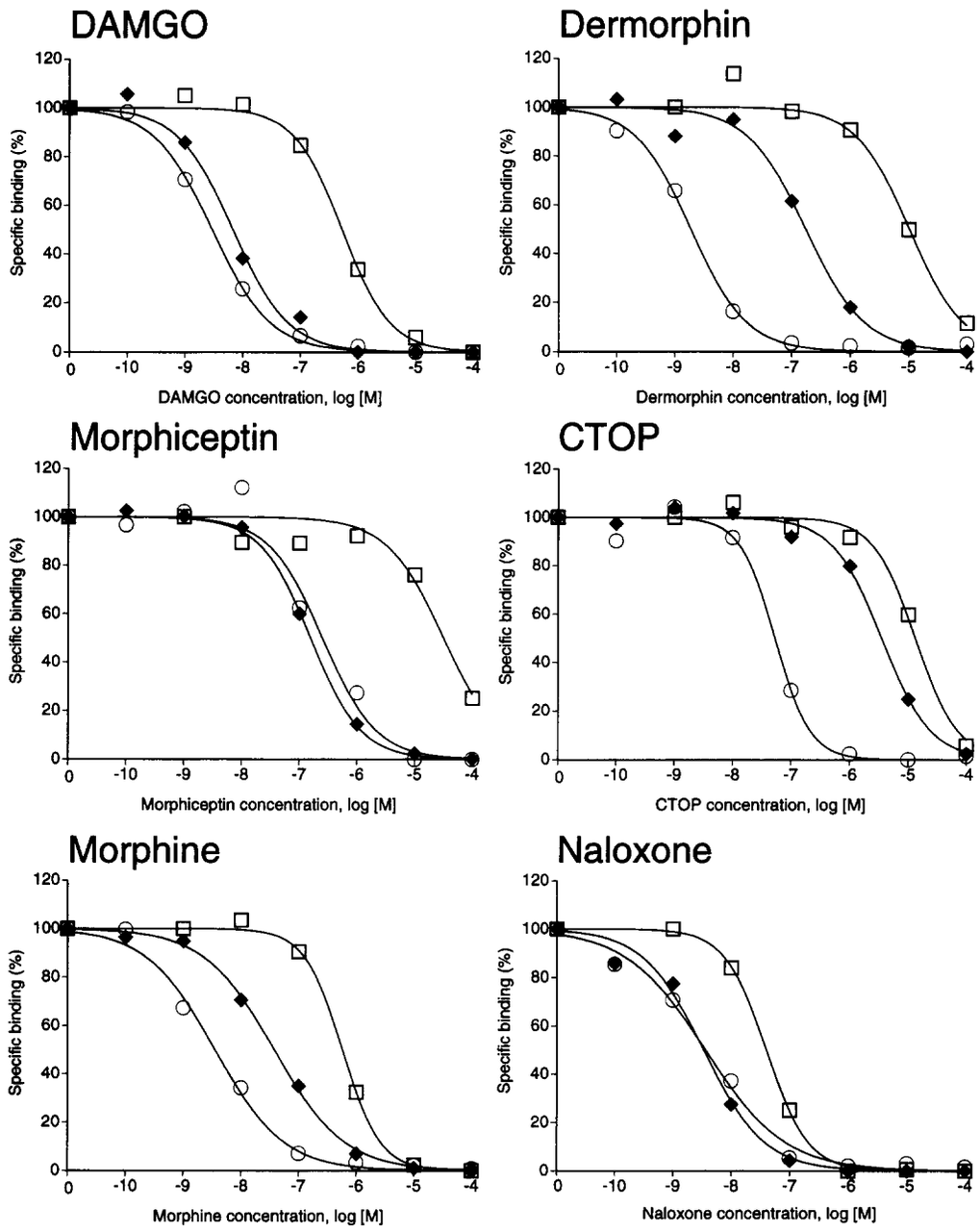

Fig. 3. Displacements of the specific binding of [^{3}H]DAMGO to the membrane of COS-7 cells expressing μ- (○) or KKKM (◆) receptor and of [^{3}H]U69,593 to the membrane of COS-7 cells expressing κ- (□) receptor with unlabeled μ-selective opioid ligands.

receptor KKKM (Fig. 3). [^{3}H]DAMGO was used to label μ- and KKKM receptors and [^{3}H]U69,593 to label κ-OPR. K_d values of [^{3}H]DAMGO to μ- and KKKM receptors and of [^{3}H]U69,593 to κ-OPR were nearly equivalent (3.46 ± 0.84 nM in μ-OPR, 3.56 ± 0.92 nM in KKKM receptor and 3.02 ± 0.41 nM in κ-OPR). Unlabeled DAMGO displaced the binding of the tritiated ligand to μ and KKKM receptors in the same degree, while the potency of unlabeled DAMGO to inhibit the specific binding of the tritiated ligand to κ-OPR was about 100 times lower than those to μ and KKKM receptors. Replacement of the region around the third extracellular loop of κ-OPR with the corresponding region of μ-OPR increased the affinity to μ-selective peptidic ligands, such as dermorphin, morphiceptin and CTOP. Particularly, a μ-selective peptidic agonist morphiceptin showed an equivalent affinity to KKKM receptor compared with that to the wild type μ-OPR, while the increment in the affinity of a μ-selective peptidic antagonist CTOP is a little. Non-peptidic ligands, morphine and naloxone, also bound to KKKM receptor with higher affinity than to κ-OPR. In particular, naloxone exhibited an equivalent affinity to KKKM receptor compared with that to the wild type μ-OPR, These results indicate that the region around the third extracellular loop is critical not only for μ-selective peptidic ligands but also for non-peptidic ligands to distinguish between μ- and κ-OPRs. This is the case quite different from that the replacement of the region around the first extracellular loop of δ-OPR with the corresponding region of μ-OPR increased the affinity of the peptidic ligands, such as DAMGO, dermorphin

and morphiceptin, but not of the non-peptidic ligands, such as morphine and naloxone [15]. To further elucidate the contribution of the region around the third extracellular loop to the binding selectivity of the non-peptidic ligands, we should examine the effect of replacement of such a region between μ- and δ-OPRs on the affinity of those ligands.

A 'message and address' concept for opioid ligands has been proposed by Portoghese [17]. Briefly, the concept is that the specific regions of opioid ligands are responsible for receptor transduction process that leads to a specific biological effect ('message'), while other ligand regions provide additional binding affinity and are not essential for the transduction process ('address'). This idea has been supported by a recent molecular pharmacological study using μ/κ chimeric receptors reported by Wang et al. [18]. They revealed that substitution of the second extracellular loop of μ-OPR for the corresponding region of κ-OPR increased the affinity for dynorphin A [1–17] by about 100-fold. In this study, we demonstrated that DAMGO distinguished between μ- and κ-OPRs at the different region from that for the distinction between μ- and δ-OPRs. These findings suggest that at least two specific regions of DAMGO play a role of 'address' for selective binding to μ-OPR. One region is responsible for the interaction with the receptor domain around the first extracellular loop and critical in the distinction between μ- and δ-OPRs. The other interacts with the domain around the third extracellular loop to play an important role in the discrimination between μ- and κ-OPRs. The interactions between the receptor and DAMGO in such domains of μ-, but not δ- nor κ-, OPR may provide additional binding affinity to produce the high affinity binding of DAMGO to μ-OPR. Alternatively, the interactions may 'negatively' act on the binding of DAMGO to δ- and κ-OPRs, that is, the domain around the first extracellular loop of δ-OPR and that around the third extracellular loop of κ-OPR may hinder the binding of DAMGO to these OPRs, respectively. Further studies are necessary to clarify the molecular basis for high selectivity of DAMGO to μ-OPR.

Acknowledgments: This study was partly supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

[1] Pasternak, G.W. (1988) J. Am. Med. Assoc. 259, 1362–1367.
[2] Childers, S.R. (1993) in: Opioid I (Herz, A. ed.), Handbook Exp. Pharmacol. vol. 104, pp. 189–216, Springer-Verlag, Berlin/Heidelberg/New York/London/Paris/Tokyo/Hong Kong/Barcelona/Budapest.
[3] North, R.A. (1993) in: Opioid I (Herz, A. ed.), Handbook Exp. Pharmacol. vol. 104, pp.773- 797, Springer-Verlag, Berlin/Heidelberg/New York/London/Paris/Tokyo/Hong Kong/Barcelona/Budapest.
[4] Evans, C.J., Keith Jr., D.E., Morrison, H., Magendzo, K. and Edwards, R.H. (1992) Science 258, 1952–1955.
[5] Kieffer, B.L., Befort, K., Gavriaux-Ruff, C. and Hirth, C.G. (1992) Proc. Natl. Acad. Sci. USA 89, 12048–12052.
[6] Minami, M., Onogi, T., Toya, T., Katao, Y., Hosoi, Y., Maekawa, K., Katsumata, S., Yabuuchi, K. and Satoh, M. (1994) Neurosci. Res. 18, 315–322.
[7] Minami, M., Toya, T., Katao, Y., Maekawa, K., Nakamura, S., Onogi, T., Kaneko, S. and Satoh, M. (1993) FEBS Lett. 329, 291–295.
[9] Kobilka, B.K., Kobilka, T.S., Daniel, K., Regan, J.W., Caron, M.G. and Lefkowitz, R.J. (1988) Science 240, 1310–1316.
[10] Frielle, T., Daniel, K.W., Caron, M.G. and Lefkowitz, R.J. (1988) Proc. Natl. Acad. Sci. USA 85, 9494–9498.
[11] Wess, J., Gdula, D. and Brann, M.R. (1991) Mol. Pharmacol. 41, 369–374.
[12] Yokota, Y., Akazawa, C., Ohkubo, H. and Nakanishi, S. (1992) EMBO J. 11, 3585–3591.
[13] Gether, U., Johansen, T.E. and Schwartz, T.W. (1993) J. Biol. Chem. 268, 7893–7898.
[14] Adachi, M., Furuichi, Y. and Miyamoto, C. (1995) Eur. J. Pharmacol. 269, 225–234.
[15] Onogi, T., Minami, M., Katao, Y., Nakagawa, T., Aoki, Y., Toya, T., Katsumata, S. and Satoh, M. (1995) FEBS Lett. 357, 93–97.
[16] Selden, R.F. (1987) in: Current Protocols in Molecular Biology (Ausubel, F.M., Brent, R., Kingston, R.E. eds.) 9.2, Wiley, New York.
[17] Portoghese, P.S. (1989) Trends Pharmacol. Sci. 10, 230–235.
[18] Wang J.B., Johnson, P.S., Wu, J.M., Wang, W.F. and Uhl, G.R. (1994) J. Biol. Chem. 269, 25966–25969.