FEBS Letters 384 (1996) 198–202 FEBS 16923

The region in the μ opioid receptor conferring selectivity for sufentanil over the δ receptor is different from that over the κ receptor

Jinmin Zhu[a], Ji-Chun Xue[a], Ping-Yee Law[b], Patricia A. Claude[b], Lai-Yi Luo[a], Jinling Yin[a], Chongguang Chen[a], Lee-Yuan Liu-Chen[a,*]

[a]*Department of Pharmacology, Temple University School of Medicine, 3420 N. Broad St., Philadelphia, PA 19140, USA*
[b]*Department of Pharmacology, University of Minnesota School of Medicine, Minneapolis, MN 55455, USA*

Received 8 February 1996

Abstract **We determined the binding domains of sufentanil and lofentanil in the μ opioid receptor by comparing their binding affinities to seven μ/δ and six μ/κ chimeric receptors with those to μ, δ and κ opioid receptors. TMHs 6 and 7 and the e3 loop of the μ opioid receptor were important for selective binding of sufentanil and lofentanil to the μ over the κ receptor. TMHs 1–3 and the e1 loop of the μ opioid receptor conferred binding selectivity for sufentanil over the δ receptor. Thus, the region that conferred binding selectivity for sufentanil differs, depending on chimeras used. In addition, the interaction TMHs 1–3 and TMHs 6–7 was crucial for the high affinity binding of these two ligands. These two regions are likely to contain sites of interaction with the ligands or to confer conformations specific to the μ receptor.**

Key words: Sufentanil; Lofentanil; Chimeric receptor; Opioid receptor; Structure-function relationship

1. Introduction

Opiate and opioid drugs, acting on membrane-bound receptors, produce many pharmacological effects. The existence of at least three types of opioid receptors – μ, δ and κ – has been demonstrated [1]. Mu opioid receptors mediate many effects of opiates and opioid compounds, including, most notably, modulation of pain perception and euphoria [1]. Activation of μ opioid receptors couples via pertussis toxin-sensitive G proteins to various effectors including adenylate cyclase and K^+ and Ca^{2+} channels [1].

Following the cloning of the δ receptor [2,3], several laboratories reported cloning of the μ opioid receptor [4–12]. In addition, the κ opioid receptor has been cloned [13–21]. All three opioid receptors contain seven putative transmembrane helices (TMHs), a common structural motif of G protein-coupled receptor superfamily. Sequence comparison among the three types of opioid receptors shows substantial divergence in the N- and C-terminal domains as well as extracellular loops, while sequences within TMHs and intracellular loops are very similar. These divergent sequences may contribute to the binding of type-selective ligands. The availability of μ, δ and κ opioid receptor clones permits identification of the structural basis of binding selectivity of these receptors at the molecular level.

*Corresponding author. Fax: (1) (215) 707-7068.

Abbreviations: aa, amino acid; CHO cells, Chinese hamster ovary cells; DAMGO, [D-Ala,MePhe^4,Gly(ol)^5]enkephalin; e1 loop, the first extracellular loop; e3 loop, the third extracellular loop; SUPERFIT, *cis*-(+)-3-methylfentanyl isothiocyanate; TMH, transmembrane helix.

Deletion of 64 N-terminal amino acids and 33 C-terminal amino acids of the μ receptor did not affect binding of agonists and antagonists [6,22], indicating that the N- and C-terminal domains do not contribute to ligand binding.

Chimeric μ/κ, μ/δ and δ/κ receptors have been very useful in delineation of ligand binding domains of μ, δ and κ receptors. For the μ receptor, using μ/κ chimeras, we [23,24] demonstrated that the TMHs 6 and 7 and the third extracellular (e3) loop of the μ receptor were important for binding of selective agonists, such as DAMGO, sufentanil, PL017 and morphine, as well as covalent binding of the irreversible ligand β-funaltrexamine. Similar results were reported for DAMGO binding by Minami et al. [25], who used μ/κ chimeras. In contrast, Onogi et al. [26] and Fukuda et al.[27] reported that the determinant of selectivity of the μ receptor for DAMGO was located in the first extracellular (e1) loop, based on analysis of binding of DAMGO to a series of chimeric μ/δ opioid receptors. The e1 loop was also partly involved in selectivity of other peptide ligands, but not nonpeptide ligands [26]. Major determinants for binding of morphine are within TMHs 5–7 as determined by binding to μ/δ chimeras [27]. Thus, in the case of DAMGO, regions conferring specificity in the μ receptor are different, as determined from μ/κ and μ/δ chimera studies. On the contrary, for morphine, the regions are similar, whether determined from μ/κ or μ/δ chimera studies.

Sufentanil and its analog lofentanil are narcotic analgesics with high potency. Both are thought to act on μ opioid receptors and display morphine-like side effects. Chemically, these two drugs are phenylpiperidines and they are distinctly different from morphine and oxymorphone, which are morphinans. In this study, we determined the region in the μ receptor that conferred selectivity for the binding of sufentanil and lofentanil by examining their binding to chimeric μ/δ receptors constructed from cloned rat μ and mouse δ opioid receptors [3,4] and to chimeric μ/κ receptors generated from cloned rat μ and κ opioid receptors [4,14].

2. Materials and methods

2.1. Construction of chimeric μ/κ and μ/δ receptors

Six chimeric μ/κ receptors, chimeras I, II, III, IV, XI, and XII, were constructed from rat μ and κ opioid receptors [4,14]. Chimera I (amino acid (aa) κ1–186/μ194–267/κ263–380) and chimera II (aa μ1–193/κ187–262/μ269–398) were generated by switching the fragments containing the C-terminal half of the TMH 4, the e2 loop and the TMH 5. Chimera III (aa κ1–141/μ151–398) and chimera IV (aa μ1–150/κ142–380) were constructed by swapping the regions from the N-terminus to the start of the TMH 3. Chimera XI (aa μ1–268/κ263–380) and chimera XII (aa κ1–262/μ269–398) were generated by exchanging the regions from the middle of the third intracellular (i3) loop to the

SSDI S0014-5793(96)00312-2

C-terminus. Details of generation of μ/κ chimeras were described previously [28]. Schematic drawings of chimeras are shown in Table 1.

Seven chimeric μ/δ receptors (δ/μ1, μ/δ1, μ/δ3, δ/μ4, μ/δ4, δ/μ5 and μ/δ3/μ67) were constructed from the mouse δ and the rat μ opioid receptors [3,4]. Chimeras δ/μ1 (aa δ1–75/μ95–398) and μ/δ1 (aa μ1–94/δ76–372) were constructed by swapping the regions from the N-terminus to the start of the first intracellular (i1) loop. Chimera μ/δ3 (aa μ1–153/δ135–372) was generated by combining the region from the N-terminus to the end of TMH 3 of the μ receptor with a fragment of the δ receptor from the end of TMH 3 to the C-terminus. Chimeras δ/μ4 (aa δ1–187/μ207–398) and μ/δ4 (aa m1–206/δ188–372) were constructed by exchanging the segments from the N-terminus to the beginning of the second extracellular (e2) loop. Details of generation of these five μ/δ chimeras has been described [29].

Chimera δ/μ5 (aa δ1–260/μ280–398) was constructed by combining the region from the N-terminus to the beginning of TMH 6 of the δ receptor with the segment from the beginning of TMH 6 to the C-terminus of the μ receptor. For this purpose, a *Mlu*I restriction enzyme site was created at Thr-279 and Arg-280 of the μ receptor and at the corresponding Thr-260 and Arg-261 of the δ receptor using the Promega pAlter-1 site-directed mutagenesis protocol.

Chimera μ/δ3/μ67 (μ1–153/δ135–260/μ280–398) was constructed on the basic structure of μ/δ3 with the fragment from the beginning of TMH 6 to the C-terminus of the μ receptor. Chimera μ/δ3/μ5 was generated from chimeras μ/δ3 and δ/μ5. Chimera μ/δ3 was treated with *Hin*dIII and *Bgl*II and chimera δ/μ5 with *Bgl*II and *Xba*I. The fragments from N-terminus to Lys-233 of the μ/δ3 and that from Ile-215 to C-terminus of the δ/μ5 were isolated and ligated into the plasmid vector pcDNA3 treated with *Hin*dIII and *Xba*I. Schematic drawings of μ/δ chimeras are shown in Table 2.

Nucleotide sequence determination [30] was performed to ensure successful generation of chimeras.

2.2. Stable expression in Chinese hamster ovary (CHO) cells

CHO cell lines stably expressing each of the seven chimeric μ/δ receptors, μ and δ receptors were established as described [31].

2.3. Transient expression in COS-1 cells

Wild types and chimeric μ/κ receptors were transfected into COS-1 cells with DEAE-dextran-chloroquine method [32] as described [28]. Cells were harvested for 48–60 h following transfection.

2.4. Membrane preparation

Membranes were prepared from CHO cells and COS-1 cells as described previously [29]. Protein contents of membranes were determined by the BCA method of Smith et al. [33] with bovine serum albumin as the standard.

2.5. Opioid receptor binding

Opioid receptor binding was conducted with [^{3}H]diprenorphine in TEL (50 mM Tris-HCl, 1 mM EGTA and 5 μM Leupeptin). (−)Naloxone (10 μM) was used to define nonspecific binding. Saturation experiments were performed with various concentrations of [^{3}H]diprenorphine (ranging from 0.05 nM to 5 nM) as described [28,29]. Inhibition of [^{3}H]diprenorphine binding was performed with [^{3}H]diprenorphine at a concentration close to its K_d for each receptor and various concentrations of sufentanil or lofentanil. Binding was conducted at 0°C for 2 h in duplicate in a volume of 1 ml with 30–60 μg protein. Bound and free ligands were separated by rapid filtration under reduced pressure over GF/B filters pre-soaked with 0.5% polyethyleneimine. Binding data were analyzed with EBDA and LIGAND programs [34].

2.6. Materials

[^{3}H]Diprenorphine (35 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Sufentanil and lofentanil were generously provided by Janssen Research Foundation (Beerse, Belgium). The vectors pRc/CMV and pcDNA3 were purchased from Invitrogen (San Diego, CA); pfu DNA polymerase and the vector pBK-CMV from Stratagene (San Diego, CA).

3. Results and discussion

Saturation binding of [^{3}H]diprenorphine to the chimeric μ/κ and μ/δ receptors was performed and compared with μ, κ and δ opioid receptors. All six chimeric μ/κ and seven μ/δ receptor bound [^{3}H]diprenorphine with high affinity with K_d values ranging from 0.14 to 1.2 nM ($K_d = 1.22 \pm 0.06$ nM for δ/μ5 and $K_d = 0.15 \pm 0.02$ nM for μ/δ3/μ5, $n = 3$ each) ([28,29] for all others). These K_d values are similar to those of μ, κ and δ receptors, suggesting that these chimeric receptors retain general structural conformation of opioid receptors.

Competitive inhibition of [^{3}H]diprenorphine binding by sufentanil or lofentanil to the six chimeric μ/κ receptors was performed and K_i values were determined and compared to those of μ and κ opioid receptors (Table 1; Fig. 1Fig. 2). Sufentanil bound to the μ receptor with K_i value of 0.71 ± 0.11 nM ($n = 3$), about 200-fold higher affinity than it did to the κ receptor ($K_i = 138 \pm 45$ nM, $n = 3$). Chimera II bound sufentanil with high affinity, similar to the μ receptor, and XII had an affinity ~7 times lower than the μ receptor. In contrast, I and XI had low affinity for sufentanil, similar to the κ receptor. Chimera III and IV had intermediate affinity for sufentanil, being 53 and 21 times lower than that of the μ receptor.

Lofentanil has a K_i value of 0.25 ± 0.08 nM ($n = 3$) for the μ receptor, about 50-fold higher than that for the κ receptor ($K_i = 11.8 \pm 0.4$ nM, $n = 3$). Like sufentanil, lofentanil bound to chimeras II and XII with similar high affinity as it bound to the μ receptor. Chimera I had low affinity, similar to the κ receptor. Chimeras III, IV and XI had intermediate affinity for lofentanil, with 3–9 times lower affinity than the μ receptor.

Thus, TMHs 6 and 7 and the e3 loop of the μ opioid receptor appear to be important for the selective binding of sufentanil and lofentanil over the κ receptor. In addition, the findings that chimera II bound sufentanil and, to a less extent,

Table 1. K_i valus of sufentanil and lofentanil for inhibition of [^{3}H]diprenorphine binding to the rat μ and κ opioid receptors and chimerec μ/κ receptors. Ki value were derived from results shown in figure 1 & 2. Data are shown as mean ± s.e.m. of three independent determinations in duplicate.

		Sufentanil K_i (nM)	Lofentanil K_i (nM)
μ		0.71±0.11[a]	0.25±0.08
κ		138 ± 45 [a]	11.8±0.4
I		153 ± 25	4.35±0.24
II		0.30±0.01	0.29±0.14
III		37.8±8.3	1.34±0.10
IV		15.0±1.0	0.82±0.18
XI		85.0±17.0 [a]	2.15±0.15
XII		4.70±0.70 [a]	0.34±0.11

a Xue et al. 1995.

Table 2. K_i values of sufentanil for inhibition of [^{3}H]diprenorphine binding to the rat μ and mouse δ opioid receptors and chimeric μ/δ receptors. K_i values were derived from results shown in Figures 3. Data are shown as mean ± s.e.m. of three independent determinations in duplicate.

		Sufentanil K_i (nM)
μ		0.61 ± 0.15
δ		148.3 ± 19.7
δ/μ1		4.6 ± 0.9
μ/δ1		153.6 ± 25.0
μ/δ3		0.83 ± 0.05
δ/μ4		6.00 ± 1.50
μ/δ4		49.0 ± 9.8
δ/μ5		26.0 ± 1.90
μ/δ3/μ67		3.17 ± 0.56

lofentanil with higher affinity than chimeras IV and XII and with the same high affinity as the μ receptor indicate that the interaction TMHs 1–3 and TMHs 6 and 7 is crucial for the high affinity binding of these two ligands. Since similar regions are important for the binding of sufentanil and lofentanil, it is likely that sufentanil and lofentanil bind to the binding pocket of the μ receptor in a similar manner.

Similar competitive inhibition of [^{3}H]diprenorphine binding by sufentanil were carried out with chimeric μ/δ receptors and μ and δ opioid receptors and K_i values were determined (Table 2 and Fig. 3). Chimera μ/δ3 displayed high binding affinity to sufentanil, similar to the μ receptor. δ/μ1, δ/μ4 and μ/δ3/μ67 exhibited 5–10-fold lower affinity than of the μ receptor, but 25–50-fold higher affinity than the δ receptor. Chimeras μ/δ4 and δ/μ5 bound sufentanil with lower affinities than the μ receptor by 40–80 fold. Chimera μ/δ1 had a K_i value similar to that of the δ receptor. Since lofentanil has only a 20-fold selectivity for the μ over δ receptor, with K_i values of 0.25 nM and 5.1 nM, respectively, its binding to μ/δ chimeras was not analyzed.

Thus, TMHs 1–3 and the e1 loop of the μ opioid receptor appear to confer binding selectivity for sufentanil over the δ receptor. While TMHs 6 and 7 do not play an important role in selective binding of sufentanil to the μ over δ receptor, the interaction between TMHs 1–3 and TMHs 6 and 7 is also important for the high affinity binding of sufentanil.

These results indicate that the region in the μ opioid receptor conferring selectivity for sufentanil over the κ receptors is TMHs 6–7 and e3 loop, whereas sufentanil distinguishes between μ and δ receptors at the region of TMHs 1–3 and e1 loop. Interestingly, we and others have observed a similar phenomenon regarding the binding of DAMGO [23,25–27]. DAMGO was found to distinguish between μ and κ receptors at the region of TMHs 6–7 and the e3 loop. In contrast, DAMGO distinguished between μ and δ receptors at the region of the e1 loop. It is, thus, likely that the binding pocket for sufentanil in the μ receptor is somehow similar to that for DAMGO. There may be a few of amino acids located in the TMHs 6–7 including the e3 loop and TMHs 1–3 including the e1 loop of the μ receptor, which are responsible for selective binding of these ligands to over the κ receptor and over the δ receptor, respectively. Alternatively, it is likely that TMHs 6–7 of the μ receptor provides the conformation of the μ opioid receptor to μ/κ chimera XII that favors high affinity binding of μ selective ligands, including morphine, DAMGO and sufentanil [23]. Similarly, TMHs 1–3 of the μ receptor may make μ/δ3 assume more μ receptor conformation that binds μ ligands with high affinity.

In addition, particularly by using μ/κ chimeric receptors, we observed that the interaction between TMHs 1–3 and TMHs 6 and 7 was important for the high affinity binding of sufentanil and lofentanil. This may be due to some inter-helical interactions creating a proper local conformation which is crucial for the high affinity binding of selective ligands to the μ receptor. Interhelical interactions have been demonstrated between an Asp in the TMH 2 and an Asn in the TMH 7 in the $5HT_{2A}$ receptor [35] and between an Asn residue in the TMH 2 and an Asp in the TMH 7 in the gonadotropin-releasing hormone receptor [36]. These interactions are shown to be important in maintaining proper receptor conformations for coupling to G proteins [35] or binding [36]. Besides interactions between specific amino acids, as pointed out by Kobilka et al. [37], the arrangement of TMHs relative to each other was most likely to be determined by interactions among various charged, uncharged polar and nonpolar amino acid residues and possible disulfide bond formation. The adjacent TMHs presumably have been evolved to have minimal steric hindrance and most favorable electrostatic interactions. Such interactions may be disrupted in some chimeric receptors.

Chimera μ/δ4 had lower affinities for sufentanil than μ/δ3, although μ/δ4 had more μ sequence than μ/δ3. This finding is reminiscent of our observation on SUPERFIT [29]. Despite the fact that δ/μ4 had more δ sequence than δ/μ3, chimera δ/μ4 had a lower affinity for SUPERFIT than δ/μ3. It is likely that the TMH 4 of δ receptor may not be as compatible with the TMH 5 of the μ receptor and vice versa. Chimeras that contain TMH 4 of one receptor and TMH 5 of the other

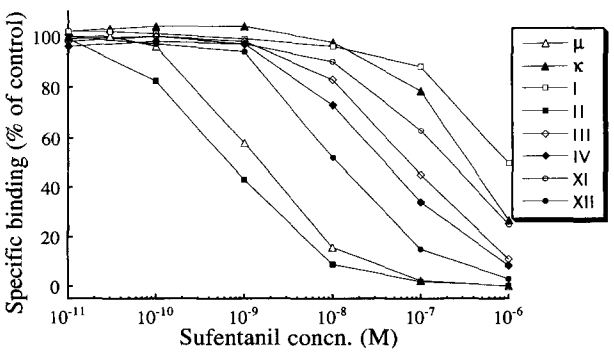

Fig. 1. Comparison of competitive inhibition by sufentanil of [^{3}H]diprenorphine binding to cloned rat μ and κ opioid receptors and μ/κ chimeras. K_i values of sufentanil are shown in Table 1. Each curve represents one of the three experiments performed.

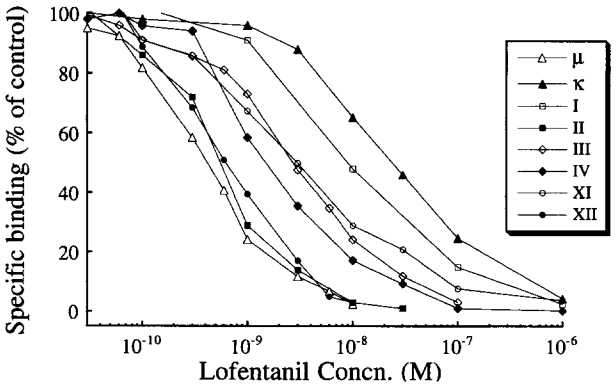

Fig. 2. Comparison of competitive inhibition by lofentanil of [^{3}H]diprenorphine binding to cloned rat μ and κ opioid receptors and μ/κ chimeras. K_i values of lofentanil are shown in Table 1. Each curve represents one of the three experiments performed.

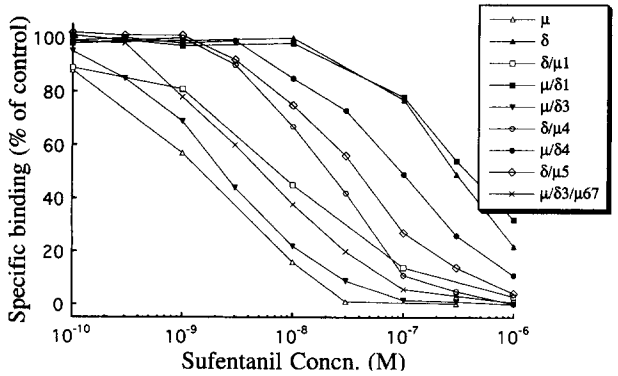

Fig. 3. Comparison of competitive inhibition by sufentanil of [^{3}H]diprenorphine binding to cloned rat μ and mouse δ opioid receptors and μ/δ chimeras. K_i values of sufentanil are shown in Table 2. Each curve represents one of the three experiments performed.

(such as μ/δ4 and δ/μ4) do not have the proper local conformation to bind sufentanil and SUPERFIT with high affinity, although both chimeras bound [^{3}H]diprenorphine, a nonselective ligand, with high affinity. Law et al. found that, in general, δ/μ4 had lower affinity for δ selective ligands than δ/μ3 (unpublished observations). However, μ/δ3 did not seem to have higher affinities for μ ligands than μ/δ4. Thus, this phenomenon might be limited to sufentanil and its analogs.

In conclusion, the regions conferring selectivity of sufentanil and lofentanil for the μ over the δ receptor are different from those for the μ over the κ receptor. These regions of the μ receptor are likely to contain sites of interaction with the ligands or to confer the μ receptor conformation. Chimeric receptor studies provide starting points for future studies on identification of amino acid residues as binding epitopes.

Acknowledgements: This work was supported by a grant from National Institute on Drug Abuse (DA 04745). J.Z. was supported by a training grant from National Institute on Drug Abuse (T32 DA07237). We thank Dr. C.J. Evans of the University of California Los Angeles for the mouse δ opioid receptor cDNA and Dr. L. Yu of the University of Indiana for the rat μ opioid receptor cDNA clone.

References

[1] Pasternak, G.W. (1988) The Opiate Receptors, Humana Press, Clifton, NJ.
[2] Kieffer, B., Befort, K., Gaveriaux-Ruff, C. and Hirth, C.G. (1992) Proc. Natl. Acad. Sci. USA 89, 12048–12052.
[3] Evans, C., Keith, D.E. Jr., Morrison, H., Magendzo, K. and Edwards, R.H. (1992) Science 258, 1952–1955.
[4] Chen, Y., Mestek, A., Liu, J., Hurley, J.A. and Yu, L. (1993) Mol. Pharmacol. 44, 8–12.
[5] Fukuda, K., Kato, S., Mori, K., Nishi, M. and Takeshima, H. (1993) FEBS Lett. 327, 311–314.
[6] Wang, J.B., Imai, Y., Eppler, C.M., Gregor, P., Spivak, C.S. and Uhl, G.R. (1993) Proc. Natl. Acad. Sci. USA 90, 10230–10234.
[7] Thompson, R.C., Mansour, A., Akil, H. and Watson, S.J. (1993) Neuron 11, 903–913.
[8] Bunzow, J.R., Zhang, G., Bouvier, C., Saez, C., Ronnekleiv, O.K., Kelly, M.J. and Grandy, D.K. (1995) J. Neurochem. 64, 14–24.
[9] Wang, J.-B., Johnson, P.S., Persico, A.M., Hawkins, A. L., Griffin, C.A. and Uhl, G.R. (1994) FEBS Lett. 338, 217–222.
[10] Bare, L.A., Mansson, E. and Yang, D. (1994) FEBS Lett. 354, 213–216.
[11] Raynor, K., Kong, H., Mestek, A., Bye, L.S., Tian, M., Liu, J., Yu, L. and Reisine, T. (1995) J. Pharmacol. Exp. Ther. 272, 423–428.
[12] Kaufman, D.L., Keith, D.E.J., Anton, B., Tian, J., Magendzo, K., Newman, D., Tran, T.H., Lee, D.S., Wen, C., Xia, Y. R., Lusis, A.J. and Evans, C.J. (1995) J. Biol. Chem. 270, 15877–15883.
[13] Yasuda, K., Raynor, K., Kong, H., Breder, C.D., Takeda, J., Reisine, T. and Bell, G.I. (1993) Proc. Natl. Acad. Sci. USA 90, 6736–6740.
[14] Li, S., Zhu, J., Chen, C., Chen, Y.-W., de Riel, J.K., Ashby, B. and Liu-Chen, L.-Y. (1993) Biochem. J. 295, 629–633.
[15] Chen, Y., Mestek, A., Liu, J. and Yu, L. (1993) Biochem. J. 295, 625–628.
[16] Nishi, M., Takeshima, H., Fukuda, K., Kato, S. and Mori, K. (1993) FEBS Lett. 330, 77–80.
[17] Minami, M., Toya, T., Katao, Y., Maekawa, K., Nakamura, S., Onogi, T., Kaneko, S. and Satoh, M. (1993) FEBS Lett. 329, 291–295.
[18] Meng, F., Xie, G.-X., Thompson, R.C., Mansour, A., Goldstein, A., Watson, S.J. and Akil, H. (1993) Proc. Natl. Acad. Sci. USA 90, 9954–9958.
[19] Xie, G., Meng, F., Mansour, A., Thompson, R.C., Hoversten, M.T., Goldstein, A., Watson, S.J. and Akil, H. (1994) Proc. Natl. Acad. Sci. USA 91, 3779–3783.
[20] Mansson, E., Bare, L. and Yang, D. (1994) Biochem. Biophys. Res. Comm. 202, 1431–1437.
[21] Zhu, J., Chen, C., Xue, J.-C., Kunapuli, S., de Riel, J.K. and Liu-Chen, L.Y. (1995) Life Sci. 56, PL201–207.
[22] Surratt, C.K., Johnson, P.S., Moriwaki, A., Seidleck, B.K., Blaschak, C.J., Wang, J.B. and Uhl, G.R. (1994) J. Biol. Chem. 269, 20548–20553.
[23] Xue, J.-C., Chen, C., Zhu, J., Kunapuli, S., de Riel, J.K., Yu, L. and Liu-Chen, L.-Y. (1995) J. Biol. Chem. 270, 12977–12979.
[24] Chen, C., Xue, J.-C., Zhu, J., Chen, Y.-W., Kunapuli, S., de Riel, J.K., Yu, L. and Liu-Chen, L.-Y. (1995) J. Biol. Chem. 270, 17866–17870.
[25] Minami, M., Onogi, T., Nakagawa, T., Katao, Y., Aoki, Y., Katsumata, S. and Satoh, M. (1995) FEBS Lett. 364, 23–27.
[26] Onogi, T., Minami, M., Katao, Y., Nakagawa, T., Aoki, Y., Toya, T., Katsumata, S. and Satoh, M. (1995) FEBS Lett. 357, 93–97.
[27] Fukuda, K., Kato, S. and Mori, K. (1995) J. Biol. Chem. 270, 6702–6709.
[28] Xue, J.-C., Chen, C., Zhu, J., Kunapuli, S., de Riel, J.K., Yu, L. and Liu-Chen, L.-Y. (1994) J. Biol. Chem. 269, 30195–30199.
[29] Zhu, J., Yin, J., Law, P.-Y., Claude, P.A., Rice, K.C., Evans, C.J., Chen, C., Yu, L. and Liu-Chen, L.-Y. (1996) J. Biol. Chem. 271, 1430–1434.
[30] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[31] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[32] Cullen, B. (1987) Methods Enzymol. 152, 684–704.
[33] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gart-

ner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[34] McPherson, G.A. (1983) Computer Prog. Biomed. 17, 107–114.
[35] Sealfon, S.C., Chi, L., Ebersole, B.J., Rodic, V., Zhang, D., Ballesteros, J.A. and Weinstein, H. (1995) J. Biol. Chem. 270, 16683–16688.
[36] Zhou, W., Flanagan, C., Ballesteros, J.A., Konvicka, K., Davidson, J.S., Weinstein, H., Millar, R.P. and Sealfon, S.C. (1994) Mol. Pharmacol. 45, 165–170.
[37] Kobilka, B.K., Kobilka, T.S., Daniel, K., Regan, J.W., Caron, M.G. and Lefkowitz, R.J. (1988) Science 240, 1310–1316.