Amino acid residues in the transmembrane domain of the type 1 sigma receptor critical for ligand binding

Hideko Yamamoto[a], Reiko Miura[b], Toshifumi Yamamoto[a,c], Keiko Shinohara[a], Masayuki Watanabe[a], Shigeru Okuyama[d], Atsuro Nakazato[d], Toshihide Nukada[b,*]

[a] *Department of Psychopharmacology, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan*
[b] *Department of Neurochemistry, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan*
[c] *Molecular Recognition, Yokohama City University, Yokohama 236-0027, Japan*
[d] *The 1st Laboratory, Medicinal Research Laboratory, Taisho Pharmaceutical, Saitama 330-8530, Japan*

Received 29 December 1998

Abstract The type 1 sigma receptor expressed in *Xenopus* oocytes showed binding abilities for the sigma-1 ligands, [^{3}H](+)pentazocine and [^{3}H]NE-100, with similar kinetic properties as observed in native tissue membranes. Amino acid substitutions (Ser99Ala, Tyr103Phe and di-Leu105,106di-Ala) in the transmembrane domain did not alter the expression levels of the type 1 sigma receptor as determined by immunoblot analysis using an anti-type 1 sigma receptor antiserum. By contrast, ligand binding was significantly suppressed by the substitutions. These findings provide evidence that the transmembrane domain of the type 1 sigma receptor plays a critical role in ligand binding of this receptor.

Key words: Type 1 sigma receptor; *Xenopus* oocyte; Ligand binding; Immunoblot analysis; Site-directed mutagenesis; Transmembrane domain

1. Introduction

Sigma receptors were initially postulated to account for the psychotomimetic actions of *N*-allylnormetazocine ((±)SKF-10.047) [1]. Subsequent biochemical and pharmacological studies using radioligands have demonstrated that the sigma receptors are distinct from opioid or phencyclidine binding sites, and then they can be classified into at least two subtypes designated 'sigma-1' and 'sigma-2' [2]. In addition, lines of evidence indicate that sigma receptors in the central nervous system are involved in drug addiction and affective disorders [3,4] and those in the immune system are involved in immunoregulations [5]. Their physiological roles, however, remain to be clarified, and their endogenous ligands have also yet to be determined.

Recently, cDNAs of the type 1 sigma receptor (SigmaR1) were cloned, and subsequently, their amino acid sequences were deduced [6–10]. The primary structure of SigmaR1 is highly conserved among a variety of mammalian species and tissues, indicating its importance in cellular functions. Interestingly, a protein targeted by SR 31747A, a sigma ligand and a novel immunosuppressant (or SR 31747A binding protein), is very similar to SigmaR1 with respect to structural and pharmacological profiles [11]. In addition, many antipsychotics, antidepressants and immunosuppressants have some affinity for sigma receptors [2]. While pharmacological characterization of sigma receptors using selective sigma-1 ligands is being in progress, the structure-affinity relationship of SigmaR1 remains to be established.

To address these issues at the molecular level, we cloned SigmaR1 cDNA from guinea pig liver, and expressed it in *Xenopus* oocytes by injection of cRNA derived from the cDNA. Using highly selective sigma-1 ligands, (+)pentazocine and *N*,*N*-dipropyl-2-(4-methoxy-3-(2-phenylethoxy)phenyl)-ethylamine monohydrochloride (NE-100) [12], the effects of site-directed mutagenesis of SigmaR1 on ligand binding were investigated.

2. Materials and methods

2.1. Materials

NE-100, [^{3}H]NE-100 (specific activity, 3145 GBq/mmol) and [^{3}H]azidoNE-100 (specific activity, 3070 GBq/mmol) were synthesized [12] in the Department of Organic Chemistry at the Research Center, Taisho Pharmaceutical (Saitama, Japan). [^{3}H](+)Pentazocine (specific activity, 2146 GBq/mmol) was purchased from New England Nuclear. All other chemical reagents were of analytical grade.

2.2. cDNA cloning and sequencing

Based on the cDNA sequence for the guinea pig SigmaR1 [6], two oligodeoxyribonucleotide primers were synthesized. The sense (S1) and the antisense (S2) primers corresponded to amino acid residues 1–7 and 217–223, respectively: S1, TTCACTCGAGGTGATGCAGTGGGC(A/G/C/T)GT(A/G/C/T)GG(A/G/C/T)CG; S2, GGCTGGTCAAGGGTCTTG(A/G/C/T)CC(A/G)AA(A/G/C/T)AG(A/G)TA. The first cDNA strands were synthesized by the S2-primed reverse transcription of total RNA from guinea pig liver using an RNA LA PCR Kit (Takara, Japan). Polymerase chain reactions (PCR) were carried out for 0.5 min at 94°C, 0.5 min at 60°C, and 1.5 min at 72°C for 30 cycles, by adding the primer S1 to the first strand reaction mixture containing the primer S2. The 700 bp products amplified by the PCR were purified, treated with T4 DNA polymerase with the four dNTPs, subcloned into the *Eco*RV site of pBluescript SK(−) (Stratagene) and then subjected to nucleotide sequence analysis using a DNA sequencer (ABI Prism 377, PE Applied Biosystems). The recombinant pBluescript plasmid carrying a cDNA encoding the entire coding sequence of SigmaR1 was referred to as 'pSR1'.

2.3. Construction of mutant SigmaR1

The genetic mutations of the transmembrane domain [7] of SigmaR1 were performed with PCR (see above) using the antisense primer S2 together with the sense primer SM1, SM2, SM3 or SM4 (see Fig. 1A). The primer SM1, CTTCTGCATGCCGCGCTGTCCGAG, contains the point mutation (G) replacing the first codon TCG coding for Ser-99 of SigmaR1 by GCG encoding Ala. The primer SM2, CTTCTGCATGCCTCGCTGTCCGAGTATGTGGCGGCCTTCGGCACC, contains the point mutations (GC) replacing the first and second codons of CTG and CTC coding for Leu-105 and Leu-106 by

*Corresponding author. Fax: (81) (3) 3329-8035.
E-mail: nukada@prit.go.jp

Abbreviations: SigmaR1, type 1 sigma receptor; NE-100, *N*,*N*-dipropyl-2-(4-methoxy-3-(2-phenylethoxy)phenyl)-ethylamine monohydrochloride; PCR, polymerase chain reactions; HRP, horseradish peroxidase; K_d, dissociation constant

PII: S0014-5793(99)00084-8

GCG and GCC encoding Ala, respectively. The primer SM3, CTTCTGCATGCCGCGCTGTCCGAGTATGTGGCGGCCTTCG-GCACC, contains the combined point mutations derived from the primers SM1 and SM2. Finally, the primer SM4, CTTCTGCAT-GCCTCGCTGTCCGAGTTTGTGCTGCTC, contains the point mutation (T) replacing the second codon TAT coding for Tyr-103 by TTT encoding Phe. The plasmid pSR1 (see above) was used as template. The 390 bp products amplified by the PCR using a set of primers of SM1/S2, SM2/S2, SM3/S2 and SM4/S2 were purified, blunted with T4 DNA polymerase, digested with *Sph*I, and cloned into *Sph*I/*Eco*RI (blunted) site of pSR1 in order to yield pSAR, pLAR, pSLAR and pYFR, respectively.

Subcloning and mutagenesis procedures were verified by restriction enzyme analysis and DNA sequencing.

2.4. Functional expression of wild-type and mutant SigmaR1

The 700 bp *Hin*dIII/*Eco*RI fragment containing the entire coding region of wild-type SigmaR1 was excised from the plasmids pSR1, blunted with T4 DNA polymerase, and inserted into the *Eco*RV site of pSPA2 [13] to synthesis cRNA specific for wild-type SigmaR1 in vitro using a MEGAscript SP6 Kit (Ambion). The 80 bp *Sph*I/*Bgl*II fragment of the resulting pSPA2 plasmid was exchanged by the 80 bp *Sph*I/*Bgl*II fragment derived from pSAR, pLAR, pSLAR or pYFR to synthesis mutant receptor cRNAs in vitro.

After removal of the follicular cell layer by treatment with 1 mg/ml collagenase for 1.5 h at 20°C [14], *Xenopus* oocytes were injected either with 0.5 μg/μl of wild-type or mutant sigma-1 receptor cRNA unless otherwise specified: the average volume of injection was ~50 nl per oocyte. The injected oocytes were incubated for 3 days, and then, subjected to immunoblot analysis and a binding assay.

2.5. Immunoblot analysis

A polyclonal antiserum (ASR1) was raised in New Zealand White rabbits against a synthetic docosapeptide (PSR1: CSEVFYP-GETVVHGPGEATAVE), corresponding to amino acid residues 143–163 of SigmaR1. An extra cysteine was added to the peptide on the N-terminus for conjugation with keyhole limpet hemocyanin.

Membrane fractions were obtained from oocytes as described in [15], and microsomal, mitochondrial and synaptosomal fractions were prepared from rat brain as described in [16]. These fractions (0.5 μg) were separated by 12.5% SDS-PAGE and transferred to a FluoroTrans membrane (Pall BioSupport) [17]. Membranes were blocked overnight at 4°C with 3% (w/v) BSA in TBS/Tw (20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.05% Tween-20). For immunostaining of SigmaR1, the antiserum ASR1 was diluted 500-fold in TBS/Tw containing 0.1% BSA. Before incubation with membranes (for 3 h at room temperature), the antiserum was pre-incubated with or without 100 μg/ml of the antigen peptide PSR1 for 1 h at 37°C. Then, membranes were incubated with horseradish peroxidase-(HRP-)conjugated goat anti-rabbit IgG (Biosource, at 1:2000 dilution). Labelled proteins were determined by diaminobenzidine-based HRP products with heavy metal intensification [18].

2.6. Binding assays

Radioligand binding assays with [^{3}H](+)pentazocine and [^{3}H]NE-100 were performed using oocyte membranes fractions according to the methods as described previously [19,20]. Saturation experiments were conducted over a concentration range of 2.0–15 nM [^{3}H](+)pentazocine, and NE-100 binding was carried out using 2.0 nM [^{3}H]NE-100. Non-specific binding was determined in the presence of 1 μM of haloperidol. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad). The dissociation constant (K_d) was determined using the computer program Ligand [21]. Statistical data are represented by the mean ± S.E.M.

Photolabelling assays with [^{3}H]azidoNE-100 were carried out using primary neuronal cells in culture obtained from rat cerebral cortex according to the procedures described [22].

3. Results and discussion

3.1. cDNA cloning of SigmaR1

Determination of the nucleotide and predicted amino acid sequences of the 690 bp insert of clone pSR1 (see Section 2) revealed three nucleotide changes from the sequence of guinea pig SigmaR1 [6], which resulted in three amino acid changes identical to the human version of SigmaR1 [7]: Val-71, Pro-114 and Arg-115 were determined as Gly, Arg and Gly, encoded by GGA, CGC and GGC, respectively. Six other nucleotide changes in the sequences were shown not to alter the coding amino acid residues: C (12), G (15), C (18), A (339), C (654) and C (657) of SigmaR1 [6] were G, T, T, C, A and T in our clones, respectively. Thus, the insert of the clone pSR1 contained the cDNA sequence encoding SigmaR1.

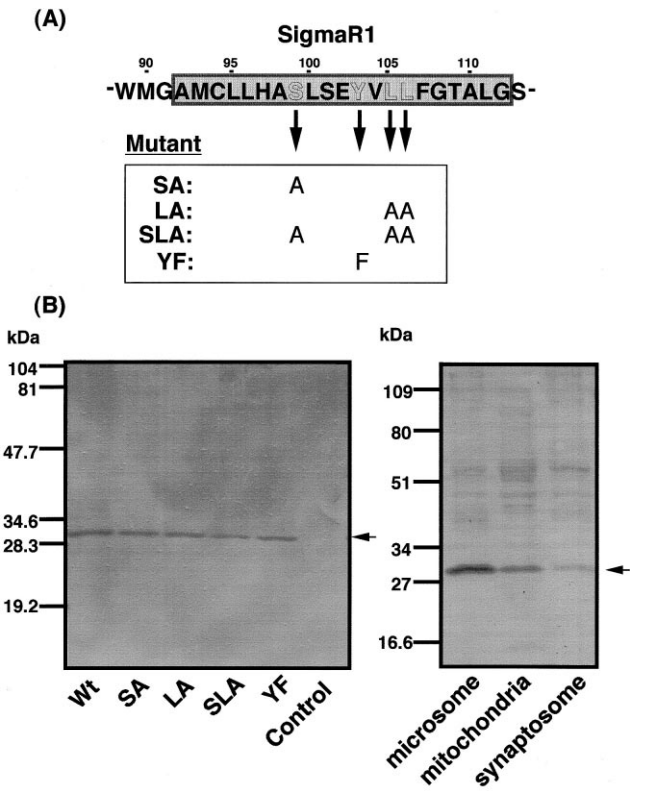

Fig. 1. A: Schematic representation of mutant SigmaR1 designated as SA, LA, SLA and YF. The putative transmembrane domain [7] is shaded in the amino acid sequence of SigmaR1. Amino acid substitutions on the mutant SigmaR1 are indicated by arrows. B: (left) Immunoreactivities with the antiserum ASR1 to the membrane fractions prepared from *Xenopus* oocytes without cRNA injection (control) or after injection of wild-type (Wt) or mutant (SA, LA, SLA or YF) SigmaR1 cRNA. Note that immunoreactive 29 kDa polypeptides (arrow) are not appreciably detectable unless cRNA for wild-type or mutant SigmaR1 is injected. B: (right) Immunoreactive 29 kDa polypeptides (arrow) with ASR1 in the microsomal, mitochondrial and synaptosomal fractions from rat brain, as indicated.

3.2. Expression of wild-type and mutant SigmaR1 in Xenopus oocytes

Aiming at identifying the regions on SigmaR1 that interact with sigma-1 ligands, four kinds of mutants SigmaR1 were generated and expressed in *Xenopus* oocytes (Fig. 1A). In mutants 'SA' and 'YF', Ser-99 and Tyr-103 were substituted by Ala and Phe, respectively. Mutant 'LA' had a substitution of di-Ala for di-Leu 105 and 106, and mutant 'SLA' had an additional substitution of Ala for Ser-99 on the mutant LA. The positions of these point mutations were assigned to the putative transmembrane domain of SigmaR1 [7] (Fig. 1A).

In oocytes injected with the wild-type SigmaR1 cRNA, the antiserum ASR1 reacted with a polypeptide of 29 kDa (Fig.

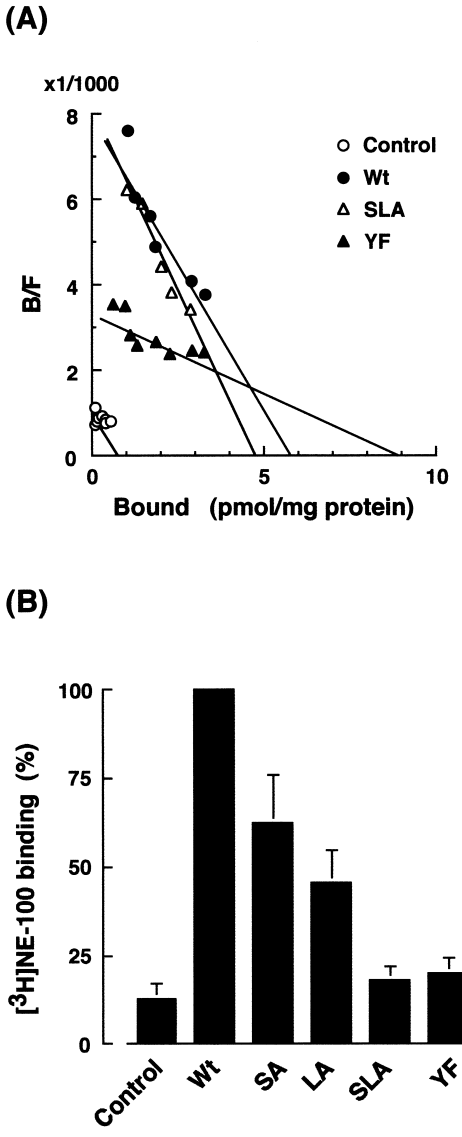

Fig. 2. A: Representative scatchard plots of [^{3}H](+)pentazocine binding using membranes (2.0–2.7 μg) prepared from *Xenopus* oocytes without cRNA injection (control) or after injection of wild-type (Wt) or mutant (SLA or YF) SigmaR1 cRNA (see Fig. 1A). B: Specific [^{3}H]NE-100 binding in membranes (0.7–14.1 μg) from *Xenopus* oocytes without cRNA injection (control) or after injection of wild-type (Wt) or mutant (SA, LA, SLA or YF) SigmaR1 cRNA (see Fig. 1A). The data are represented as the ratio of [^{3}H]NE-100 binding to that obtained from oocytes injected with wild-type SigmaR1 cRNA (Wt). The original bindings before normalization were 2.43 ± 0.59, 26.8 ± 10.7, 19.0 ± 9.2, 10.6 ± 4.5, 4.0 ± 1.4 and 4.3 ± 1.9 pmol/mg protein for the control, Wt, SA, LA, SLA and YF, respectively ($n = 5$). To lessen seasonal or individual variations [14], a set of oocytes injected with cRNAs for wild-type and mutant SigmaR1 was prepared at the same time for binding assays.

1B, left, Wt: arrow). This immunoreactivity was abolished by pre-incubation of ASR1 with the peptide antigen PSR1 ($n = 3$). Moreover, ASR1 scarcely reacted with the polypeptide unless oocytes were injected with SigmaR1 cRNA (Fig. 1B, left, control). These results indicate that an immunoreactive polypeptide of 29 kDa was recognized specifically by the antiserum against the synthetic SigmaR1 peptide. In addition, the antiserum ASR1 mainly reacted with a polypeptide of 29 kDa (Fig. 1B, right, arrow) in rat brain microsomal (microsome), mitochondrial (mitochondria) and synaptosomal (synaptosome) fractions, the reactivities that were also abolished by the pre-incubation of ASR1 with the peptide PSR1 ($n = 3$). The 29 kDa polypeptide was most predominant in the microsomal fraction. The size of this immunoreactive polypeptide corresponded well with the size of SigmaR1 reported [6] or the size (29–30 kDa) of a native sigma-1 ligand binding protein determined by selective covalent labelling of rat cortical neuronal culture using [^{3}H]azidoNE-100, an azido analog of the sigma-1 specific ligand NE-100 [12], as a photoactive radioligand ($n = 3$).

As shown in Fig. 1B (left: SA, LA, SLA and YF), the antiserum ASR1 reacted with a 29 kDa polypeptide in oocytes injected with each mutant SigmaR1 cRNA as well. These immunoreactivities were also abolished by pre-absorption of ASR1 with the antigen peptide PSR1 ($n = 3$). Moreover, levels of the immunoreactivities in the various mutant SigmaR1 were comparable with those in wild-type SigmaR1 (Fig. 1B, Wt). Thus, it is suggested that mutations in the transmembrane domain of SigmaR1 do not affect the expression of the receptors per se.

3.3. Ligand binding activities in wild-type and mutant SigmaR1

To test whether mutations in the transmembrane domain affect binding activities for sigma-1 ligands, saturation binding assays with [^{3}H](+)pentazocine, a prototype agonist of sigma-1 receptors, on *Xenopus* oocyte membranes were carried out after injection of wild-type or mutant SigmaR1 cRNA. Binding of [^{3}H](+)pentazocine to membranes prepared from oocytes injected with wild-type SigmaR1 cRNA was markedly increased (Fig. 2A, Wt) as compared with those from oocytes without injection of SigmaR1 cRNA (Fig. 2A, control). When [^{3}H]NE-100, a sigma-1 antagonist, was used as a radioligand instead of [^{3}H](+)pentazocine, an increase in the amount of binding was also produced by injection of wild-type SigmaR1 cRNA (Fig. 2B, control and Wt). Therefore, these findings were consistent with the results that the immunoreactivities with the SigmaR1 antiserum were not observed in *Xenopus* oocytes unless SigmaR1 cRNA was injected (Fig. 1B, left).

Scatchard analysis of the binding to membranes prepared from oocytes expressing wild-type SigmaR1 resulted in a linear plot (Fig. 2A, Wt) suggesting a single binding site for the exogenously-expressed SigmaR1 in *Xenopus* oocytes as reported in native tissue membranes [23]. The dissociation constant (K_d) was 11.7 ± 1.2 nM ($n = 6$) (Table 1). This K_d value was also comparable with that (14.2 ± 2.3 nM, $n = 3$) obtained

Table 1
Effects of amino acid substitutions in the transmembrane domain of SigmaR1 on the binding affinity for [^{3}H](+)pentazocine

SigmaR1	K_d (nM)
None (control)	30.4 ± 12.6 (3)
Wt	11.7 ± 1.2 (6)
SA	36.6 ± 3.9* (5)
LA	42.0 ± 4.2* (5)
SLA	9.9 ± 1.6 (5)
YF	49.0 ± 8.0* (6)

The dissociation constants (K_d) were determined by saturation binding of [^{3}H](+)pentazocine using membrane fractions prepared from *Xenopus* oocytes without cRNA injection (none (control)) or after injection of wild-type (Wt) or mutant (SA, LA, SLA or YF) SigmaR1 cRNA (see Figs. 1A and 2A). The number of experiments is indicated in parentheses. *$P < 0.01$ relative to wild-type SigmaR1.

from binding of [^{3}H](+)pentazocine to rat crude synaptosomal membranes prepared from rat cerebral cortex. Thus, all these findings indicate that exogenous wild-type SigmaR1 was functionally expressed in *Xenopus* oocytes.

As in the case of wild-type SigmaR1, binding analysis with [^{3}H](+)pentazocine was performed using membranes from oocytes expressing each mutant receptor. A decrease in the affinity of [^{3}H](+)pentazocine for SigmaR1 was produced in these three kinds of mutants, SA, LA and YF (Table 1, Fig. 2A: SA, LA and YF) as compared with Wt SigmaR1. The rank order of binding affinity for [^{3}H](+)pentazocine among these mutant SigmaR1 was SA > LA > YF (Table 1). By contrast, the mutant SLA, having combined mutations in SA and LA, did not show a marked change in the affinity (Table 1, Fig. 2A). Considering the results that both wild-type and four kinds of mutant SigmaR1 were expressed to a similar extent as immunoreactive polypeptides, it seems likely that these changes in the ligand binding affinity by the mutations are due to direct perturbation of the sigma ligand-SigmaR1 interactions.

To further investigate the ligand interactions, the sigma-1 antagonist [^{3}H]NE-100 was used as a radioligand for the binding assay. These mutations in the transmembrane domain of SigmaR1 also diminished specific [^{3}H]NE-100 binding (Fig. 2B: SA, LA and YF) as compared with wild-type SigmaR1 (Fig. 2B, Wt). The rank order of binding ability for [^{3}H]NE-100 was SA > LA > YF, which was identical to that for [^{3}H](+)pentazocine, indicating that these three kinds of mutations exert similar effects on both agonist and antagonist binding to SigmaR1. Among these mutations, the single YF mutation yielded a most appreciable decrease in binding activities for both [^{3}H](+)pentazocine and [^{3}H]NE-100. Therefore, it is indicated that the amino acid residue, Tyr-103, in the transmembrane domain is critical for ligand binding to SigmaR1.

Unlike [^{3}H](+)pentazocine binding, [^{3}H]NE-100 binding was almost completely abolished in the mutant SLA (Fig. 2B, SLA). This discrepancy between (+)pentazocine and NE-100 binding in the mutant SLA implies the presence of different recognition site(s) in the transmembrane domain of SigmaR1 for different ligands such as agonist and antagonist. The amino acid residues, Ser-99, Leu-105 and Leu-106 in the domain might play an important role in distinguishing each ligand through interaction with the ligand. Further studies using additional sigma-1 ligands and site-directed mutagenesis will be necessary to determine the direct interaction between specific amino acid residues on SigmaR1 and specific sigma-1 ligands.

Taken together, all these findings indicate that the segment of amino acid residues Ser-99–Leu-106 in the putative transmembrane domain of SigmaR1 serves as a crucial structural determinant in receptor-ligand interaction without changing the expression level of SigmaR1. By analogy to G protein-coupled receptors including β-adrenergic [24], muscarinic acetylcholine [25,26] and dopamine D_2 [27] receptors, it is plausible to assume that a ligand binding pocket for signal transduction lies in the transmembrane domain of SigmaR1.

Acknowledgements: The authors are grateful to Dr. Yoshiyuki Wada for providing us useful technical advice for preparing oocytes. We also thank Dr. Mitsunobu Yoshii for the critical reading of the manuscript. This investigation was supported in part by Research Grants from the Ministry of Education, Science and Culture of Japan to H.Y. (#09680781) and to T.N. (#08680855).

References

[1] Martin, W.R., Eades, C.G., Thomson, J.A., Hoppler, R.E. and Gilbert, P.E. (1976) J. Pharmacol. Exp. Ther. 197, 517–532.
[2] Walker, J.M., Bowen, W.D., Walker, F.O., Matsumoto, R.R., De Costa, B. and Rice, K.C. (1990) Pharmacol. Rev. 42, 355–402.
[3] Weissman, A.D., Casanova, M.F., Kleinman, J.E., London, E.D. and De Souza, E.B. (1991) Biol. Psychiatry 29, 41–54.
[4] Witkin, J.M. (1994) Neurosci. Biobehav. Rev. 18, 121–142.
[5] Liu, Y., Whitlock, B.B., Pultz, J.A. and Wolfe Jr., S.A. (1995) J. Neuroimmunol. 59, 143–154.
[6] Hanner, M., Moebius, F.F., Flandorfer, A., Knaus, H.-G., Striessnig, J., Kempner, E. and Glossmann, H. (1996) Proc. Natl. Acad. Sci. USA 93, 8072–8077.
[7] Kekuda, R., Prasad, P.D., Fei, Y.-J., Leibach, F.H. and Ganapathy, V. (1996) Biochem. Biophys. Res. Commun. 229, 553–558.
[8] Seth, P., Leibach, F.H. and Ganapathy, V. (1997) Biochem. Biophys. Res. Commun. 241, 535–540.
[9] Prasad, P.D., Li, H.W., Fei, Y.-J., Ganapathy, M.E., Fujita, T., Plumley, L.H., Yang-Feng, T.L., Leibach, F.H. and Ganapathy, V. (1998) J. Neurochem. 70, 443–451.
[10] Seth, P., Fei, Y.-J., Li, H.W., Huang, W., Leibach, F.H. and Ganapathy, V. (1998) J. Neurochem. 70, 922–931.
[11] Jbilo, O., Vidal, H., Paul, R., De Nys, N., Bensaid, M., Silve, S., Carayon, P., Davi, D., Galiègue, S., Bourrié, B., Guillemot, J.-C., Ferrara, P., Loison, G., Maffrand, J.-P., Le Fur, G. and Casellas, P. (1997) J. Biol. Chem. 272, 27107–27115.
[12] Okuyama, S., Imagawa, Y., Ogawa, S., Araki, H., Ajima, A., Tanaka, M., Muramatsu, M., Nakazato, A., Yamaguchi, K., Yoshida, M. and Otomo, S. (1993) Life Sci. 53, PL285–290.
[13] Nakamura, K., Nukada, T., Haga, T. and Sugiyama, H. (1994) J. Physiol. 474, 35–41.
[14] Takao, K., Yoshii, M., Kanda, A., Kokubun, S. and Nukada, T. (1994) Neuron 13, 747–755.
[15] Goodhardt, M., Ferry, N., Buscaglia, M., Baulieu, E.-E. and Hanoune, J. (1984) EMBO J. 3, 2653–2657.
[16] Jones, D.H. and Matus, A.I. (1974) Biochim. Biophys. Acta 356, 276–287.
[17] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[18] Adams, J.C. (1981) J. Histochem. Cytochem. 29, 775.
[19] Chaki, S., Tanaka, M., Muramatsu, M. and Otomo, S. (1994) Eur. J. Pharmacol. 251, R1–R2.
[20] Sagi, N., Yamamoto, H., Yamamoto, T., Okuyama, S. and Moroji, T. (1996) Eur. J. Pharmacol. 304, 185–190.
[21] Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107, 220–239.
[22] Schuster, D.I., Arnold, F.J. and Murphy, R.B. (1995) Brain Res. 670, 14–28.
[23] DeHaven-Hudkins, D.L., Fleissner, L.C. and Ford-Rice, F.Y. (1992) Eur. J. Pharmacol. 227, 371–378.
[24] Strader, C.D., Sigal, I.S., Candelore, M.R., Rands, E., Hill, W.S. and Dixon, R.A.F. (1988) J. Biol. Chem. 263, 10267–10271.
[25] Fraser, C.M., Wang, C.-D., Robinson, D.A., Gocayne, J.D. and Venter, J.C. (1989) Mol. Pharmacol. 36, 840–847.
[26] Page, K.M., Curtis, C.A.M., Jones, P.G. and Hulme, E.C. (1995) Eur. J. Pharmacol. 289, 429–437.
[27] Javitch, J.A., Li, X., Kaback, J. and Karlin, A. (1994) Proc. Natl. Acad. Sci. USA 91, 10355–10359.