A Single Residue, Aspartic Acid 95, in the δ Opioid Receptor Specifies Selective High Affinity Agonist Binding

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The enkephalins, dynorphins, and endorphins are endogenous opioids which function as neurotransmitters, neuromodulators, and hormones and are involved in the perception of pain, modulation of behavior, and regulation of autonomic and neuroendocrine function. Pharmacological studies have defined three classes of opioid receptors, designated as δ, κ, and μ. Investigate mechanisms by which agonists and antagonists interact with the δ opioid receptor, we have substituted aspartic acid 95 in the transmembrane segment 2 of the cloned mouse δ opioid receptor with an asparagine (D95N). The D95N mutant receptor had reduced affinity for δ receptor-selective agonists such as enkephalin, D-Pen2,D-Pen5
[3H]enkephalin and [D-Ser2,Leu5]enkephalin-Thr6 such that it did not bind these peptides even at micromolar concentrations. The binding of δ-selective non-peptide agonists was also reduced. In contrast, the δ receptor-selective antagonists, such as naltrindole, the benzo-furan analog of naltrindole, and 7-benzylidenediethylorphan, which interact with δ, κ, and μ opioid receptors, showed no difference in binding to the wild-type and mutant δ receptor. The D95N mutant remained coupled to G proteins, and the receptor was functionally active since it mediated agonist inhibition of cAMP accumulation. These results indicate that selective agonists and antagonists bind differently to the δ receptor and show that Asp-95 contributes to high affinity δ-selective agonist binding. The identification of a key residue involved in selective agonist binding to the δ opioid receptor will facilitate the development of novel therapeutic reagents that can be used for the treatment of chronic pain and other conditions.

The pentapeptides methionine- and leucine-enkephalin induce diverse actions in the central nervous system, including modulation of locomotor activity and analgesia (1-3). They induce their biological effects by interacting with δ opioid receptor. The ligand binding domain of several G protein-coupled receptors is generally believed to be composed of several charged residues within a hydrophobic pocket created by transmembrane spanning regions (4-6). A conserved aspartate in the second transmembrane spanning region of the α2-adrenergic receptor has been shown to be critical for agonist binding (6). The recently cloned mouse δ opioid receptor (7-9) has within its structure an aspartate at residue 95 in the second transmembrane spanning region which may be associated with the ligand binding domain. To determine the role of Asp-95 in ligand binding, we converted this residue in the cloned mouse δ receptor to an asparagine by site-directed mutagenesis and compared the ligand binding characteristics of the mutant and wild-type proteins after expression in COS-7 cells. Our findings show that this mutation alters the receptor so that it has very low affinity for δ-selective agonists but binds antagonists and non-selective agonists as well as the normal receptor. These findings indicate that selective agonists and antagonists bind differently to the δ receptor and reveal that this aspartic acid residue may contribute to stabilizing enkephalin binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Met-enkephalin, [d-Pen2,d-Pen5]enkephalin (DPDPE), [d-Ser2,Leu5]enkephalin-Thr6 (DSLET), d-Ala4
detorphin II, bre- mazocine, and naltrindole were obtained from Research Biochemicals Inc. (Natick, MA). (-)Buprenorphine (10) and BW373U86 (11) were prepared by Dr. Alan Cowan, Department of Pharmacology, Temple University. 7-Spiroindino-oxymorphone (SIOM), 7-benzylidenediethylorphan (BNTX), and the benzo-furan analog of naltrindole (NTB) were synthesized by Dr. P. Portoghese (12, 13). [3H]DPDPE (specific activity, 47.4 Ci/mmol) and [3H]naltrindole (specific activity, 31.2 Ci/mmol) were obtained from Du Pont-New England Nuclear.

**Mutagenesis of the Cloned Mouse δ Opioid Receptor**—The mouse δ opioid receptor cDNA (9) was mutated using the Altered Sites™ in vitro mutagenesis system (Promega LKB Biotechnology Inc.). To mutate aspartic acid 95 to an asparagine, the δ receptor cDNA was subcloned into the phagemid pALTERTM, and with the helper phage R408, a single-stranded template was produced. The 21-mer oligonucleotide (GCCTTGCTAATGGCCTGGCC) containing the desired mutation (GAT to AAT) was annealed to the single-stranded template and elongated with T4 DNA polymerase. The heteroduplexes

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1. The abbreviations used are: DPDPE, [d-Pen2,d-Pen5]enkephalin; DSLET, [d-Ser2,Leu5]enkephalin-Thr6; SIOM, 7-spiroindino-oxymorphone; BNTX, 7-benzylidene-dialtrazon; NTB, benzofuran analog of naltrindole; GTPγS, guanosine 5'-O-(thiotriphosphate).

2. Portoghese, P. S., Moe, S. T., and Takemori, A. E. (1993) J. Med. Chem., in press.
DNA was used to transform the repair-defective Escherichia coli strain BMH 71-18 mut S. Transformants were selected by growth in LB plates containing 125 μg/ml ampicillin. The mutation was confirmed by DNA sequencing. The cDNA was excised from pALTER with EcoRI and Saff and subcloned into the corresponding sites in the mammalian expression vector pCMV6c (14).

Expression of the Mouse 6 Opioid Receptor cDNA in COS-7 Cells—

The mutated and wild-type cDNAs were transfected into COS-7 cells by the calcium-phosphate-mediated procedure as previously described (9, 14). For the receptor binding studies, COS-7 cells expressing the δ receptor were harvested 72 h after transfection in 50 mM Tris-HCl (pH 7.8) containing 1 mM EGTA, 5 mM MgCl₂, 10 μM leupeptin, 10 μg/ml pepstatin, 200 μg/ml bacitracin, and 0.5 μg/ml apronitin (Buffer 1) and centrifuged at 24,000 × g for 7 min at 4 °C. The pellet was homogenized in Buffer 1 using a polytron. The homogenate was centrifuged at 46,000 × g for 20 min at 4 °C and the pellet resuspended in Buffer 1 and used in the radioligand binding assay. Cell membranes (20-30 μg of protein) were incubated with the δ-selective agonist [³H]DPDPE (15) (2 nM) or the δ selective antagonist [³H]naltrindole (3, 12, 13) (1 nM) in a final volume of 200 μl for 40 min at 25 °C in the presence or absence of competing agents. Nonspecific binding was defined as radioactivity remaining bound in the presence of 1 μM naltrindole. The binding reaction was terminated by the addition of ice-cold 50 mM Tris-HCl (pH 7.8) and rapid filtration over Whatman GF/B glass fiber filters that were pretreated with 0.5% polyethylene-imine and 0.1% bovine serum albumin. The filters were washed with 12 ml of ice-cold buffer, and the bound radioactivity was determined using a liquid scintillation counter. Data from radioligand binding studies were used to generate inhibition curves. IC₅₀ values were obtained by curve-fitting performed by the program FITCOMP.

CAMP Accumulation—CAMP accumulation in COS-7 cells expressing the δ receptors were measured as previously described (9, 14). Briefly, COS-7 cells were subcultured in 12-well culture plates. The cells were transfected 72 h prior to the CAMP experiments. Culture medium was removed from the wells and replaced with 500 μl of fresh medium containing 0.5 mM isobutylmethylxanthine. Cells were incubated for 20 min at 37 °C. Medium was removed and replaced with fresh medium containing 0.5 mM isobutylmethylxanthine, with or without 10 μM forskolin and various opioid agonists. The cells were incubated for 30 min at 37 °C. Medium was removed and cells sonicated in the wells in 50 mM Tris-HCl. The HCl was evaporated off in a Speed-Vac and the CAMP analyzed using a radioimmunoassay kit from Du Pont-New England Nuclear.

RESULTS

Both the wild-type and D95N mutant receptors expressed in COS-7 cells could be labeled with the δ-selective agonist [³H]DPDPE (15). However, the binding of [³H]DPDPE to the mutant receptor was reduced approximately 75% compared to the wild-type receptor. The binding of 2.0 nM [³H]DPDPE to the wild-type receptor was 261 fmol/mg protein and 68 fmol/mg protein for the D95N mutant. The reduced binding to the D95N mutant could be due to uncoupling of the receptor from G proteins, low levels of expression of the receptor, or an alteration in the ligand binding properties of the receptor. The mutant receptor remained coupled to G proteins since GTPγS reduced [³H]DPDPE binding to 83% of the wild-type receptor. The binding of 2.0 nM [³H]DPDPE to the wild-type receptor was 261 fmol/mg protein. Reduced binding to the D95N mutant was not altered, consistent with the aspartate 95 being the site of Na⁺ regulation of agonist binding to this receptor.

The D95N mutant receptor was expressed at higher levels than the wild-type receptor as determined by the binding of the antagonist [³H]naltrindole. Scatchard analysis of [³H]naltrindole binding (Fig. 3) revealed a Bmax and Kra for binding to the wild-type receptor of 6.4 pmol/mg protein and 0.14 nM, respectively. The Bmax and Kra for binding to the D95N mutant was 14.0 pmol/mg protein and 0.082 nM, respectively. These results indicate that the mutant receptor was expressed at a higher density than the wild-type receptor. Furthermore, the affinity of the mutant and wild-type receptors for the δ-selective antagonist naltrindole and the δ₁ and δ₂-selective antagonists (12, 13) BNTX and NTB, respectively, were similar (Table I). The normal binding affinity of the D95N mutant for antagonists indicates that the substitution of Asp-95 by Asn in the second transmembrane domain did not cause a gross conformational change in the structure of the receptor.

The results imply that the mutant receptor has a selective reduction in affinity for agonists. This is further indicated by the diminished potencies of the δ-selective agonists Met-enkephalin, DPDPE, N-Ala³-deltorphin II (17), and DSLET to inhibit [³H]naltrindole binding to the mutant receptor compared to the wild-type receptor (Fig. 4, Table I). The δ-selective agonists tested above are all peptides. To determine whether the differences in ligand binding properties of the mutant and wild-type receptors were due to the peptide nature of the agonists or their receptor selective characteristics, we tested two recently developed selective antagonists.

Fig. 1. Regulation of agonist binding to the wild-type δ opioid and D95N mutant receptor by GTP analogs and Na⁺. COS-7 cells were transfected with either the wild-type (dark bars) or D95N mutant (open bars) cDNA. [³H]DPDPE (2 nM) binding was inhibited with either GTPγS or Na⁺. 90 mM N-methyl-D-glucamine (NMDG) was used as a control for changes in ionic strength. These experiments, [³H]DPDPE binding to the wild-type and D98N mutants are 240 ± 34 and 85 ± 17 fmol/mg protein, respectively. These are the means ± S.E. of three different experiments.

Fig. 2. Inhibition of forskolin-stimulated CAMP formation in COS cells expressing the wild-type and D98N mutant δ receptor. COS-7 cells were transfected with either the wild-type (dark bar) or D98N mutant (open bar) δ receptor cDNA. Cells were stimulated with 10 μM forskolin in the presence or absence of 1 μM opioid agonists. Values are the means of three different experiments with S.E. less than 10% of the average.
potent, non-peptide δ-selective agonists, BW373U86 (11) and SIOM,2 for their interaction with the mutant δ receptor. Both compounds inhibit forskolin-stimulated cAMP formation in cells expressing either the wild-type or D95N mutant δ receptor (Fig. 2), indicating that they are full agonists at the δ receptor. BW373U86 and SIOM potently inhibit [3H]naltrindole binding to the wild-type δ receptor (Fig. 4, Table I). In contrast, they are much less potent at binding to the mutant δ receptor. Both the non-selective opioid receptor agonist bremazocine and the alkaloid buprenorphine also interact potently with μ, κ, and δ opioid receptors. It is a partial μ opioid agonist (10) and a full agonist at the δ receptor, since it inhibits forskolin-stimulated cAMP formation in COS cells expressing either the mutant δ or the wild-type receptor (Fig. 2). Buprenorphine binds to both the mutant and wild-type δ opioid receptors with similar potencies (Table I). The similar potency of bremazocine and buprenorphine at binding to the wild-type and mutant δ receptors indicates that non-selective agonists do not bind in the same manner to the cloned δ receptor as do δ-selective agonists.

**DISCUSSION**

The most important finding of this study is that δ-selective agonists bind differently to the cloned δ opiate receptor than do δ-selective antagonists or non-selective opioid agonists. The reduction in affinity of the mutant receptor for δ-selective agonists could be explained by the necessity of Asp-95 for selective agonist binding. The similar affinity of the mutant and wild-type receptors for antagonists indicate that Asp-95 is not necessary for antagonist binding. These findings indicate that there are distinct agonist and antagonist binding domains of the δ receptor and that Asp-95 is associated with the agonist binding domain. Alternatively, there are distinct conformational requirements for agonist and antagonist binding to the same ligand binding domain of the receptor and mutation of the Asp-95 to an Asn shifts the receptor to a conformation favoring antagonist binding. This latter possibility seems unlikely since non-selective agonists bind with equal potency to the mutant and wild-type δ receptors, indicating that the mutant receptor does not favor antagonist binding.

If the negative charge provided by Asp-95 serves as a counterion to the positive charge of the nitrogen atom in enkephalin and δ-selective agonist to stabilize binding, then it is likely that the Asp-95 is in close proximity to the agonist binding domain. This would allow the negative charge of the aspartic acid to be in contact with the positive charge of selective agonists bound to the receptor. However, it is unlikely that this residue is entirely responsible for selective agonist binding to the δ receptor since it is conserved in the...
cloned δ opioid receptor (9) which has agonist specificities distinct from those of the δ receptor.

Conversion of the Asp at residue 79 in the second transmembrane spanning region of the α2-adrenergic receptor to an Asn has been reported to induce a modest reduction in agonist affinity (6). A similar mutation of the somatostatin receptor subtype SSTR2 resulted in no change in agonist binding (19). The dramatic reduction in δ-selective agonist binding to the D95N mutant indicates that Asp-95 of the receptor is a site of action of Na+ in regulating agonist affinity (6). A similar mutation of the somatostatin receptor subtype SSTR2 resulted in no change in agonist affinity (6, 19). Na+ has been proposed to regulate agonist binding (6, 19). These findings have been based on the use of the selective antagonist BNTX and the 6z-selective antagonist NTB. Our findings that the cloned receptor is a unique role in agonist binding to this receptor.

Interestingly, the conserved Asp of the δ opioid, α2-adrenergic and somatostatin receptors is necessary for Na+ regulation of agonist binding (6, 19). Na+ has been proposed to affect agonist binding by directly interacting with receptors to cause G protein uncoupling, thereby converting the receptor into a low affinity state for agonists (6). This effect of sodium to diminish agonist binding was first identified for opioid receptors (20). The findings of the present study suggest that the Asp-95 is the site of action of Na+ in regulating agonist binding to the δ opioid receptor.

Previous behavioral studies have suggested that subtypes of δ opioids are expressed in the central nervous system (12, 13). These findings have been based on the use of the δ-selective antagonist BNTX and the δ-selective antagonist NTB. Our findings that the δ receptor subtype-selective antagonist NTB has 500-fold higher potency at binding to the cloned δ receptor than the δ receptor-selective antagonist BNTX, suggests that the cloned receptor is a δ2 subtype. Similar antagonist binding specificities have also been observed for δ receptors endogenously expressed in NG108 cells. Recently, Evans et al. (7) cloned a δ receptor from an NG108 cDNA library, with identical amino acid sequence as the one examined in the present study, further indicating that the cloned opioid receptor is a δ2 subtype.

Our findings that selective agonists and antagonists bind differently to the δ receptor may facilitate the development of more selective δ agonists that could be used for the treatment of chronic pain. Furthermore, recent studies have suggested that δ receptor antagonists may be useful in the treatment of cocaine (21) and ethanol (22) abuse as well as in the prevention of graft rejection (23). Identification of the unique antagonist binding domain in the δ receptor could facilitate further development of selective antagonists at the δ receptor and its subtypes for the treatment of these disorders.

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REFERENCES
1. Koo, G., and Bloom, F. (1992) Science 242, 715-720
2. Gilman, A. Bell, J., Nies, A., and Taylor, P. (1990) The Pharmacological Basis of Therapeutics, 8th Ed., pp. 483-521, Pergamon Press, New York
3. Simon, E. (1991) Med. Res. Rev. 11, 357-374
4. Strader, C., Sigal, I., Register, R., Candelore, M., Rands, E., and Dixon, R. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4584-4588
5. Chung, F., Wang, C., Porter, P., Venter, J., and Fraser, C. (1988) J. Biol. Chem. 263, 4555-4555
6. Horstman, D., Brandon, S., Wilson, A., Guyer, C., Cragoe, E. Jr., and Limbird, L. (1990) J. Biol. Chem. 265, 21590-21685
7. Evans, C., Keith, D., Morrison, H., Magendzo, K., and Edwards, R. (1992) Science 258, 1952-1955
8. Kieffer, B., Befort, K., Gaveriaux-Ruff, C., and Hirth, C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 12048-12052
9. Yasuda, K., Raynor, K., Kong, H., Breder, C., Takeda, J., Reisine, T., and Bell, G. I. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6736-6740
10. Cowan, A., and Lewis, J. (1993) Buprenorphine: A Unique Opioid, Wiley-Liss, New York, in press
11. Lee, F. H., McNutt, R., and Chang K-J. (1992) International Narcotics Research Conference, Keystone, CO, Abstract 34
12. Sofuoglu, M., Portoghese, P., and Takemori, A. (1991) J. Pharmacol. Exp. Ther. 257, 676-686
13. Portoghese, P., Sultana, M., Nagase, H., and Takemori, A. (1992) Eur. J. Pharmacol. 218, 195-196
14. Yasuda, K., Reza-Domiano, S., Breder, C., Law, S., Saper, C., Reisine, T., and Bell, G. I. (1992) J. Biol. Chem. 267, 20422-20428
15. Mosberg, H. I., Hutson, R., Huby, V. J., Gao, K., Yamamura, H. I., Galligan, J., and Burke, T. F. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5871-5874
16. Gacel, G., Fournie-Zaluski, M., and Roques, B. P. (1980) FEBS Lett. 118, 245-247
17. Erspamer, V., Melchiorri, R., Falconieri-Erspamer, G., Negri, L., Corsi, R., Severini, C., Barra, D., Simmaco, M., and Kreil, G. (1980) Proc. Natl. Acad. Sci. U. S. A. 89, 12048-12052
18. Herz, A. (1993) Opioids I, p. 6, Springer-Verlag, Berlin
19. Kong, H., Raynor, K., Yasuda, K., Bell, G. I., and Reisine, T. (1993) Mol. Pharmacol., in press
20. Pert, C., and Snyder, S. H. (1974) Mol. Pharmacol. 10, 868-879
21. Meekins, K., Bilyar, E., Wild, K., Portoghese, P., Reid, L., and Porreca, F. (1992) Eur. J. Pharmacol. 218, 340-346
22. Froehlich, J., Zweifel, M., Li, T., and Portoghese, P. (1991) Research Society of Alcoholism Conference, Marco Island, Fl, Abstract 20
23. Arakawa, K., Akami, T., Okamoto, M., Oka, T., Nagase, H., and Matsuzato, S. (1992) Transplantation 53, 802-803

3 K. Raynor, G. I. Bell, and T. Reisine, unpublished findings.