Irreversible Binding of cis-(+)-3-Methylfentanyl Isothiocyanate to the \( \delta \) Opioid Receptor and Determination of Its Binding Domain*

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Jinmin Zhu, Jiling Yin, Ping-Yee Law‡, Patricia A. Claude‡, Kenner C. Rice§, Christopher J. Evans¶, Chongguang Chen, Lei Yu* and Lee-Yuan Liu-Chen**

From the Department of Pharmacology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140, ‡Department of Pharmacology, University of Minnesota School of Medicine, Minneapolis, Minnesota 55455, §Laboratory of Medicinal Chemistry, NIDDK, National Institutes of Health, Bethesda, Maryland 20892, ¶Neuropsychiatry Institute, University of California, Los Angeles, California 90024, and Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana 46202

Binding of cis-(+)-3-methylfentanyl isothiocyanate (SUPERFIT) to cloned opioid receptors stably expressed in Chinese hamster ovary cells was characterized. SUPERFIT inhibited \([\text{3}H] \text{diprenorphine binding with much higher affinity for the } \delta \text{ than the } \mu \text{ or } \kappa \text{ receptor. Pretreatment with SUPERFIT followed by extensive} \] washing reduced \( \delta \) binding with an IC\(_{50}\) value of 7.1 nM, yet it did not affect \( \mu \) and \( \kappa \) binding up to 0.1 \( \mu \). The reduction in \( \delta \) binding by SUPERFIT pretreatment was due to a decrease in \( B_{\text{max}} \) with no change in \( K_{d} \). These results indicate that SUPERFIT is a highly selective \( \delta \) irreversible ligand. We then determined the region in the \( \delta \) receptor that conferred binding selectivity for SUPERFIT by examining its binding to six \( \mu/\delta \) chimeric receptors. SUPERFIT bound to \( \delta, \mu/\delta1 \) (amino acids 1-94/676-372), \( \delta/\mu3 \) (61-134/154-398), and \( \delta/\mu4 \) (61-187/207-398) receptors with high affinity but to \( \mu, \delta/\mu1 \) (61-75/95-398), \( \mu/\delta3 \) (1-153/335-372), and \( \mu/\delta4 \) (1-206/188-372) receptors with low affinity. Pretreatment with SUPERFIT potently inhibited \([\text{3}H] \text{diprenorphine binding to } \delta, \mu/\delta1, \delta/\mu3, \text{ and } \delta/\mu4 \text{ but affected binding to } \mu, \delta/\mu1, \mu/\delta3, \text{ and } \mu/\delta4 \text{ only at much higher concentrations. Thus, the segment from the beginning of the first intracellular loop to the middle of the third transmembrane helix of the } \delta \text{ receptor is important for selective binding of SUPERFIT.}

Opiate and opioid drugs, acting on membrane-bound receptors, have been widely used as analgesics. They, however, also produce side effects such as respiratory depression, decreased gastrointestinal motility, sedation, and mood changes (1). At least three types (\( \mu, \delta, \text{ and } \kappa \)) of opioid receptor are present in the nervous system (1). Many structurally diverse opiates and opioid compounds have been synthesized aiming to minimize the side effects and to understand the structure-function relationship. Some drugs act nonselectively on all three types of opioid receptors, yet there are ligands selective for each receptor.

Among the agents synthesized are affinity ligands or irreversible ligands. An affinity ligand is thought to bind to the receptor and then form a covalent bond on or near the binding site, resulting in irreversible attachment (2, 3). Affinity ligands have been very useful in the purification of receptors such as the \( \delta \) opioid receptor (4) and in the elucidation of receptor structure. Specific incorporation of radiolabeled affinity ligand into the receptor followed by polyacrylamide gel electrophoresis and fluorography or autoradiography has been used to identify the receptor, to determine molecular mass of the receptor without purification, and to examine the nature of carbohydrate moieties (for example, see Refs. 5 and 6). Because of the covalent nature of the bond between the ligand and the receptor, the site of incorporation, thus part of the binding domain, can be precisely determined by peptide mapping and/or determination of amino acid sequences of labeled fragments (for example, see Ref. 7).

SUPERFIT, \(^1\) an isothiocyanate derivative of cis-(+)-3-methylfentanyl (Fig. 1), was synthesized by Burke et al. (8) and found to bind irreversibly to the \( \delta \) receptor. Incubation of NG108-15 cell membranes with SUPERFIT (1-5 nm) followed by extensive washing reduced \( \delta \) binding by 60-90%. The reduction in \( \delta \) binding was due to a decrease in \( B_{\text{max}} \) with no change in \( K_{d} \) (8). Similar preincubation of rat brain membranes with SUPERFIT (1-5 nm) greatly reduced \( \delta \) receptor binding, without affecting \( \mu \) receptor binding. The (+)-enantiomer (SUPERFIT) was 50 times more potent than its (−)-enantiomer (9). The high affinity and enantioselectivity of SUPERFIT indicate that the irreversible binding is the result of selective interaction with the \( \delta \) receptor. \(^{1} \text{[\text{3}H]SUPERFIT was shown to label the } \delta \text{ receptor in NG108-15 cells as a } 58-\text{kDa} \text{ protein band in a denaturing gel (4).} \) \(^{1} \text{[\text{3}H]SUPERFIT-labeled } \delta \text{ receptor was purified} \) to homogeneity (4).

\( \delta \) opioid receptors have been cloned from several species (10-16). In addition, \( \mu \) and \( \kappa \) opioid receptors have been cloned (Refs. 17 and 18 and references therein). All three opioid receptors contain seven putative transmembrane helices (TMHs), a common structural motif of a 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.

Chimeric \( \mu/\kappa, \delta/\kappa, \text{ and } \mu/\delta \) receptors have been used to delin-
peptide antagonists, including naltrindole, (5 in the vicinity of points Dots receptor.

receptors. For instance, by examining the binding characteristics of μκ or δ/μ chimeric receptors, we (19), Wang et al. (20), and Meng et al. (21) demonstrated that the second extracellular loop of the κ receptor was essential for the high affinity binding of the dynorphin family peptides. For the δ receptor, by using chimeric κ/δ receptors, Meng et al. (21) found that replacement of TMHs 5–7 of the δ receptor with those of the κ receptor greatly reduced binding of δ-selective peptide ligands such as cyclic [d-Pen2,4-D-Phe1]enkephalin (DPDPE), [d-Pen2]enkephalin-Thr, and ICI174,864, as well as non-peptide antagonists, including naltrindole, (5α)-17-(cyclopropylmethyl)-4,5-epoxy-3,14-dihydroxy-7-(phenylmethylene)-morphinan-6-one (BNTX), and naltriben. In addition, by examining binding of μ/δ chimeras, Fukuda et al. (22) reported that the major determinant for selective binding of DPDPE was in TMHs 5–7.

In this study, we characterized the binding of SUPERFIT to cloned μ, δ, and κ receptors (11, 23, 24). In addition, we determined the region in the δ receptor that conferred selectivity for SUPERFIT binding by examining its binding to chimeric μ/δ receptors constructed from cloned rat μ and mouse δ opioid receptors (11, 23).

**EXPERIMENTAL PROCEDURES**

Materials—[3H]Diprenorphine (35 Ci/mmol) was purchased from Amersham Corp. Naloxone was generously provided by DuPont Merck Pharmaceutical Co. (Wilmington, DE).

Construction of Chimeric μ δ Receptors—Six chimeric μ δ receptors ([δ/μ]1, [δ/μ]3, [δ/μ]4, and [μ/δ]4) (Fig. 2) were constructed from the mouse δ opioid receptor (11) and the rat μ opioid receptor (23). Chimeras were constructed either by generating receptor fragments with polymerase chain reaction followed by ligation of the fragments or by introducing a specific restriction site with site-directed mutagenesis. Chimera [δ/μ]1 (amino acids (aa) 61–75/μ95–398) and chimera [μ/δ]1 (aa 1–94/967–372) were constructed by swapping the regions from the N terminus to the start of the first intracellular loop. A SbaI site was introduced at Val-94/Arg-95 of the μ receptor and at Val-75/Arg-76 of the δ receptor. Chimera [δ/μ]3 (aa 134/154–398) and chimera [μ/δ]3 (aa 1–153/135–372) were generated by exchanging the regions from the N terminus to the middle of the third TMH. A SpeI site was introduced at Thr-134/5er-135 of the δ receptor and Thr-153/ser-154 of the μ receptor, and fragments were generated by polymerase chain reaction. Chimera [δ/μ]4 (aa 1–187/μ207–372) and chimera [μ/δ]4 (aa 1–206/6188–372) were constructed by exchanging the regions from the N terminus to the beginning of the second extracellular loop. A NheI site was introduced at Ala-206/Thr-207 of the μ receptor and at Ala-187/Val-188 of the δ receptor.

For each chimera, DNA sequence of the fragment generated by polymerase chain reaction and that in the transitional region between two receptors was determined to ensure successful in-frame construction.

Stable Expression in CHO Cells—CHO cell lines stably expressing the μ receptor, the δ receptor, or each of the six chimeras were established as described (25).

Cell Membrane Preparation—Membranes were prepared by hypotonic lysis with 20 mM Tris-HCl buffer (pH 7.5), 5 mM MgCl2, followed
SUPERFIT Binds Irreversibly to the Cloned δ Receptor

| Table I |
|----------|
| K<sub>d</sub> and B<sub>max</sub> of [³H]diprenorphine binding to rat μ, δ, and κ opioid receptors and μ/δ chimeric receptors expressed in CHO cells. |
| Data are shown as mean ± S.E. of three independent experiments in duplicate. |
|  |
|  |
| K<sub>d</sub> | B<sub>max</sub> |
| [nM] | fmol/ mg protein |
| δ | 0.45 ± 0.04 | 1024 ± 80 |
| μ | 0.33 ± 0.03 | 3062 ± 98 |
| κ | 0.26 ± 0.03 | 1343 ± 74 |
| μ/δ1 | 0.44 ± 0.03 | 693 ± 14 |
| μ/δ2 | 0.38 ± 0.03 | 548 ± 30 |
| μ/δ3 | 0.22 ± 0.08 | 459 ± 21 |
| δ/μ3 | 0.12 ± 0.01 | 272 ± 55 |
| μ/δ4 | 0.15 ± 0.01 | 417 ± 37 |
| δ/μ4 | 0.13 ± 0.01 | 721 ± 13 |

Results and Discussion

Phosphate-based buffer, in place of Tris buffer, was used in binding experiments (10 mM KH₂PO₄, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, pH 8.0) to avoid reaction of the isothiocyanate group of SUPERFIT with Tris. This buffer was used in previous SUPERFIT studies (4, 8). Saturation binding of [³H]diprenorphine to the membranes of CHO cells stably transfected with δ, μ, or κ receptor was carried out (Table I). K<sub>d</sub> values were determined to be 0.33, 0.45, and 0.26 nm for μ, δ, and κ receptors, respectively. These values are similar to those determined in Tris-HCl buffer (11, 19, 23, 24).

Binding of SUPERFIT to cloned μ, δ, and κ opioid receptors was investigated. SUPERFIT inhibited [³H]diprenorphine (37°C, 60 min) binding to cloned δ, μ, and κ opioid receptors with IC₅₀ values of 3.4, 680, and 747 nm, indicating that SUPERFIT had ≈200-fold selectivity in this assay for δ over μ or κ receptors, respectively (Fig. 3; also see Table III). Pretreatment of the δ receptor with various concentrations of SUPERFIT (37°C, 45 min) followed by extensive washing reduced binding in a concentration-dependent fashion with an IC₅₀ value of 7.1 nM, but it did not affect μ or κ binding at ≈0.1 μM (Fig. 4; also see Table III). In contrast, pretreatment with 1 μM fentanyl (Fig. 1) did not reduce δ binding, as compared with the untreated control. Reduction in the δ binding by SUPERFIT was examined further. The δ receptor was treated with 5 nm
SUPERFIT Binds Irreversibly to the Cloned δ Receptor

**Table III**

IC₅₀ values (in nM) of SUPERFIT for inhibition of [³H]diprenorphine binding to mouse δ and rat µ opioid receptors and chimeric µ/δ receptors.

| Receptors       | δ  | µ  | δ/µ1 | δ/µ1 | δ/µ3 | δ/µ3 | δ/µ4 | δ/µ4 |
|-----------------|----|----|------|------|------|------|------|------|
| IC₅₀ reversibly | 3.4±0.7 | 680±49 | 743±47 | 4.1±2.2 | 3.1±0.5 | 599±102 | 36.0±3.8 | 319±41 |
| IC₅₀ irreversibly | 7.1±1.3 | >1000 | >1000 | 6.9±1.1 | 2.3±0.4 | >1000 | 53.5±7.0 | >1000 |

**Fig. 6. Effect of pretreatment with various concentrations of SUPERFIT on chimeric µ/δ receptors.** Membranes were pretreated with various concentrations of SUPERFIT or 1 μM fentanyl (control), and binding was performed with [³H]diprenorphine as described under **“Experimental Procedures.”** Each point represents mean ± S.E. of three experiments. IC₅₀ values are shown in Table III.

SUPERFIT or 1 μM fentanyl (control) and washed three times, and saturation experiments with [³H]diprenorphine were performed. SUPERFIT pretreatment decreased the B_max by 40% without changing K_d compared with fentanyl pretreatment (Table II). These results are consistent with previous observations of Burke et al. (8) and Kim et al. (9) that SUPERFIT was a highly selective δ irreversible ligand. It thus can be used to probe the structure of binding pocket of the δ receptor.

To determine the structural basis in the δ receptor for selective binding of SUPERFIT, we examined its binding to three pairs of chimeric µ/δ receptors (Fig. 5, Table III). All chimeric µ/δ receptors bound [³H]diprenorphine with high affinity with K_d values ranging from 0.13 to 0.44 nM (Table I), indicating that these chimeric receptors retain opioid receptor conformation to some extent. B_max values of µ/δ chimeric receptors stably expressed in CHO cells varied from 200 to 700 fmol/mg of protein, which were lower than µ and δ receptors (1024 and 3062 fmol/mg of protein, respectively). In all binding experiments, we used [³H]diprenorphine at a concentration close to the K_d and kept the receptor concentration at <10% of the K_d value. Chimeras µ/δ1 and µ/δ3 bound SUPERFIT with high affinity, similar to the δ receptor. In contrast, δ/µ1, µ/δ3, and µ/δ4 had low affinity, similar to the µ receptor. Chimera δ/µ4 had intermediate affinity for SUPERFIT (Fig. 5, Table III).

In addition, µ/δ chimeras were examined for their abilities to bind SUPERFIT irreversibly. Pretreatment with SUPERFIT followed by three washes potently inhibited [³H]diprenorphine binding to µ/δ1 and δ/µ3 with IC₅₀ values of 6.9 and 2.3 nM, respectively, but did not inhibit [³H]diprenorphine binding to δ/µ1, µ/δ3, and µ/δ4 (IC₅₀ values >1 μM) (Fig. 6, Table III). The potency for δ/µ4 was moderate (IC₅₀ = 53.5 nM). These data indicate that the segment from the N-terminal portion of the first intracellular loop to the middle of the third TMH is important for reversible and irreversible binding of SUPERFIT to the δ receptor. Since the sequences within the first intracellular loop, the TMH 2 and TMH 3, are highly homologous between the two receptors, the selectivity to SUPERFIT is most likely conferred by the first extracellular loop of the δ receptor.

Two possibilities may exist in terms of the irreversible binding site of SUPERFIT. First, the site of SUPERFIT irreversible binding is within this region. It has been thought that the isothiocyanate group of SUPERFIT reacts with a cysteine or lysine residue to form a covalent bond. Second, chimeras µ/δ1 and µ/δ3 may assume favorable conformations for SUPERFIT to form a covalent bond with the receptor, whereas chimeras δ/µ1, µ/δ3, and µ/δ4 do not. When [³H]SUPERFIT is available, we will be able to differentiate these two possibilities by limited proteolysis of [³H]SUPERFIT-labeled δ receptor and determination of the size of labeled peptide fragments. Chimeric receptor studies, nonetheless, provide starting points for future studies on identification of smaller fragments and, ultimately, amino acid residues as binding epitopes.

Although SUPERFIT is a δ selective irreversible ligand, its parent compound, cis-(-)-3-methylfentanyl is a selective µ agonist (27). Similarly, fentanyl is a selective agonist for the µ receptor (28), yet its isothiocyanate derivative, FIT, is a selective irreversible ligand for the δ receptor (29). Thus, the isothiocyanate functional group confers not only the irreversible binding characteristics but also the δ selectivity of both SUPERFIT and FIT. Determination of the site of SUPERFIT incorporation with δ receptor will provide insights into how binding pockets of µ and δ receptors differ.

Kong et al. (30) reported that an aspartate residue in the TMH 2 of the δ receptor was critical for high affinity binding of selective agonists. This Asp may also be important for the binding of SUPERFIT. However, this residue is unlikely to be the site of irreversible binding of SUPERFIT since 1) it is conserved among most G-coupled receptors including µ and κ opioid receptors (23, 24); 2) it is also important for agonist binding of the µ opioid receptors (31); and 3) the isothiocyanate group does not react with aspartate.

In this study, within each of the three pairs of chimeras, one is the "mirror image" of the other. We demonstrated that for the two pairs of δ/µ1-µ/δ1 and δ/µ3-µ/δ3, there was not only a reduction in SUPERFIT affinity in one of the pair but also acquisition or preservation of high affinity binding in the other, compared with the δ receptor. By use of such pairs, our conclusion is strengthened.

Chimera δ/µ4 had a lower affinity for SUPERFIT than δ/µ3, although δ/µ4 had more δ sequence than δ/µ3. This finding is in...
agreement with that of Law et al., who found that, in general, 
\( \delta/4 \) had lower affinity for \( \delta \) selective ligands than \( \delta/3 \). It is likely that interactions among TMHs are important in determining the conformation of the receptor binding pocket. The TMH 4 of \( \delta \) receptor may not be as compatible with the TMH 5 of the \( \mu \) receptor, thus creating a chimera (\( \delta/4 \)) that does not bind SUPERFIT as well as the \( \delta/3 \) chimera, although both chimeras bound [\(^3\)H]diprenorphine, a nonselective ligand, with high affinity.

The region from the first intracellular loop to the TMH 3 of the \( \delta \) receptor confers binding selectivity for SUPERFIT. In contrast, the specific binding sites of the \( \delta \) receptor for DPDPE, naltrindole, naltriben, and BNTX are located between the TMH 5 and TMH 7 (21, 22). It is likely that depending on stereochemical features, ligands are oriented differently in the binding pocket.

In conclusion, SUPERFIT binds specifically and irreversibly to the cloned mouse \( \delta \) opioid receptor. The region between the N-terminal portion of the first intracellular loop to the middle of the TMH 3 is necessary for selective binding of SUPERFIT. We will determine the amino acid residue that forms a covalent bond with SUPERFIT. Such information will be very useful for molecular modeling of the \( \delta \) receptor.

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