J. Biochem. 120, 459-465 (1996)

Reversible Affinity Labeling of Opioid Receptors *via* Disulfide Bonding: Discriminative Labeling of μ and δ Subtypes by Chemically Activated Thiol-Containing Enkephalin Analogs

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Received for publication, May 9, 1996

The 3-nitro-2-pyridinesulfenyl (Npys) group bound to a mercapto group is a highly activated electrophilic reagent, which only reacts with a free mercapto group to form a disulfide bond *via* the thiol-disulfide exchange reaction. We incorporated the Npys group into enkephalin analogs to affinity label μ and δ opioid receptors. When rat brain membranes were incubated with [D-Ala^2,Leu(CH$_2$SNpys)^5]enkephalin, and assayed for the inhibition of binding of DAGO and DSLET enkephalin analogs to opioid receptors, the number of receptors decreased sharply, depending upon the concentration of this SNpys-containing enkephalin. It was found that this enkephalin analog occupies μ receptors highly specifically (EC_{50}=51 nM) and almost 100 times more selectively than δ receptors. In contrast, [D-Ala^2,Leu^5]enkephalyl-Cys(Npys)^6 attached covalently to δ receptors (EC_{50}= 34 nM) about 150 times more selectively than to μ receptors. Although *N*-ethylmaleimide also inhibited the binding of DAGO and DSLET, four to six orders of magnitude higher concentrations were required as compared to SNpys-containing enkephalins. When enkephalin-bound rat membranes were treated with dithiothreitol, the loss of receptors was reversed, depending upon the concentration of and incubation time with dithiothreitol. The recovery was much faster (about 1,000 times) for δ receptors than for μ receptors. The present results indicated that both μ and δ receptors in rat brain consist of a free mercapto group near the enkephalin binding site and that SNpys-containing enkephalins can label these mercapto groups discriminatively. The disulfide bond between [D-Ala^2,Leu^5]enkephalyl-Cys^6 and δ receptors appears to be exposed, while that between [D-Ala^2,Leu(CH$_2$-SNpys)^5]enkephalin and μ receptors is shielded.

Key words: activated thiol, affinity labeling, disulfide bond, enkephalins, opioid receptors.

Affinity techniques are very important in receptor biochemistry. In particular, affinity labeling is now an important method for identifying a ligand binding site (*1*). The ligands for affinity labeling should have two different essential structural elements: *i.e.*, (i) an affinity core to bind to the specific binding site and (ii) a reactant for nucleophiles in the receptor protein. Affinity labeling usually results in irreversible cross-linking of a ligand to a receptor. The most utilized reactants are electrophiles such as Michael acceptors, halomethylketones, and isocyanates, which react with nucleophiles in the receptor protein. Carbenes and nitrenes have been utilized as reactants for photoaffinity labeling. However, these reactants are not so specific because of their high reactivity, reacting with various amino acid residues. Photo-irradiation often damages a protein, resulting in destruction of the receptor protein. The best affinity ligand for identifying a ligand binding site is a ligand that can label predominantly one of the functional groups which belongs to a certain amino acid residue.

Various types of enkephalin analogs have been designed and synthesized for affinity labeling of opioid receptors. Selective affinity labeling of μ opioid receptors was achieved with enkephalin analogs with C-terminal chloromethyl ketones, for example, chloromethyl ketones of [Leu^5]-, [D-Ala^2,Leu^5]-, and [D-Ala^2,D-Leu^5]enkephalins (*2, 3*) and H-Tyr-D-Ala-Gly-MePhe-chloromethyl ketone (*4*). The chloromethyl ketone group can react with the ε-amino group of lysine, the β-hydroxyl group of serine, the β-mercapto group of cysteine, and the imidazole group of histidine. Attempts to photoaffinity label opioid receptors were also carried out with enkephalin analogs having azidophenylalanine at position 4 or a 2-nitro-4-azidophenyl

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Abbreviations: AcOH, acetic acid; Boc, *tert*-butoxycarbonyl; *n*-BuOH, 1-butabol; DAGO, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin; DCC, *N,N*-dicyclohexylcarbodiimide; DMF, *N,N*-dimethylformamide; DSLET, [D-Ser^2,Leu^5]enkephalyl-Thr^6; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTT, ditiothreitol; EtOAc, ethyl acetate; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; MBzl, *p*-methoxybenzyl; MVD, mouse vas deferens; NEM, *N*-ethylmaleimide; Npys, 3-nitro-2-pyridinesulfenyl; Npys-Cl, 3-nitro-2-pyridinesulfenyl chloride; TFA, trifluoroacetic acid.

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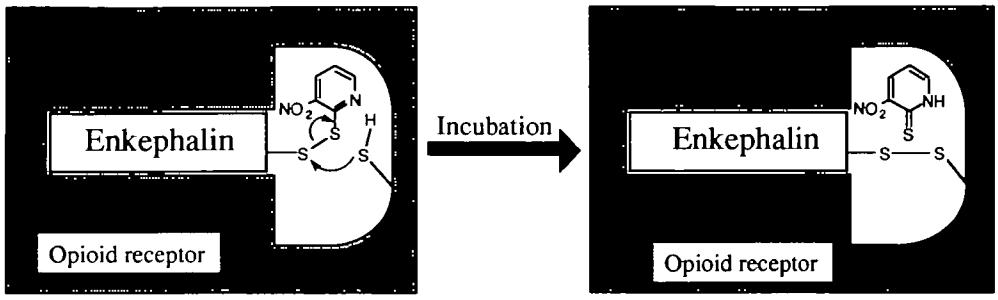

Fig. 1. **Reaction mode of an SNpys-containing enkephalin analog with the receptor mercapto group.**

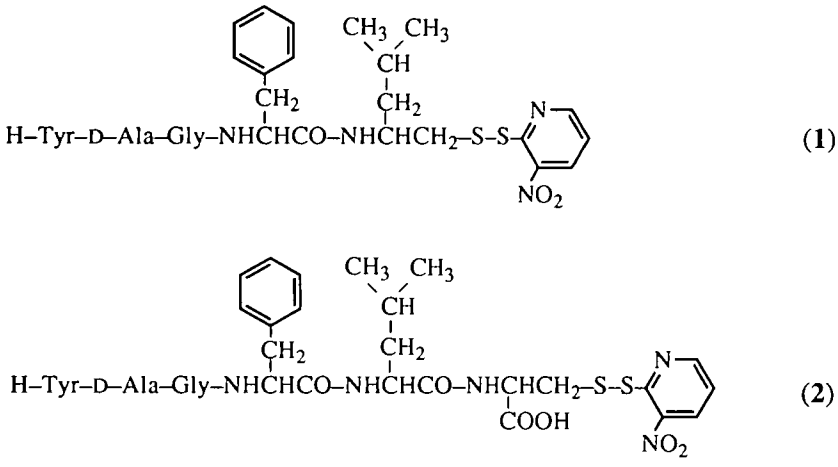

Fig. 2. **Structures of SNpys-containing enkephalin analogs.** (A) [D-Ala^2, Leu(CH_2SNpys)^5]enkephalin (**1**), and (B) [D-Ala^2,Leu^5]enkephalyl-Cys(Npys)^6 (**2**).

group at the C-terminus (*5-7*). These azide groups can also react with various amino acid side chains.

The 3-nitro-2-pyridinesulfenyl (Npys) group attached to a mercapto group, namely the SNpys group, is a highly activated electrophilic reagent, which only reacts with a free mercapto group *via* the thiol-disulfide exchange reaction (*8-10*). If the SNpys group is loaded into the affinity core, this group only reacts with the β-mercapto group of cysteine, forming a disulfide covalent bond between the ligand and the receptor (Fig. 1). Several lines of evidence have indicated the existence of mercapto group(s) in opioid receptors (*11-13*). At least two different types of mercapto groups sensitive to *N*-ethylmaleimide (NEM) were suggested (*12*): *i.e.*, the cysteine β-mercapto group in GTPase-binding protein (G_i), and the one at or near the binding site of the receptor protein. Alkylation of β-mercapto of the G_i protein inhibits the binding of a receptor to G_i, and thus prevents the ligand-receptor interaction.

We incorporated the Npys group into enkephalins, and obtained [D-Ala^2,Leu(CH_2SNpys)^5]enkephalin (**1**) and [D-Ala^2,Leu^5]enkephalyl-Cys(Npys)^6 (**2**) (Fig. 2). When these SNpys-containing enkephalins, **1** and **2**, were utilized as affinity probes for opioid receptors in peripheral tissues, they were found to label the opioid receptors in guinea pig ileum (GPI) and mouse vas deferens (MVD), respectively (*14, 15*). The G_i mercapto groups in the isolated muscle preparations are unexposed, and thus the mercapto groups labeled by these compounds were assumed to be at or near the ligand binding sites. GPI contains predominantly μ receptors, while MVD contains δ receptors. These results suggested that SNpys-containing enkephalins **1** and **2** preferentially label μ and δ receptors, respectively. However, it is difficult to evaluate the selectivity of these affinity ligands for opioid receptors in the peripheral tissues of GPI and MVD. In the present study, in order to explore the binding characteristics and receptor selection of these SNpys-containing enkephalin analogs in the central nervous system, we examined their ability to cross-link μ and δ opioid receptors in rat brain. Another important advantage of thiol-directed affinity labeling is the reversibility of the disulfide bond, which can be cleaved reductively by thiol compounds. Thus, the treatment of labeled receptors with dithiothreitol was carried out to demonstrate the disulfide bonding on affinity labeling and also to evaluate its reversibility.

MATERIALS AND METHODS

Peptide Syntheses—All melting points were uncorrected. Thin-layer chromatography was carried out on silica gel 60 GF_{254} (Merck) with the following solvent systems (by volume): R_f^1, $CHCl_3$-MeOH (5 : 1); R_f^2, $CHCl_3$-MeOH (9 : 1); R_f^3, $CHCl_3$-MeOH-AcOH (50 : 10 : 2); R_f^4, $CHCl_3$-MeOH-AcOH (95 : 5 : 1); R_f^5, *n*-BuOH-AcOH-H_2O (4 : 1 : 2); R_f^6 *n*-BuOH-AcOH-pyridine-H_2O (4 : 1 : 1 : 2). Compounds possessing a free amino group were detected by spraying ninhydrin and those with a protected amino group were carbonized after spraying 10% H_2SO_4. Compounds containing the mercapto group were detected by spraying with an ethanolic buffer solution of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and those containing the disulfide bond by spraying with a $NaBH_4$-EtOH solution, followed by the DTNB solution. Optical rotations were measured with a JASCO DIP-370 spectropolarimeter (JASCO, Tokyo).

[*D-Ala^2,Leu(CH_2SNpys)^5]Enkephalin* (**1**)—For the synthesis of compound **1**, *tert*-butoxycarbonyl (Boc)-L-leucine-

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thiol was prepared from L-leucinol and dimerized, converting the unstable SH group into a stable disulfide bond. A protected dimeric enkephalin analog [Boc-Tyr-D-Ala-Gly-Phe-Leu(CH_2S-)]$_2$ (**3**) was synthesized as reported previously (*16*). To a solution of compound **3** (417 mg, 0.31 mmol) in MeOH-H_2O (4 : 1) (5 ml) was added *n*-tributyl phosphine (*n*-Bu)$_3$P (0.08 ml, 0.31 mmol) at room temperature. After 3 h, 3-nitro-2-pyridinesulfenyl chloride (Npys-Cl) (71.0 mg, 0.37 mmol) in dioxane (3 ml) was added at 0°C, and then the reaction mixture was further stirred for 2 h at 0°C and for 18 h at room temperature. After evaporation *in vacuo*, the residual oil was purified by gel filtration on a Sephadex LH-20 column (1.5×137 cm) eluted with *N,N*-dimethylformamide (DMF) to afford Boc-Tyr-D-Ala-Gly-Phe-Leu(CH_2SNpys) (**4**); yield 142 mg (28%); m.p. 123–124°C; $[\alpha]_D^{20}$ +20.9° (*c* 0.5, DMF); R_f^1 0.49, R_f^4 0.46.

Compound **4** (134 mg, 0.16 mmol) was dissolved in trifluoroacetic acid (TFA) (2 ml) at 0°C. After 30 min, the solution was evaporated and the residual oil was purified on a Sephadex G-15 column (2.0×138 cm) eluted with 30% AcOH. The fractions containing pure compound **1** were pooled, evaporated *in vacuo*, and then lyophilized; yield 123 mg (97%); m.p. 124–126°C; $[\alpha]_D^{20}$ +36.2° (*c* 1.0, DMF); R_f^5 0.74.

[D-Ala^2,Leu^5]Enkephalyl-Cys(Npys)[6] (**2**)—In the previous study (*15*), the title compound was prepared by the solid phase method using *N,N*-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt). However, the yields were usually low, and it has been reported that the Npys group attached to a mercapto group is unstable against HOBt (*17, 18*). Thus, in the present study we first synthesized a Boc-protected [D-Ala^2,Leu^5]enkephalyl-Cys-(MBzl)[6] ethyl ester, in which the *p*-methoxybenzyl (MBzl) group was converted to Npys by treatment with Npys-Cl.

Boc-Phe-Leu-Cys(MBzl)-OEt (**5**)—To a solution of H-Cys(MBzl)-OEt·TosOH (2.21 g, 5.0 mmol) and Boc-Phe-Leu-OH (*19*) (1.89 g, 5.0 mmol) in DMF (20 ml) and Et_3N (0.70 ml, 5.0 mmol) were added HOBt (0.92 g, 6.0 mmol) and DCC (1.13 g, 5.5 mmol) at 0°C. After stirring for 1 h at 0°C and overnight at room temperature, the reaction mixture was evaporated *in vacuo*, and the residue was dissolved in EtOAc. The solution was washed successively with H_2O, 0.5 M $NaHCO_3$, and 5% $KHSO_4$, and then dried over Na_2SO_4. After evaporation, the residue was crystallized from ether-petroleum ether; yield 2.95 g (94%); m.p. 138–139°C; $[\alpha]_D^{20}$ −35.5° (*c* 1.0, DMF); R_f^2 0.42, R_f^4 0.28. Found: C, 62.88; H, 7.53; N, 6.88%. Calcd. for $C_{33}H_{47}N_3O_7S$: C, 62.93; H, 7.52; N, 6.67%.

Boc-Tyr-D-Ala-Gly-Phe-Leu-Cys(MBzl)-OEt (**7**)—Compound **5** (1.26 g, 2.0 mmol) was dissolved in TFA (2 ml) at 0°C for 30 min to obtain H-Phe-Leu-Cys(MBzl)-OEt·TFA (**6**·TFA); yield 0.81 g (63%); R_f^5 0.87, R_f^6 0.89. To a solution of **6**·TFA (0.81 g, 1.3 mmol), Et_3N (0.2 ml, 1.3 mmol), and Boc-Tyr-D-Ala-Gly-OH (0.53 g, 1.3 mmol) in DMF (5 ml) were added HOBt (0.24 g, 1.6 mmol) and EDC·HCl (0.27 g, 1.4 mmol) at 0°C. The reaction mixture was treated as described for compound **5**. The crude product was purified by Sephadex LH-20 gel filtration (column: 3.5×114 cm) with elution with DMF. The product was crystallized from ether-petroleum ether; yield 1.14 g (95%); m.p. 192–193°C; $[\alpha]_D^{20}$ −24.6° (*c* 1.0, DMF); R_f^1 0.73, R_f^3 0.69. Found: C, 60.59; H, 7.02; N, 9.11%. Calcd. for $C_{47}H_{64}N_6O_{11}S$: C, 60.69; H, 7.04; N, 9.04%.

Boc-Tyr-D-Ala-Gly-Phe-Leu-Cys(MBzl)-OH (**8**)—To a solution of **7** (0.92 g, 1.0 mmol) in DMF (4 ml) was added 2 M NaOH (1.0 ml), and the reaction mixture was stirred for 1.5 h at 30°C. After evaporation, the residue dissolved in a small amount of water was acidified with citric acid, and the separated oil was extracted with EtOAc. The solution was washed with water and then dried over Na_2SO_4. The residue after evaporation was crystallized from ether-petroleum ether; yield 0.70 g (78%); m.p. 176–177°C; $[\alpha]_D^{20}$ −23.7° (*c* 1.0, DMF); R_f^1 0.38, R_f^3 0.67. Found: C, 59.74; H, 6.67; N, 9.35%. Calcd. for $C_{45}H_{60}N_8O_{11}S\cdot 1/2H_2O$: C, 59.92; H, 6.82; N, 9.32%.

Boc-Tyr-D-Ala-Gly-Phe-Leu-Cys(Npys)-OH (**9**)—To a solution of **8** (89 mg, 0.1 mmol) in MeOH (2 ml) was added Npys-Cl (29 mg, 0.15 mmol) in dioxane (2 ml) at 0°C. After 1 h, the reaction mixture was evaporated, and the residual oil was purified by Sephadex LH-20 (1.8×150 cm) with elution with DMF, and by silica gel column chromatography (2.8×22 cm) with elution with $CHCl_3$-MeOH (9 : 1); yield 31 mg (33%); m.p. 178–179°C; $[\alpha]_D^{20}$ −40.4° (*c* 0.2, DMF); R_f^1 0.13, R_f^3 0.63. Found: C, 53.73; H, 5.93; N, 11.64%. Calcd. for $C_{42}H_{54}N_8O_{12}S_2\cdot 1/2H_2O$: C, 53.89; H, 5.92; N, 11.97%.

H-Tyr-D-Ala-Gly-Phe-Leu-Cys(Npys)-OH (**2**)—Compound **9** (20 mg, 0.02 mmol) was treated with TFA (2 ml) at 0°C for 30 min. After evaporation, the residual oil was purified by Sephadex G-15 gel filtration (1.5×110 cm) with elution with 30% AcOH, and by preparative HPLC (Wakopak WS-II, 2×25 cm); yield 13 mg (77%); m.p. 172–174°C; $[\alpha]_D^{20}$ −42.8° (*c* 0.4, MeOH); R_f^5 0.68, R_f^6 0.51. Found: C, 49.06; H, 5.00; N, 11.17%. Calcd. for $C_{37}H_{46}N_8O_{10}S_2\cdot TFA\cdot H_2O$: C, 48.95; H, 5.15; N, 11.38%.

Receptor Binding Assays—Radio-ligand receptor binding assays involving rat brain preparations were carried out essentially as described previously (*20*). [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]DAGO) (1.80 TBq/mmol; New England Nuclear, Boston, MA, USA) and [^{3}H]-[D-Ser^2,Leu^5]enkephalyl-Thr[6] ([^{3}H]DSLET) (1.51 TBq/mmol; New England Nuclear) were used as tracers selective for μ and δ opioid receptors, respectively, at the final concentration of 0.25 nM. Incubations were carried out at 25°C for 60 min in 50 mM Tris-HCl buffer (pH 7.5) containing 0.1% bovine serum albumin. Bacitracin (100 μg/ml) was added as an enzyme inhibitor. Dose-response curves were constructed utilizing seven to ten doses, and the results were analyzed with the computer program, ALLFIT (*21*). The data were used to construct least-square estimates of the logistic curves relating the binding of labeled ligands [^{3}H]DAGO and [^{3}H]DSLET to the concentrations of the non-labeled ligands.

Affinity Labeling of Opioid Receptors—Rat brain membranes in 50 mM Tris-buffer (pH 7.5) were incubated with SNpys-containing enkephalins in the presence of bacitracin (100 μg/ml) at 25°C for 30 min. The concentrations of SNpys-containing enkephalins were 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM. The assay was also carried out without ligands (controls). After incubation, the membranes were centrifuged (40,000×*g*) for 15 min and then resuspended in the same buffer for homogenization (Polytron homogenizer). This washing process was repeated successively four times, and the washed membranes were finally assayed for competitive binding of DAGO and [^{3}H]-

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DAGO, or of DSLET and [^{3}H]DSLET. Direct binding assays using [^{3}H]DAGO and [^{3}H]DSLET were also carried out for μ and δ receptors.

Reduction of Disulfide Bonds by Dithiothreitol—Rat brain membrane preparations were first incubated with SNpys-containing enkephalin **1** or **2** (1 μM) or without a ligand (control) in the presence of bacitracin (100 μg/ml) at 25°C for 30 min. The membranes were then washed as described above and the washed membranes were incubated with 1 mM dithiothreitol (DTT) at 37°C for 2 h in the presence of bacitracin (100 μg/ml). At intervals, membranes were centrifuged and resuspended for washing. After four washings, DTT-treated membranes were finally assayed for competitive binding of DAGO and [^{3}H]DAGO or of DSLET and [^{3}H]DSLET, as described above. The results were compared with the results without DTT treatment. The loss of μ or δ receptors was estimated from the amount of DAGO or DSLET that occupied the receptors.

As reference experiments, rat brain membranes were also treated with NEM instead of SNpys-containing enkephalins. After washing, the membranes were assayed for DAGO/[^{3}H]DAGO or DSLET/[^{3}H]DSLET binding. Binding assays were carried out with and without DTT pretreatment.

RESULTS

Receptor Binding Affinity of SNpys-Containing Enkephalins—Radio-ligand receptor binding assays were carried out for Npys-containing enkephalins **1** and **2** using rat brain and [^{3}H]DAGO or [^{3}H]DSLET. The IC_{50} values, which are the half-maximal concentrations for inhibition of the binding of radio-labeled ligands, of compound **1** were 1.74 nM for [^{3}H]DAGO and 2.56 nM for [^{3}H]DSLET, indicating that compound **1** is almost equally potent for μ and δ receptors. Those of compound **2** were 42.7 nM for [^{3}H]DAGO and 8.01 nM for [^{3}H]DSLET. Compound **2** is therefore more potent for δ than μ receptors, and its selectivity as to δ over μ receptors is about fivefold.

Selective Affinity Labeling of μ Opioid Receptors by SNpys-Containing Enkephalin 1—When SNpys-containing peptide **1** was incubated with rat brain membranes, they bound to the ligand binding sites of receptors in the first instance. Meanwhile, if there is a receptor mercapto group near the peptide C-terminus, the Npys group would react with this free mercapto group, resulting in the formation of a disulfide bond. Such affinity-labeling of receptors would reduce the number of receptors available for the ligand added afterwards. Thus, after preincubation of membranes with SNpys-containing enkephalins, receptor binding assays would reveal the loss of μ and δ receptors, and consequently the amounts of the receptors labeled.

In the present study, rat brain membranes were first incubated with **1** for 30 min at 25°C. After four consecutive washings of the membranes by centrifugation, they were further incubated with DAGO and then with radio-labeled [^{3}H]DAGO to determine the amount of μ receptors remaining unlabeled. When membranes were incubated with 10 nM **1**, about 20% μ receptors were reduced, indicating that 20% μ receptors were occupied by **1** (Fig. 3). With increasing concentrations of compound **1**, the receptors available for DAGO decreased sharply (Fig. 3). At concentrations of more than 1 μM, **1** appeared to occupy almost all the μ receptors. Scatchard analyses (Fig. 4) gave a monophasic straight line in all cases, and the affinity constants of DAGO were estimated to be about 7×10^{10} M^{-1} in all DAGO/[^{3}H]DAGO binding assays. These results indicate that the receptors occupied by compound **1** are homogenous and highly specific for DAGO-enkephalin.

Similar results were obtained in the direct binding assays involving [^{3}H]DAGO (Fig. 5). Evaluating the receptors available for DAGO, the number of receptors which were occupied by Npys-enkephalin **1** was estimated in each assay performed with a different concentration of **1**. When the amount of receptors labeled (% labeling) was plotted against the concentrations of compound **1** for preincubation, a sigmoid curve was obtained, as shown in Fig. 6A.

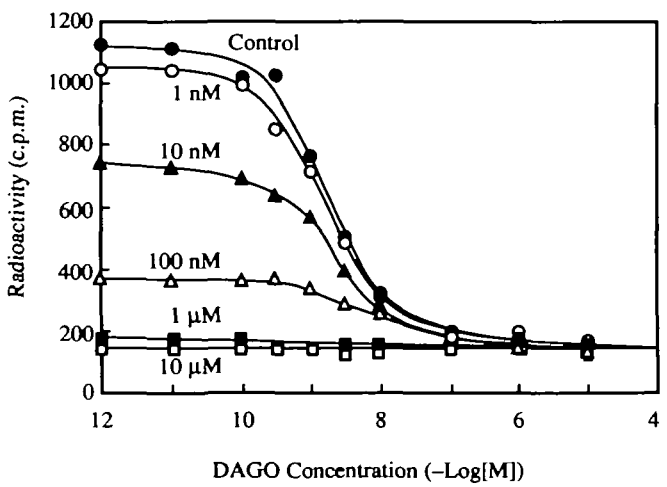

Fig. 3. **Loss of receptor binding sites on preincubation of rat brain membranes with [D-Ala^2,Leu(CH$_2$SNpys)^5]enkephalin (1).** Dose-response curves of [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGO) displacing [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]-DAGO) after preincubation with compound **1** at different concentrations are shown. The concentrations are the concentrations of [D-Ala^2,Leu(CH$_2$SNpys)^5]enkephalin (1) used for preincubation.

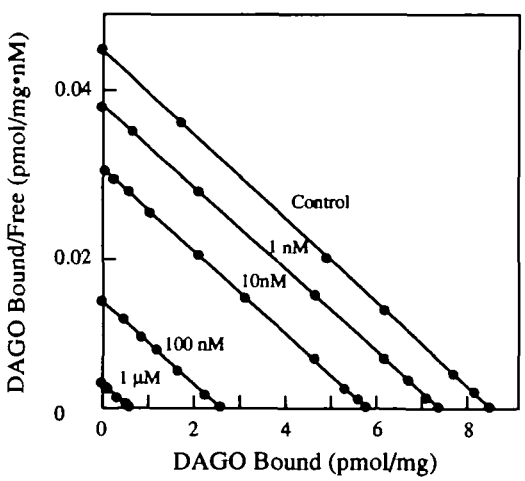

Fig. 4. **Scatchard analyses of the binding of [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGO) to μ opioid receptors after preincubation of rat brain membranes with [D-Ala^2,Leu(CH$_2$SNpys)^5]enkephalin (1).** The concentrations are the concentrations of [D-Ala^2,Leu(CH$_2$SNpys)^5]enkephalin (1) used for preincubation.

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From this curve, the effective concentration (EC_{50}) which is enough to label the half-maximal amount of total receptors was estimated to be 51 nM. When the binding assay was carried out using [^{3}H]DSLET, the labeling of δ receptors was found to be considerably less effective as compared to that of μ receptors (EC_{50} = 5,000 nM) (Fig. 6A).

Selective Affinity Labeling of δ Opioid Receptors by SNpys-Containing Enkephalin 2—When rat brain membranes were preincubated with compound **2**, and then assayed for direct binding of [^{3}H]DAGO and [^{3}H]DSLET, the reverse profile of affinity labeling was obtained. From the curves of % labeling *versus* the concentrations of compound **2**, it was found that **2** labels δ receptors much more effectively than μ receptors (Fig. 6B). The EC_{50} values were 5,100 nM for labeling of μ receptors and 34 nM for δ receptors, indicating that δ receptors were preferred over μ receptors about 150-fold by compound **2**. This receptor selectivity in affinity labeling is much higher than that in binding affinity (5-fold).

Recovery of Opioid Receptors on Enkephalin Labeling—In order to demonstrate the occurrence of disulfide bonding between the receptor and enkephalin molecules, labeled membranes were treated with DTT. If the disulfide bond is cleaved by DTT, the receptor might recover from occupation by enkephalin and again become open for ligands. This would increase the number of receptors available for ligands added afterwards. Thus, after incubation of membranes with DTT, the number of receptors was estimated by means of ordinary receptor binding assays. Incubation of rat brain membranes with 1 μM compound **1** eliminated about 95% of the total binding of DAGO for μ receptors. When these membranes were treated with 1 mM DTT for 4 h at 37°C, more than 30% DAGO binding was recovered. DTT was examined in terms of concentration (0.1-5.0 mM)

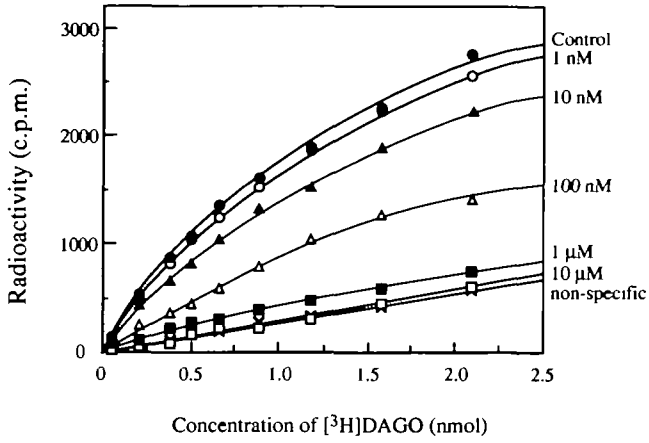

Fig. 5. **Dose-response curves on direct binding assays with [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin for rat brain membranes which had been preincubated with [D-Ala^2,Leu(CH$_2$SNpys)^5]-enkephalin (1).** The concentrations are the concentrations of [D-Ala^2,Leu(CH$_2$SNpys)^5]enkephalin (1) used for preincubation.

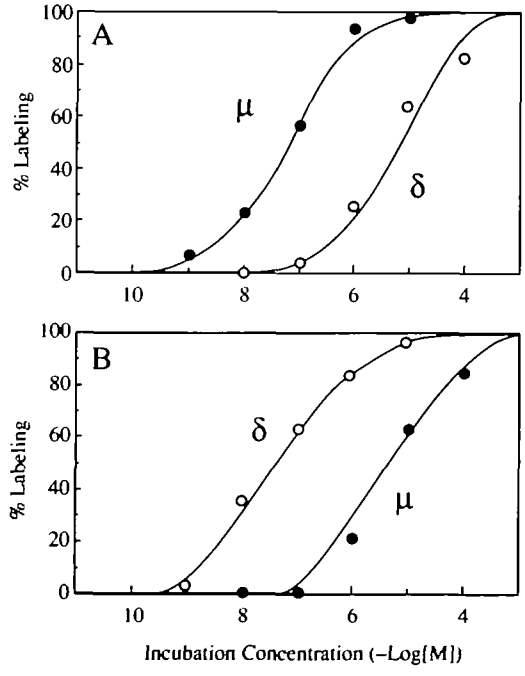

Fig. 6. **Affinity labeling of opioid receptors by [D-Ala^2,Leu(CH$_2$SNpys)^5]enkephalin (1) (A) and [D-Ala^2,Leu^5]enkephalyl-Cys(Npys)^6 (2) (B).** The percentage labeling was calculated by subtracting the total binding of DAGO or DSLET from that without preincubation with SNpys-containing enkephalins. The affinity labeling of μ receptors was determined by evaluating the binding of DAGO (●), and that of δ receptors by evaluating the binding of DSLET (○).

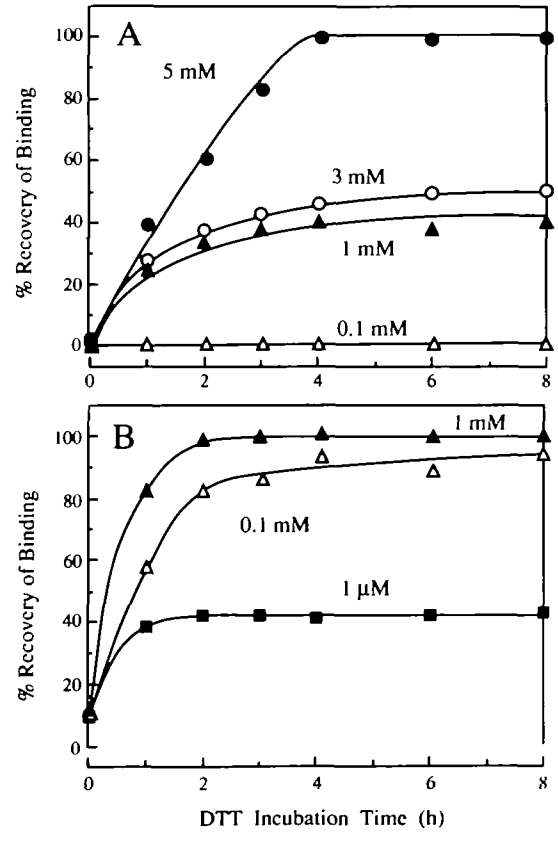

Fig. 7. **Recovery of opioid receptors from affinity labeling on treatment with dithiothreitol.** Rat membranes were preincubated with 1 μM compound 1 (A) or compound 2 (B) to label 90-95% μ and δ opioid receptors, respectively. The relationship between the percentage recovery and the incubation time with DTT is shown for different concentrations of DTT.

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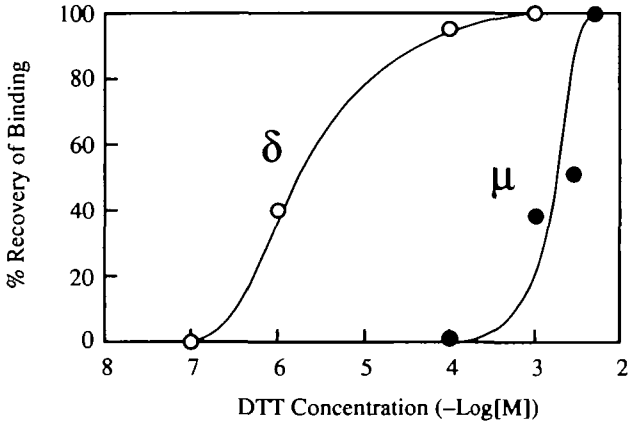

Fig. 8. **Recovery of opioid receptors from affinity labeling on treatment with dithiothreitol.** In Fig. 7, the recovery reached steady a state after certain incubation time, and the level of maximal recovery was assessed and plotted against the concentrations of DTT. The recovery of μ and δ receptors is shown by the lines connecting closed and open circles, respectively.

and incubation time (1–8 h) (Fig. 7A). No recovery was observed with 0.1 mM DTT. On treatment with 1 or 3 mM DTT, the recovery was also incomplete even after 8 h. However, on incubation with 5 mM DTT, complete recovery of μ receptors was achieved after 4 h (Fig. 8).

When the membranes were treated with 1 μM compound **2**, about 90% of the total binding of DSLET for δ receptors was eliminated. Interestingly, this loss of receptor binding was easily and completely recovered on treatment with 1 mM DTT for 4 h. Even on treatment with 0.1 mM DTT, the recovery was complete (Fig. 7B), although 1 μM DTT was insufficient. When the levels of recovery (%) were plotted against the concentrations of DTT, the difference in susceptibility to DTT became very prominent between δ and μ receptors (Fig. 8). δ receptors are almost a thousand times more sensitive to DTT than μ receptors.

Non-Specific Irreversible Labeling of Opioid Receptors by N-Ethylmaleimide—*N*-Ethylmaleimide (NEM), an alkylating reagent for the mercapto group, was incubated with rat brain membranes instead of SNpys-containing enkephalins. When NEM-treated membranes were assayed for competitive binding of DAGO/[^{3}H]DAGO or DSLET/[^{3}H]DSLET, it was found that NEM can also inhibit the binding of enkephalin analogs. However, it was found that an extremely high concentration is required to block all the receptors (Fig. 9). On treatment with DTT, no recovery of receptors was observed for NEM-treated membranes (data not shown).

DISCUSSION

The mercapto group is among the most important functional residues in protein and enzyme chemistry (*22*). Accumulating data have indicated that it is also important in receptor chemistry (*23*). The existence of a mercapto group at or near the ligand binding site of opioid receptors has been suggested by irreversible inactivation with NEM or DTNB (*11–13*). The present results clearly demonstrated that both μ and δ opioid receptors contain a free mercapto group near their enkephalin binding site. Affinity labeling with SNpys-containing enkephalins and subsequent recovery of receptors on DTT treatment demonstrated the existence of a disulfide bond between the enkephalin and the receptor.

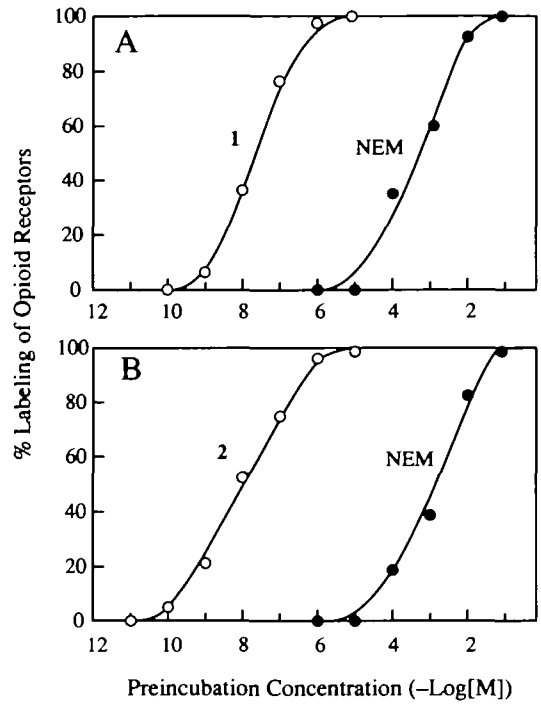

Fig. 9. **Specific and non-specific labeling of opioid μ and δ receptors with Npys-containing enkephalins and *N*-ethylmaleimide.** In A, preincubation of rat brain membranes was carried out with [D-Ala^2,Leu(CH$_2$SNpys)^5]enkephalin (**1**) or *N*-ethylmaleimide (NEM), and then the binding assay was performed using DAGO/[^{3}H]DAGO. In B, [D-Ala^2,Leu^5]enkephalyl-Cys(Npys)^6 (**2**) or NEM was used for the assays using DSLET/[^{3}H]DSLET. Thus, A represents the labeling of μ receptors, while B shows the labeling of δ receptors.

[D-Ala^2,Leu(CH$_2$SNpys)^5]enkephalin (**1**) and [D-Ala^2, Leu^5]enkephalyl-Cys(Npys)^6 (**2**) are unique among enkephalin analogs synthesized to date in that they contain a mercapto group modified so as to form a unsymmetrical disulfide bond. This activated mercapto group, namely the SNpys group, is only able to react with a free mercapto group. This substantiates the formation of a disulfide bond with the receptor mercapto group. SNpys-containing enkephalin **1** binds strongly and almost equally well to both μ and δ receptors, being non-selective in μ/δ receptor selection. However, it is notable that **1** labeled μ receptors 100 times more effectively than δ receptors. This clearly indicates that the SNpys group in Leu(CH$_2$SNpys) is in close proximity to the receptor mercapto group when bound to μ receptors.

Similar affinity labeling of δ receptors was achieved with compound **2**. Compound **2** labeled δ receptors much more effectively (about 150-fold) than μ receptors, although it exhibited moderate affinity for μ receptors and saturated μ receptors at submicromolar concentrations (data not shown). Compound **2** could label μ receptors only weakly. These results indicate that **2** binds to μ receptors in such a way that the receptor mercapto group cannot interact with the SNpys group of **2**. In contrast, the SNpys group of **2** and the mercapto group of δ receptors are in close proximity when **2** binds to the specific binding site.

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The structural requirements for irreversible attachment should be quite specific. However, SNyps-containing enkephalins **1** and **2** possess rather similar structures, as shown in Fig. 2. The N-terminal moieties including leucine residues are exactly the same. The difference lies in the distance between the SNpys group and the leucine α-carbon atom: only one methylene in **1**, but four atoms in **2**. Furthermore, compound **2** has a carboxyl group at position 6, which is known to be important for interaction with δ receptors. These structural diversities are crucial for discriminative affinity labeling of μ and δ receptors. Most of the C-terminal chloromethyl ketone derivatives of enkephalin analogs have been reported to be selective for μ receptors (*2-4*). It is likely, as judged on structural comparison among these analogs and **1**, that these enkephalin chloromethyl ketone derivatives bind to the mercapto group to which compound **1** binds.

Another notable difference in the affinity labeling of μ and δ receptors is the susceptibility of the resulting disulfide bond to DTT treatment. It should be noted that the disulfide bond formed with δ receptors is much more sensitive to DTT than that with μ receptors. The recovery of receptors was much faster (about 1,000 times) for δ receptors than μ receptors (Fig. 8). This certainly reflects the structural difference of ligand binding sites of δ and μ receptors, and suggests that the disulfide bond between compound **2** and δ receptors is exposed on the surface of the receptor molecule.

The functional roles of the receptor mercapto groups are not known yet. Our previous results indicated that enkephalins cross-linked irreversibly to peripheral tissues such as GPI and MVD exhibit long-lasting activities (*14, 15*). Thus, the receptor mercapto groups appears not to be in charge of receptor activation. The present results also suggest that the mercapto groups labeled by SNpys-containing enkephalins appear not to be involved in the ligand binding sites. We found that the amount of receptors increases (20-100%) when membranes were treated with DTT before incubation with SNpys-containing enkephalins (unpublished data). This increase in the receptor population on DTT treatment was dependent upon the individual rat brain utilized. These results suggest that in some receptors the mercapto group participates in the disulfide bonding, which may construct an inactive form of receptor, or low affinity conformation. It might be possible that the thiol-disulfide exchange reaction regulates the high and low affinity conformations of opioid receptors.

The present results indicate that the opioid receptor subtypes in the central nervous system possess a free mercapto group near the ligand binding site, and that properly designed SNpys-containing enkephalins can affinity-label these thiols discriminatively. It is now important to determine which cysteine residue is affinity-labeled and what the role of this cysteine is.

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