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Exploration of Universal Cysteines in the Binding Sites of Three Opioid Receptor Subtypes by Disulfide-Bonding Affinity Labeling with Chemically Activated Thiol-Containing Dynorphin A Analogs

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A ligand containing an SNpys group, *i.e.* 3-nitro-2-pyridinesulfenyl linked to a mercapto (or thiol) group, can bind covalently to a free mercapto group to form a disulfide bond *via* the thiol-disulfide exchange reaction. This SNpys chemistry has been successfully applied to the discriminative affinity labeling of μ and δ opioid receptors with SNpys-containing enkephalins [Yasunaga, T. *et al.* (1996) *J. Biochem.* 120, 459-465]. In order to explore the mercapto groups conserved at or near the ligand binding sites of three opioid receptor subtypes, we synthesized two Cys(Npys)-containing analogs of dynorphin A, namely, [D-Ala^2,Cys(Npys)^8]dynorphin A-(1-9) amide (1) and [D-Ala^2,Cys(Npys)^{12}]dynorphin A-(1-13) amide (2). When rat (μ and δ) or guinea pig (κ) brain membranes were incubated with these Cys(Npys)-containing dynorphin A analogs and then assayed for inhibition of the binding of DAGO (μ), deltorphin II (δ), and U-69593 (κ), the number of receptors decreased sharply, depending upon the concentrations of these Cys(Npys)-containing dynorphin A analogs. It was found that dynorphin A analogs 1 and 2 effectively label μ receptors (EC_{50}=27-33 nM), but also label δ receptors fairly well (160-180 nM). However, for κ receptors they showed drastically different potencies as to affinity labeling; *i.e.*, EC_{50}=210 nM for analog 1, but 10,000 nM for analog 2. Analog 2 labeled κ receptors about 50 times more weakly than analog 1. These results suggested that dynorphin A analog 1 labels the Cys residues conserved in μ, δ, and κ receptors, whereas analog 2 only labels the Cys residues conserved in μ and δ receptors.

Key words: affinity labeling, dynorphin A, opioid receptors, disulfide bond, enkephalin.

The affinity labeling technique is a very important method in receptor biochemistry, especially for identifying a ligand binding site (*1*). Affinity labeling usually results in irreversible cross-linking of a ligand to a receptor. Affinity ligands usually load two different structural elements, namely, an affinity core to bind to the specific binding site and a reactant for nucleophiles in the receptor protein. The most utilized reactants, such as electrophiles (Michael acceptors, halomethylketones, and isocyanates) and photoaffinity reactants (carbenes and nitrenes), are not so specific because of their high reactivity with various structural elements in proteins. The best affinity ligand for identifying a ligand binding site is a ligand that can predominantly label one of the functional groups which belongs to a certain amino acid residue such as cysteine.

Three major subtypes of opioid receptors, μ, δ, and κ, have been identified by means of cDNA cloning techniques (*2-5*). They are members of the G-protein-coupled receptor family, which has seven-transmembrane domains (TM 1-7). The alignment of these opioid receptor subtypes revealed approximately 60% sequence similarity, and several characteristic amino acid residues were found to be conserved. In particular, cysteine residues present in the first and second extracellular loops have been suggested to participate in disulfide bonding, although their functional role in the construction of a bioactive conformation is still under discussion. There are several other cysteine residues conserved among these subtypes, for example, Cys on the proximal side of TM 5 and 7, and Cys in a relatively distal portion of TM 4 and 6. Previous studies have shown that the receptor binding of opioid agonists is affected with by sulfhydryl reagents such as *N*-ethylmaleimide (NEM) (*6, 7*). Preincubation with opioid ligands prevents the receptor inactivation by NEM. These results suggest that free thiol group(s) exist at or near the ligand binding sites of opioid receptors.

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Abbreviations: Boc, *tert*-butoxycarbonyl; DAGO, [D-Ala^2,MePhe^4, Gly-ol^5]enkephalin; DEL, deltorphin II; Dyn A, dynorphin A; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MBHA, *p*-methylbenzhydrylamine; NEM, *N*-ethylmaleimide; Npys,3-nitro-2-pyridinesulfenyl; RP-HPLC, reversed-phase high performance liquid chromatography; TM, transmembrane domain; TFA, trifluoroacetic acid; and U-69593, *N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro-(4,5)-dec-8-yl]-(5*a*,7*a*,8*b*)-(−)-benzeneacetamide.

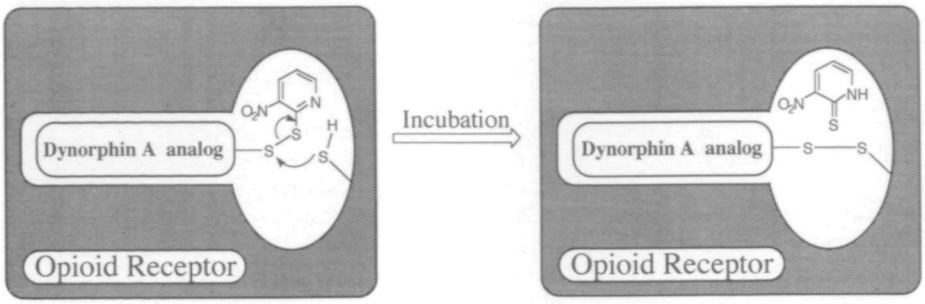

Fig. 1. **Mode of reaction of the Cys-(Npys)-containing dynorphin A analog with a free thiol group in an opioid receptor.**

(1) **H-Tyr-D-Ala-Gly-Phe-Leu-Arg-Arg-Cys(Npys)-Arg-NH$_2$**

(2) **H-Tyr-D-Ala-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Cys(Npys)-Lys-NH$_2$**

Fig. 2. **Amino acid sequences of the Cys(Npys)-containing dynorphin A analogs.**

The identification of free cysteine(s) by affinity labeling would help to define the ligand binding site in a receptor. This structural information is important for determining the functional structure of a receptor and for elucidating the molecular mechanism underlying receptor activation. The 3-nitro-2-pyridinesulfenyl (Npys) group is utilized as a protecting group for the mercapto (SH) group, resulting in the formation of a mixed disulfide bond (*8, 9*). This chemically activated Npys group, namely, the SNpys group, predominantly forms a disulfide bond with a free cysteine residue in a receptor *via* the thiol-disulfide exchange reaction (Fig. 1). In the previous studies, we showed that SNpys-containing enkephalin analogs can be used to specifically affinity-label the μ and δ subtypes in rat brain. The results indicated that both μ and δ receptors contain a free mercapto group near the enkephalin binding site and that SNpys-containing enkephalins can label these mercapto groups discriminatively (*10*).

In the present study, we examined whether or not a mercapto group also exists in the κ receptor, as in the μ and δ receptors, and whether or not such a cysteine residue is conserved among the receptor subtypes. Dynorphin A is an endogenous ligand of the κ receptor and its 17-peptide sequence is YGGFLRRIRPKLKWDNQ, in a one-letter amino acid code (*11*). Dynorphin A *per se* is highly selective for the κ receptor, although analogs lacking the C-terminal 4-9 residues are still fully active (*12, 13*). Shortened dynorphin A analogs, however, become able to bind to the μ and δ receptors reasonably strongly. In order to capture the mercapto group(s) conserved among opioid receptors, two shortened [D-Ala^2]dynorphin A (Dyn A) analogs containing Cys(Npys), namely [D-Ala^2,Cys(Npys)^8]Dyn A-(1-9) amide (1) and [D-Ala^2,Cys(Npys)^{12}]Dyn A-(1-13) amide (2) (Fig. 1) were designed and synthesized, and their binding characteristics as to opioid receptors were assessed using rat and guinea pig brains.

MATERIALS AND METHODS

Materials—Boc-Cys(Npys)-OH was purchased from Kokusan Chemical Works (Tokyo), and all other Boc-amino acids were from Watanabe Chemical (Hiroshima). *p*-Methylbenzhydrylamine (MBHA) resin was obtained from Watanabe. All amino acids had the L-configuration except for Gly and D-Ala. All other chemicals were of the best grade available.

Peptide Syntheses—The peptide syntheses were carried out by means of manual solid-phase methodology using Boc-amino acids and *p*-methylbenzhydrylamine resin on a 0.1 mmol scale. Coupling reactions were carried out with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) in the presence of 1-hydroxybenzotriazole (HOBt). After introduction of Cys(Npys), HOBt was not utilized to prevent the removal of the Npys group. Each coupling reaction was checked by means of the ninhydrin test for completion. Peptides were liberated from the resin by treatment with anhydrous liquid hydrogen fluoride containing 10% *p*-cresol at 0°C for 1 h. The products were purified by gel filtration on a column (1×60 cm) of Sephadex G-15, followed by preparative reversed-phase high performance liquid chromatography (RP-HPLC) (Cica-Merck, LiChrospher RP-18 (e) (5 μ): 25×250 mm). The elution conditions employed were as follows: solvent system, 0.1% aqueous trifluoroacetic acid (TFA)-(A solution) and acetonitrile containing 20% A solution-(B solution); flow rate, 3 ml·min^{-1}; temperature, 25°C; UV detection, 225 nm. Elution was performed with a linear concentration gradient of the B solution (20-60%) over 40 min.

The purity of peptides was verified by analytical RP-HPLC [LiChrospher RP-18 (e) (5 μ): 4.0×250 mm], under the same conditions except for a flow rate of 0.75 ml·min^{-1}. For amino acid analyses, the hydrolysis of peptide samples was carried out in constant-boiling hydrochloric acid (110°C, 24 h). Amino acid analyses were carried out with a Hitachi (model 835) amino acid analyzer.

Receptor Binding Assays—Radio-ligand receptor binding assays involving rat or guinea pig brain preparations were carried out essentially as described previously (*14*). [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]DAGO) (1.80 TBq/mmol; New England Nuclear), [^{3}H]-deltorphin II ([^{3}H]DEL) (1.85-3.18 TBq/mmol; Amersham Pharmacia

Biotech), and [^{3}H]-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro-(4,5)-dec-8-yl]-(5a,7a,8b)-(−)-benzenacetamide ([^{3}H]U-69593) (1.11-2.22 TBq/mmol; New England Nuclear) were used as tracers selective for the μ, δ, and κ 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 inhibitor for opioid peptide-degrading peptidases such as aminopeptidases and enkephalinases (*15*). Dose-response curves were constructed with seven to ten doses, and the results were analyzed with the computer program, ALLFIT (*16*). The data were used to obtain least-square estimates of the logistic curves relating the binding of labeled ligands [^{3}H]DAGO, [^{3}H]DEL, and [^{3}H]U-69593 to the concentrations of the non-labeled ligands. The binding data were also used for Scatchard analyses with the computer program, LIGAND (*17*).

Affinity Labeling of Opioid Receptors—Rat or guinea pig brain membranes in 50 mM Tris-buffer (pH 7.5) were incubated with Cys(Npys)-containing dynorphin A analogs in the presence of bacitracin (100 μg/ml) at 25°C for 30 min. The concentrations of Cys(Npys)-containing dynorphin A analogs 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]DAGO, of deltorphin II and [^{3}H]DEL, or of U-69593 and [^{3}H]U-69593.

In order to assure the cross-linking by formation of the disulfide bond, membranes incubated with SNpys-containing compounds **1** and **2** were treated dithiothreitol (DTT) as described previously (*10*). Briefly, membrane preparations were first incubated with SNpys-containing compound **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 DTT at 37°C for 2 h in the presence of bacitracin (100 μg/ml). After four washings, DTT-treated membranes were finally assayed for competitive binding as described above. The results were compared with the results without DTT treatment.

RESULTS

Receptor Binding Affinity of Cys(Npys)-Containing Dynorphin A Analogs—In order to prevent the enzymatic degradation during experiments on receptor binding and affinity labeling, particular cave was taken for the design of dynorphin A, Gly-2 being substituted by D-Ala and the amide structure being placed at the C-terminus. Radioligand receptor binding assays were carried out for Cys-(Npys)-containing [D-Ala^2]dynorphin A analogs **1** and **2** using [^{3}H]DAGO (μ receptors) and [^{3}H]DEL (δ receptors) in rat brain, and [^{3}H]U-69593 (κ receptors) in guinea pig brain. The IC_{50} values, *i.e.* the half-maximal concentrations for inhibition of the specific binding of radio-labeled ligands, of compound **1** were 26 nM for [^{3}H]DAGO, 210 nM for [^{3}H]DEL, and 340 nM for [^{3}H]U-69593, indicating that compound **1** is 8 and 13 times more potent for the μ receptor than for δ and κ receptors, respectively. These values of compound **2** were 29 nM for [^{3}H]DAGO, 130 nM for [^{3}H]DEL, and 330 nM for [^{3}H]U-69593. Compound **2** is also more potent for the μ receptor (4-fold more than for the δ and 11-fold more than for κ receptor).

Dynorphin A itself is highly specific and selective for the κ receptor, while C-terminal shortened analogs of it such as dynorphin A-(1-8-13) were found to bind to the μ and δ subtypes moderately (*11-13*). It should be noted that Cys(Npys)-containing dynorphin analogs **1** and **2** showed weaker affinity than the parent dynorphin A (1.12 nM) for the κ opioid receptor. In contrast, their binding affinities for the μ and δ subtypes were not so weak. Indeed, the affinities of analogs **1** and **2** for the μ receptor are fairly high. Despite the varying receptor binding affinities, dynorphin A analogs **1** and **2** were expected to exhibit reasonably high ability as to affinity-labeling of all three opioid receptor subtypes.

Affinity Labeling of Opioid Receptors with [D-Ala^2, Cys(Npys)^8]Dyn A-(1-9) Amide (1)—When Cys(Npys)-containing peptide **1** was incubated with brain membranes, they bound to the ligand binding sites of receptors at first. Meanwhile, if there is a receptor mercapto group near the peptide C-terminal portion, the SNpys group of compound **1** 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 dynorphin A analog **1**, receptor binding assays would reveal the loss of μ, δ, and κ receptors, and consequently decreases in the amounts of the receptors labeled.

In the present study, guinea pig 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 U-69593 and then with radio-labeled [^{3}H]U-69593 to determine the amount of κ receptors remaining unlabeled. When membranes were incubated with 10 nM **1**, about 40% κ receptors were lost, indicating that 40% κ receptors were occupied by **1** (Fig. 3A). The occupation became about 60% on incubation with 100 nM **1**. With increasing concentrations of compound **1**, the receptors available for U-69593 decreased sharply (Fig. 3A). At concentrations of more than 1 μM, **1** appeared to occupy almost all the μ receptors. Scatchard analyses gave a monophasic straight line in all cases (data not shown), and the affinity constants of U-69593 were estimated to be about $3.7 \pm 0.53 \times 10^8$ M^{-1} in all U-69593/[^{3}H]-U-69593 binding assays. These results indicate that the U-69593-specific receptors occupied by compound **1** are homogenous.

In the assay involving rat brain membranes for the δ and μ receptors, similar receptor binding profiles were observed, as shown in Fig. 3, B and C. When membranes were incubated with 100 nM 1 and then assayed for deltorphin II/[^{3}H]deltorphin II, about 65% of the δ receptors were found to be occupied by compound **1**. When assayed for DAGO/[^{3}H]DAGO, the occupation became about 80%. It appeared that the extent of the reduction in the receptor population differs with the receptor subtype.

When the amount of receptors labeled (% labeling) was plotted against the concentration of compound 1 for preincubation, sigmoid curves were obtained, as shown in Fig. 4.

From these curves, the effective concentrations (EC_{50}) which are enough to label the half-maximal amount of total receptors can be estimated. The values calculated were 210 nM for κ receptors, 160 nM for δ receptors, and 33 nM for μ receptors (Table I). Compound 1 is several times more selective as to the labeling of μ receptors than δ and κ receptors. It is of note that the ability of compound 1 as to affinity labeling of μ receptors is satisfactory, even as compared with SNpys-containing enkephalins (*10*). As to the time of incubation of Cys(Npys)-containing peptides with membrane preparations, it was found that it takes at least 20 min to attain the maximal labeling with each concentration of a peptide. Although the extent to which Cys(Npys)-containing peptides label the receptors decreased with incubation for less than 20 min, the ability to affinity-label was found to be not changed between peptides and also among the receptor subtypes. Thus, the incubation

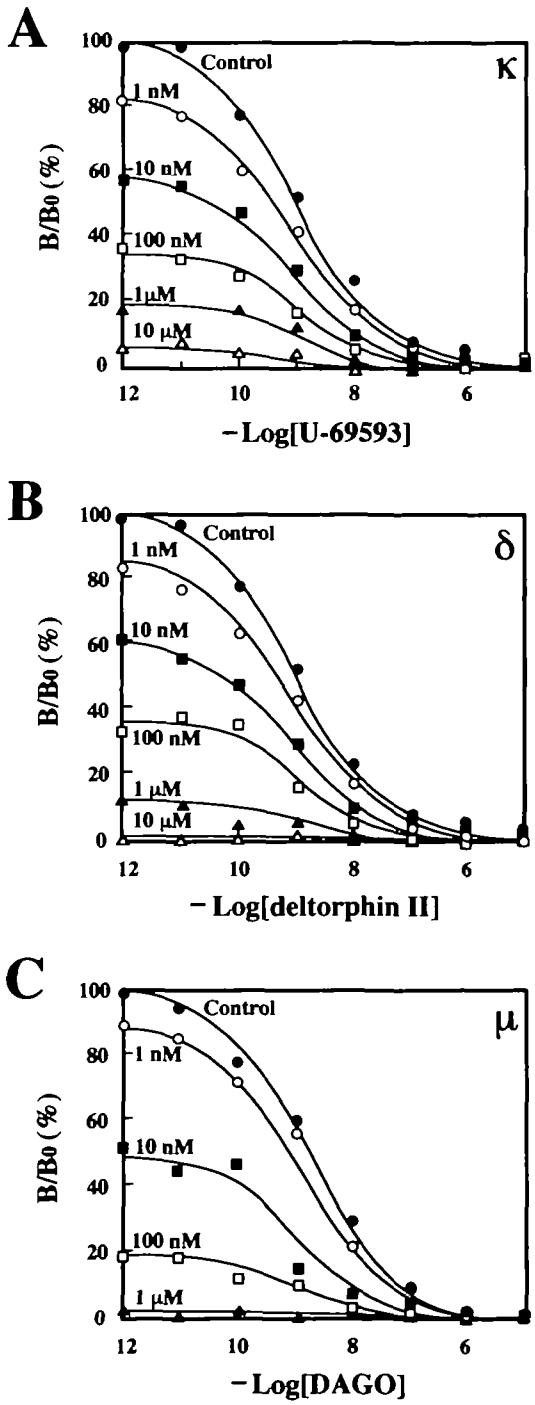

Fig. 3. **Loss of receptor binding sites on preincubation of rat brain membranes with Cys(Npys)[8]-dynorphin A analog.** Each binding assay was carried out with U-69593/[^{3}H]U-69593 for κ opioid receptors (A), deltorphin II/[^{3}H]DEL for δ opioid receptors (B), or DAGO/[^{3}H]DAGO for μ opioid receptors (C), respectively. The concentrations are the concentrations of the Cys(Npys)[8]-containing dynorphin A analog (1) used for preincubation. The assays were repeated at least three times and each data point is the average of these experiments (SE<20%).

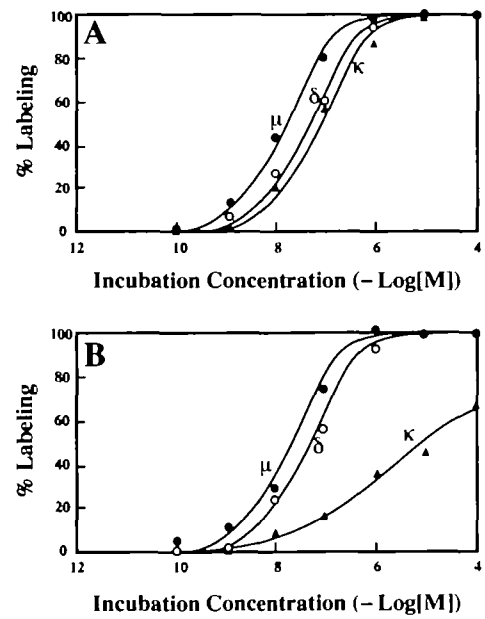

Fig. 4. **Affinity labeling of opioid receptors with Cys(Npys)[8]-dynorphin A analog (1) (A), and Cys(Npys)[12]-dynorphin A analog (2) (B).** The labeling percentage was calculated by subtracting the total binding of DAGO (μ), deltorphin II (δ), or U-69593 (κ) from that without preincubation with a Cys(Npys)-containing dynorphin A analog. The assays were repeated at least three times and each % labeling value is the average of these experiments (SE<8%).

TABLE I. **Effective concentrations of Cys(Npys)-containing dynorphin A analogs for affinity labeling of opioid receptor subtypes.**

Dynorphin A analog	Receptor assay	Receptor binding[a] IC_{50} (nM)	Affinity labeling[b] EC_{50} (nM)
1	μ-assay	26±11	33±13
	δ-assay	210±45	160±52
	κ-assay	340±42	210±35
2	μ-assay	29±9.6	27± 9.0
	δ-assay	130±28	180±28
	κ-assay	330±67	10,000[c]

[a]Receptor binding assays were carried out using [^{3}H]DAGO, [^{3}H]-DEL, and [^{3}H]U-69593 for the μ, δ, and κ subtypes, respectively. [b]Receptor affinity labeling experiments were performed by means of binding assays involving the combinations of DAGO/[^{3}H]DAGO, deltorphin II/[^{3}H]DEL, and U-69593/[^{3}H]U-69593 for the μ, δ, and κ subtypes, respectively. [c]SE was not determined because of the incomple of the % affinity labeling curve at concentrations more than 100 μM, as shown Fig. 4.

time was set at 30 min for maximal labeling, as described above.

In order to demonstrate the occurrence of disulfide bonding between the receptors and Cys(Npys)-containing peptides, labeled membranes were treated with dithiothreitol (DTT). If a disulfide bond is cleaved by DTT, a receptor might recover from occupation by dynorphins 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 determined by means of ordinary receptor binding assays. For instance, the incubation of membranes with 1 μM compound **1** eliminated about 95% of the total binding of DAGO for μ receptors. When the membranes were treated with 1 mM DTT, most of the DAGO binding was recovered.

Affinity Labeling of Opioid Receptors with [D-Ala², Cys(Npys)¹²]Dyn A-(1-13) Amide (2)—Compound **2**, [D-Ala²,Cys(Npys)¹²]Dyn A-(1-13) amide, labeled μ and δ receptors considerably strongly. The potency, expressed as EC_{50} values, was almost the same as that of compound **1** (Table I). Despite such equipotent ability as to labeling of μ and δ receptors, however, compound **2** only labeled κ receptors extremely weakly. The EC_{50} value of 10,000 nM is approximately 50 times larger than that of compound **1** for κ receptors, indicating that compound **2** is about 50 times weaker than compound **1** as to labeling of κ receptors.

DISCUSSION

Several lines of evidence have indicated the existence of mercapto group(s) in opioid receptors (*6, 7, 18*), and a mercapto group sensitive to *N*-ethylmaleimide (NEM) was suggested to exist at or near the binding site of a receptor protein (*19*). The present results clearly demonstrated that all three types of opioid receptors, namely, the μ, δ, and κ receptors, contain a free mercapto group near their ligand binding sites. The affinity labeling of opioid receptors with SNpys-containing dynorphin A analogs occurred through the formation of a disulfide bond between the dynorphin peptide and the receptor. The SNpys group is only able to react with a free mercapto group, which substantiates the formation of a disulfide bond with the receptor mercapto group. This was demonstrated by the experiment to evaluate the recovery of receptors after treatment with DTT.

[D-Ala²,Cys(Npys)⁸]Dyn A-(1-9) amide (**1**) and [D-Ala², Cys(Npys)¹²]Dyn A-(1-13) amide (**2**) are characteristic among dynorphin A analogs synthesized to date in that they contain a mercapto group modified so as to form a nonsymmetrical disulfide bond. SNpys-containing compounds **1** and **2** bind strongly to μ receptors (IC_{50}: 26 nM by **1** and 29 nM by **2**) and affinity-labeled them with almost the same potency (EC_{50}: 33 nM for **1** and 27 nM for **2**) (Table I). Similar receptor responses were observed for δ receptors with compounds **1** and **2** (IC_{50} on binding: 210 nM for **1** and 130 nM for **2**, and EC_{50} on affinity-labeling: 160 nM for **1** and 180 nM for **2**), although their potencies as to δ receptors were lower than those as to μ receptors. The ability to label opioid receptors appears to reflect the binding ability, namely, the receptor affinity. Collectively, these results suggested that the mercapto groups in μ and δ receptors are located near the Cys(Npys) residues in compounds **1** and **2**, causing spontaneous cross-linking after binding.

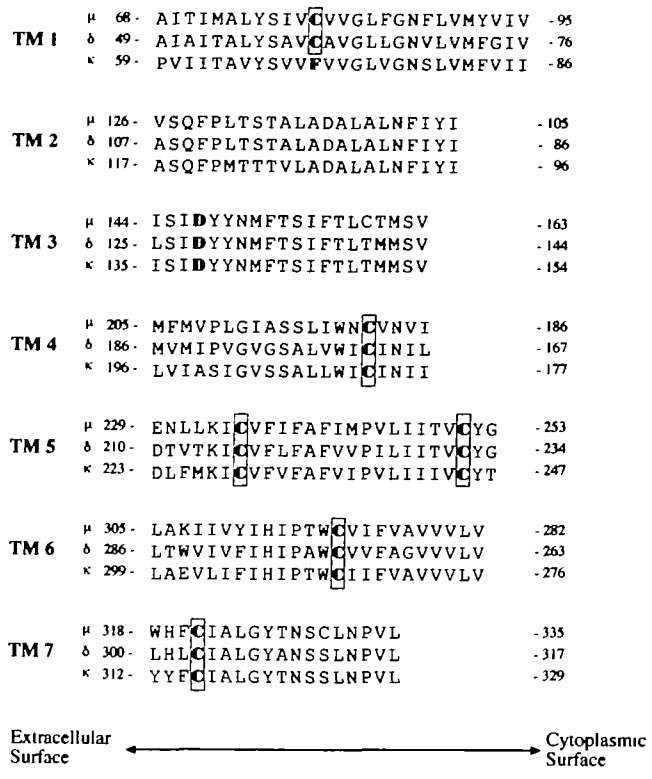

Fig. 5. **Alignment of the amino acid sequences of the seven transmembrane regions of μ, δ, and κ opioid receptors.** The boxed residues are the cysteines conserved among opioid receptor subtypes. Highlighted residue D (aspartic acid in TM 3) is the putative electrostatic interaction site of the quaternary ammonium cation in the opioid peptide. Notably, Cys in TM 1 is present in the μ and δ receptors, but not in the κ receptor.

The most striking finding in the present affinity labeling experiments on opioid receptors was that, unlike compound **1**, compound **2** only labeled κ receptors extremely weakly. For the labeling of κ receptors, about 60-370-fold higher concentrations of compound **2** were required than for labeling of μ and δ receptors. This is in contrast to the receptor selectivity of compound **1**. Compound **1** exhibited almost the same ability as to the labeling of δ and κ receptors on affinity labeling, although it showed about 5-6-fold higher selectivity for μ receptors (Table I). The potencies of compound **2** as to the labeling μ and δ receptors are almost equal to those of compound **1**. Apparently, the difference in receptor selection between analogs **1** and **2** is only in the ability to label κ receptors. The potency discrepancy for compound **2** between receptor binding (IC_{50} = 330 nM) and affinity labeling (EC_{50} = 10,000 nM) clearly indicates that κ receptors do not have a mercapto group at the right position, *i.e.* where the Cys(Npys) residue of compound **2** binds. In contrast, the mercapto group in κ receptors labeled with compound **1** appears to be located near the Cys(Npys) residue of **1**, since compound **1** exhibited almost the same receptor responses on binding (IC_{50} = 340 nM) and affinity labeling (EC_{50} = 210 nM) (Table I).

The structural differences between compounds **1** and **2** are in the position of the Cys(Npys) residue and the peptide length (Fig. 2). Since the binding affinities of compounds **1** and **2** for the three subtypes of opioid receptors are very

similar, an influence of the peptide length on affinity labeling is unlikely. Instead, the difference in the position of Cys(Npys) appears to be critical, and this strongly suggests that the Cys(Npys) residues in compounds **1** and **2** affinity-label different mercapto groups of opioid receptors.

The structures of the μ, δ, and κ receptors are very similar to each other, as shown in Fig. 5, which shows alignment of the amino acid sequences of seven-transmembrane domains. There are several Cys residues conserved among opioid receptors. The previous study on molecular modeling of opioid receptors suggested that opioid receptors contain a conserved aspartate residue in TM 3 and that this residue is the primary binding site for opioid ligands having a protonated nitrogen (*20, 21*). In this model, the binding site of opioid receptors appears to be in the cleft centered around this TM 3 aspartate (μ, Asp-147; δ, Asp-128; and κ, Asp-138). Three-dimensional models of opioid receptors suggested that, among the conserved Cys residues, only two are involved in this binding site (*21*). These include the Cys residue in TM 1 (μ, Cys-79; and δ, Cys-60) and that in TM 7 (μ, Cys-321; δ, Cys-303; and κ, Cys-315). It should be noted that the Cys residue in TM 1, corresponding to Cys-79 in μ receptors and Cys-60 in δ receptors, is not present in κ receptors. The residue is replaced by Phe (Fig. 5). Since [D-Ala^2,Cys(Npys)^{12}]Dyn A-(1-13) amide (**2**) in the present study only labeled κ sites extremely weakly as compared to its labeling abilities as to μ and δ sites, it is highly likely that analog **2** affinity labels the Cys residue in TM 1. The Cys(Npys) residue in analog **2** might be in close proximity to the Cys residue in TM 1 of the μ and δ receptors, where no Cys exists in the κ receptor.

Although the efficiency of labeling was very low, analog **2** also labeled κ receptors. The residue labeled by compound **2** in κ receptors might be the Cys residue in a transmembrane domain other than TM 1. In contrast, [D-Ala^2, Cys(Npys)^8]Dyn A-(1-9) amide (**1**) appears to affinity-label the Cys residue conserved in all three opioid receptor subtypes. As mentioned above, in the opioid receptor model the Cys residue in TM 7 was suggested to be in the binding site, and it is likely that the Cys residue affinity-labeled by compound **1** is that in TM 7.

The present results suggested that all three opioid receptor subtypes contain one or two free Cys residues near or in the ligand binding site. It is feasible that our Cys-(Npys)-containing dynorphin A analogs can covalently cross-link these subtypes *via* the thiol-disulfide exchange reaction. Further biochemical approaches would enable determination of the exact location of the cysteine residues affinity-labeled to elucidate their roles and functions.

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