Specific Affinity Labeling of μ Opioid Receptors by S-Activated Enkephalin Analog Containing *p*-Nitrophenylalanine

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(Received July 23, 1993)

Synopsis. *p*-Nitrophenylalanine (Phe(p-NO_2)) was incorporated in place of Phe^4 of [D-Ala^2, Leu(CH_2S-Npys)^5]-enkephalin. In the rat brain receptor binding assay, Npys-containing Phe(p-NO_2)^4- and Phe^4-enkephalins exhibited almost unchanged affinities for μ receptors (IC_{50}=16—21 nM). However, when the membrane was incubated with each analog, Phe(p-NO_2)^4-enkephalin (EC_{50}=6.90 nM) could label the μ receptors about three times more effectively than Phe^4-enkephalin.

G-protein-coupling opioid receptors contain the two different mercapto groups which can be alkylated with *N*-ethylmaleimide.[1] Both of these thiols are originated from the cysteine residues, and the one is present in the C-terminal portion of G-protein and the other in the ligand binding site of receptor protein.[1] We have previously reported that the enkephalin analogs containing a mixed disulfide bond of leucinethiol, Leu(CH_2SH),[2] could bind covalently to a free mercapto (SH) group in the binding site of the μ opioid receptors via the thiol–disulfide exchange reaction.[3,4] Leucinethiol activated by the 3-nitro-2-pyridinesulfenyl (Npys) group was very effective for such an affinity labeling. When [D-Ala^2, Leu(CH_2S–Npys)^5]enkephalin was incubated with the isolated muscle tissue from the guinea pig ileum, the enkephalin covalently bound to the tissue and exhibited a continuous activation of muscle contraction. For the complete labeling of receptors, however, considerably higher concentrations were required, and it was thought that many other mercapto groups present in membrane proteins would also be modified under these conditions. Such modifications of proteins might affect the molecular responses of opioid receptors. Furthermore, several subtypes of opioid receptors usually coexist in the tissues such as brain and guinea pig ileum.[5] For discriminative affinity labeling of only a single type of receptors, it is requisite to obtain more specific and more selective ligands.

Castell et al.[6] reported that the affinity of enkephalins for μ receptors increases very much by replacement of the phenylalanine residue at position 4 by *p*-nitrophenylalanine, Phe(p-NO_2). In the present study, to attain a more specific affinity ligand we have synthesized the Phe(p-NO_2)^4 analog (**1**) of [D-Ala^2, Phe^4, Leu(CH_2S–Npys)^5]enkephalin (**2**) (Fig. 1). The binding ability to label the μ opioid receptors was examined by incubating the analog with rat brain membrane.

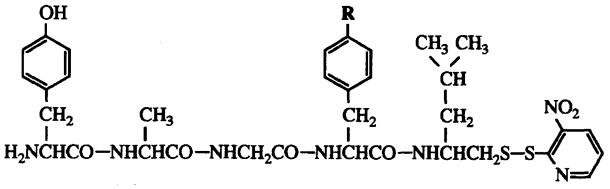

Fig. 1. Structure of Npys-containing enkephalins. [D-Ala^2, Phe(p-NO_2)^4, Leu(CH_2S–Npys)^5]enkephalin (**1**, R=NO_2) and [D-Ala^2, Phe^4, Leu(CH_2S–Npys)^5]-enkephalin (**2**, R=H).

Results and Discussion

The schematic profile of synthesis of [D-Ala^2, Phe(p-NO_2)^4, Leu(CH_2S–Npys)^5]enkephalin (**1**) is shown in Fig. 2. The disulfide-bridged leucinethiol dimer **3** was prepared from leucinol as previously reported.[7] Dimeric pentapeptide (Boc–Tyr–D-Ala–Gly–Phe(p-NO_2)–Leu(CH_2S-))$_2$ (**5**) was obtained by the stepwise elongations of **3** with Boc–Phe(p-NO_2)–OH (to afford compound **4**) and then with Boc–Tyr–D-Ala–Gly–OH using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in the presence of 1-hydroxybenzotriazole (HOBt). After reduction of the disulfide bond in **5** using tributylphosphine ((n-Bu)$_3$P), the resulting Boc–pentapeptide-thiol was reacted with 3-nitro-2-pyridinesulfenyl chloride (Npys–Cl) to afford S-activated peptide **6** (Fig. 2).

Specific binding affinity of [D-Ala^2, Phe(p-NO_2)^4, Leu(CH_2S–Npys)^5]enkephalin (**1**) for μ opioid receptors was assessed using rat brain membrane and [^{3}H]–[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin ([^{3}H]–DAGO) as a ligand selective for μ receptors,[8] and compared with that of [D-Ala^2, Phe^4, Leu(CH_2S–Npys)^5]enkephalin (**2**). Figure 3 shows the dose-response curves analyzed by the computer program ALLFIT,[9] which constructs the least-square estimates of the logistic curves relating binding of labeled ligand to concentrations of unlabeled ligand. It is clear that both enkephalins are almost equipotent for μ receptors. The IC_{50} values, the half-maximal concentration of unlabeled ligands for inhibition of binding of lableled ligand, were 15.6±1.4 nM for Phe(p-NO_2)^4-enkephalin **1** and 20.9±2.0 nM for Phe^4-enkephalin **2**. For δ opioid receptors using [^{3}H]–[D-Ser^2, Leu^5]enkephalyl–Thr^6 ([^{3}H]–DSLET) as a tracer,[10] **1** exhibited the IC_{50} value of 13.5±1.7 nM and thus it

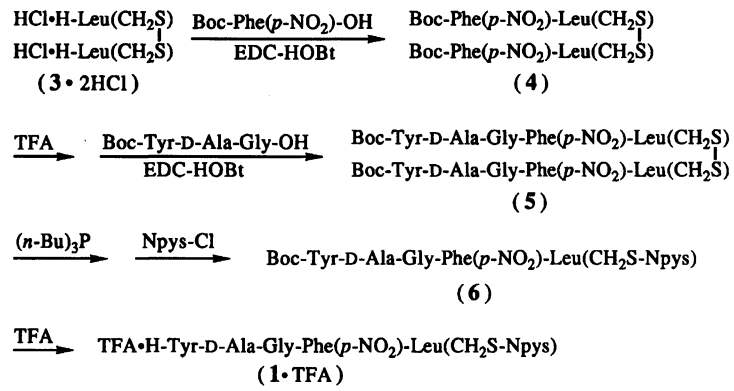

Fig. 2. Synthetic scheme of [D-Ala^2, Phe(p-NO_2)^4, Leu(CH_2S–Npys)^5]enkephalin (**1**).

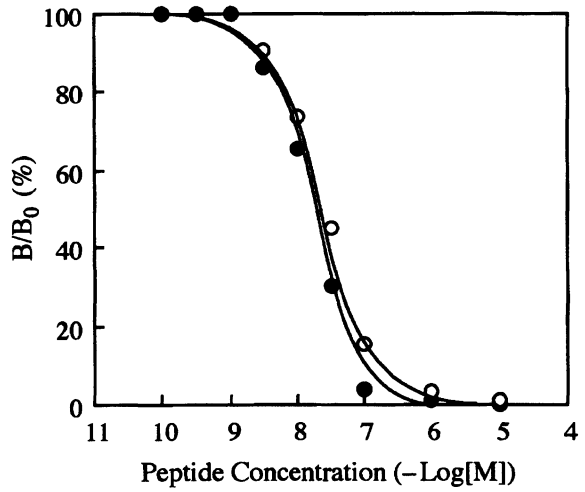

Fig. 3. Dose-response curves of Npys–enkephalins in rat brain membrane preparations using [^{3}H]–DAGO for μ opioid receptors. [D-Ala^2, Phe(p-NO_2)^4, Leu(CH_2S–Npys)^5]enkephalin (**1**, ●—●) and [D-Ala^2, Phe^4, Leu(CH_2S–Npys)^5]enkephalin (**2**, ○—○).

appears to be non-selective in interaction with μ or δ receptors. Phe^4-enkephalin **2** was slightly μ-selective with a selectivity ratio of 2. Although Castell et al.[6] reported that the incorporation of Phe(p-NO_2) increases the binding affinity of Phe^4-enkephalin for μ receptors, such a modification for [D-Ala^2, Phe^4, Leu(CH_2S–Npys)^5]enkephalin **2** enhanced only slightly the affinity for δ receptors, but not for μ receptors.

The ability of enkephalin analogs to affinity-label the opioid receptors can be examined by their incubation with membranes and subsequent assays for biological activity or receptor binding. Since the assessment of biological activities usually requires to evaluate the efficacy between receptor binding and activity, in the present study the capability of enkephalin analogs for affinity labeling was examined by the radioligand receptor binding assays. When Npys-containing analogs [D-Ala^2, Phe(p-NO_2)^4 or Phe^4, Leu(CH_2S–Npys)^5]enkephalin (**1** or **2**) were incubated with rat brain membrane, they will first bind to the ligand binding site of the receptors. However, if a mercapto group is present near the peptide bound in the receptor, the Npys group will react with this free thiol, resulting in the formation of a disulfide bond. Such affinity labeling of receptors would substantially reduce the number of receptors available for binding of the ligands added afterwards. Thus, after incubation of membranes with Npys–peptides, the ordinary receptor binding assay would estimate the amount of receptors unlabeled and consequently the amount of labeled receptors.

In order to estimate the total amount of the free receptors, the amount of DAGO–enkephalin that displaces radio-labeled [^{3}H]–DAGO was measured.[11] This binding assay evaluates the extent of affinity labeling of μ receptors, because DAGO binds exclusively to the μ receptors.[8] Since [D-Ala^2, Phe^4, Leu(CH_2S–Npys)^5]-enkephalin **2** exhibited no labeling of δ receptors in mouse vas deferens,[3] no assays were carried out by utilizing [^{3}H]–DSLET in the present study. For the complete removal of peptides bound non-specifically to the membrane, at least four times washings were required, and the wash was performed by repeated operations of suspension with buffer and successive centrifugation. When [D-Ala^2, Phe(p-NO_2)^4, Leu(CH_2S–Npys)^5]enkephalin **1** was incubated with rat brain membrane, the amount of free receptors diminished sharply, depending upon the concentrations of Npys–enkephalin (Fig. 4). As shown in Fig. 4B, when the rat brain membrane was incubated with 100 nM of peptide **2**, about 25% of the whole μ receptors were available for binding of DAGO–enkephalin. On the other hand, only 6% were available when incubated with peptide **1** (Fig. 4A). This difference in the amount of free receptors is the reflection of difference in their ability to bind covalently to the receptors.

When the extent (%) of affinity-labeling was plotted against the concentrations of Npys–enkephalins incubated, the typical sigmoidal curves were depicted as shown in Fig. 5. At the concentration near the IC_{50} (about 20 nM), [D-Ala^2, Phe(p-NO_2)^4, Leu(CH_2S–Npys)^5]enkephalin **1** seems to occupy approximately 80% of μ receptors. Whilst, about 50% of the receptors were labeled by [D-Ala^2, Phe^4, Leu(CH_2S–Npys)^5]-enkephalin **2**. From the analysis of Fig. 5, it became

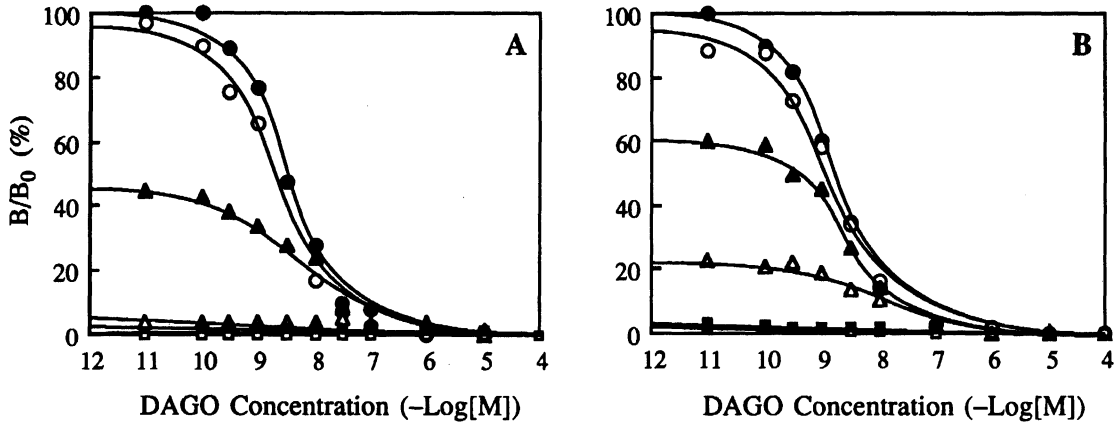

Fig. 4. Dose-response curves of DAGO–enkephalin in rat brain membrane preparations using [^{3}H]–DAGO for μ opioid receptors. (A) Rat brain membrane was incubated with [D-Ala^2, Phe(p-NO$_2$)^4, Leu(CH$_2$S–Npys)^5]enkephalin (**1**) before binding assays, while in (B) incubations were carried out with [D-Ala^2, Phe^4, Leu(CH$_2$S–Npys)^5]enkephalin (**2**). The concentrations of Npys-containing enkephalins incubated (**1** and **2**) are (1 nM, ○—○), (10 nM, ▲—▲), (100 nM, △—△), (1 μM, ■—■), (10 μM, □—□), and (control, ●—●).

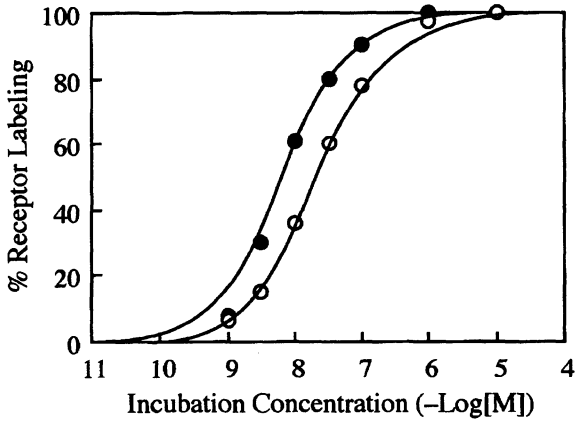

Fig. 5. The concentration-dependent affinity labeling of μ opioid receptors by Npys-containing enkephalins. [D-Ala^2, Phe(p-NO$_2$)^4, Leu(CH$_2$S–Npys)^5]enkephalin (**1**, ●—●) and [D-Ala^2, Phe^4, Leu(CH$_2$S–Npys)^5]-enkephalin (**2**, ○—○).

apparent that, for labeling of μ receptors, [D-Ala^2, Phe(p-NO$_2$)^4, Leu(CH$_2$S–Npys)^5]enkephalin **1** (EC$_{50}$=6.90 nM, which denotes the effective concentration to occupy the half-maximal amount of receptors) is almost three times more effective than its Phe^4 derivative **2** (EC$_{50}$=20.3 nM). This increment in labeling of μ receptors is due definitely to the incorporation of Phe(p-NO$_2$) at position 4. In spite of unchanged binding affinity for μ receptors between Phe(p-NO$_2$)^4– and Phe^4–enkephalins, their ability to label the receptors by a disulfide formation differed very much. Although the exact reason is not clear for such differences in their receptor interactions, the present study implies the availability of Phe(p-NO$_2$)^4-containing enkephalin analogs for the specific labeling of μ receptors and the attempts to isolate μ receptors using these analogs are in progress in our laboratories.

Experimental

General. Melting points were uncorrected. TLC was carried out on silica gel 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$_2$O (4:1:2); and R_f^6, n-BuOH–AcOH–pyridine–H$_2$O (4:1:1:2). Boc–Phe(p-NO$_2$)–OH was purchased from Watanabe Chemical Ind. (Hiroshima). Compounds with free amino group were detected by spraying ninhydrin, and other compounds were detected by carbonization after spraying 10% H$_2$SO$_4$. Optical rotations were measured with a JASCO DIP-370 digital spectropolarimeter.

(Boc–Phe(p-NO$_2$)–Leu(CH$_2$S–))$_2$ (4): To a solution of Boc–Phe(p-NO$_2$)–OH(1.37 g, 4.42 mmol), (H–Leu-(CH$_2$S–))$_2$·2HCl (**3**) (675 mg, 2.00 mmol)[7] and Et$_3$N (0.560 ml, 4.00 mmol) in DMF (15 ml) were added HOBt (713 mg, 5.28 mmol) and EDC·HCl (928 mg, 4.84 mmol) at 0 °C. The reaction mixture was stirred for 3 h at 0 °C and for a day at room temperature. The solution was evaporated and the residue was solidified by addition of water. The crude product was purified by Sephadex LH-20 (3.5×114 cm) eluted with DMF. The product was recrystallized from DMF–ether–pet. ether; yield 1.51 g (89%); mp 221—222 °C; $[\alpha]_D^{20}$ +36.0° (c 1.01, DMF); R_f^2 0.71, R_f^4 0.56. Found: C, 56.42; H, 7.04; N, 9.90%. Calcd for C$_{40}$H$_{60}$N$_6$O$_{10}$S$_2$: C, 56.58; H, 7.12; N, 9.90%.

(Boc–Tyr–D-Ala–Gly–Phe(p-NO$_2$)–Leu(CH$_2$S–))$_2$ (5): Compound **4** (679 mg, 0.800 mmol) was treated with trifluoroacetic acid (TFA) (2 ml) at 0 °C for 30 min. The solution was evaporated and the residue of (H–Phe(p-NO$_2$)–Leu(CH$_2$S–))$_2$ was solidified by adding ether; yield 702 mg (100% as a TFA salt); R_f^2 0.19. To a solution of this TFA salt (702 mg, 0.800 mmol) in Et$_3$N (0.220 ml, 1.60 mmol) and DMF (15 ml) was added Boc–Tyr–D-Ala–Gly–OH (721 mg, 1.76 mmol), HOBt (285 mg, 2.11 mmol), and EDC·HCl (372 mg, 1.94 mmol) at 0 °C. The reaction mixture was stirred for 3 h at 0 °C and overnight at room temperature. The solution was then treated as described for

compound **4**. The purification was carried out by gel filtration on Sephadex LH-20 (3.5×114 cm) eluted with DMF; yield 475 mg (42%); mp 148—149 °C; $[\alpha]_D^{20}$ +19.3° (*c* 1.00, DMF); R_f^1 0.62, R_f^3 0.72. Found: C, 56.32; H, 6.55; N, 11.60%. Calcd for $C_{68}H_{94}N_{12}O_{18}S_2 \cdot H_2O$: C, 56.34; H, 6.67; N, 11.59%.

Boc–Tyr–D-Ala–Gly–Phe(*p*-NO$_2$)–Leu(CH$_2$S–Npys) (6): To a solution of compound **5** (143 mg, 0.100 mmol) in MeOH–H_2O (4:1, 5 ml) were added (n-Bu)$_3$P (0.030 ml, 0.120 mmol) at 0 °C. After 3 h with continuous stirring, Npys–Cl (46.0 mg, 0.240 mmol) in dioxane (2 ml) was added at 0 °C. The reaction mixture was stirred overnight at room temperature, and evaporated in vacuo to leave an oily residue. The residue was purified by gel filtration using a Sephadex LH-20 column (1.5×137 cm) eluted with DMF; yield, 117 mg (67%); mp 121—122 °C; $[\alpha]_D^{20}$ +67.1° (*c* 1.02, MeOH); R_f^2 0.44, R_f^4 0.32. Found: C, 53.23; H, 5.83; N, 12.67%. Calcd for $C_{39}H_{52}N_8O_{11}S_2 \cdot 1/2H_2O$: C, 53.23; H, 5.84; N, 12.73%.

H–Tyr–D-Ala–Gly–Phe(*p*-NO$_2$)–Leu(CH$_2$S–Npys)·TFA (1·TFA): Compound **6** (44 mg, 0.050 mmol) was treated with TFA (2 ml) at 0 °C for 30 min. The reaction mixture was evaporated and the oily residue was crystallized by the addition of ether; yield, 33 mg (75%); mp 128—129 °C; $[\alpha]_D^{20}$ +35.1° (*c* 1.00, DMF); R_f^5 0.68, R_f^6 0.85.

Receptor Binding Assay. Radio-ligand receptor binding assays using rat brain membrane preparations were carried out essentially as described previously.[12] [^{3}H]–DAGO ([^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin) (1.8 TBq/mmol) and [^{3}H]–DSLET ([^{3}H]-[D-Ser^2, Leu^5]-enkephalyl-Thr^6) (1.5 TBq/mmol) were used as tracers specific 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^{-1}) was also added to the buffer as an enzyme inhibitor. The results were analyzed by the computer program ALLFIT.[9]

Affinity Labeling of μ Opioid Receptors. Rat brain membranes in 50 mM Tris–HCl buffer (pH 7.5) were incubated with Npys–peptides (0.1 nM—10 μM) or without ligands (control) in the presence of bacitracin (100 μg ml^{-1}) at 25 °C for 30 min. Membranes were then centrifuged (40000 g, 15 min) and suspended in the same volume of buffer to homogenize with Polytoron homogenizer for a few minutes. These washing operations were repeated successively four times. Washed membranes were finally assayed for binding of DAGO using [^{3}H]–DAGO as a tracer as described above.

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2) The abbreviations according to biochemical nomenclature by IUPAC-IUB Joint Commission, *Eur. J. Biochem.*, **138**, 9-37 (1984), are used throughout. Unless otherwise specified, the amino acids are L-stereoisomers. Additional abbreviations are as follows: Boc, *t*-butoxycarbonyl; DAGO, [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin; DSLET, [D-Ser^2, Leu^5]enkephalyl–Thr^6; DMF, *N*,*N*-dimethylformamide; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carobodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; leucinethiol, 2-amino-4-methyl-1-pentanethiol; Npys, 3-nitro-2-pyridinesulfenyl or 3-nitro-2-pyridylthio; TFA, trifluoroacetic acid.

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