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Discriminative affinity labelling of opioid receptors by enkephalin and morphiceptin analogues containing 3-nitro-2-pyridinesulphenyl-activated thiol residues

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ABSTRACT

The thiol groups of leucinthiol, cysteamine and cysteine incorporated into opioid peptides enkephalin and morphiceptin were activated by the 3-nitro-2-pyridinesulphenyl (Npys) group to form mixed disulphides highly reactive to a free thiol. Enkephalin analogues containing Npys-leucinthiol or -cysteine at positions 4, 5 and 6 exhibited high affinities for both μ and δ receptors, while morphiceptin analogues containing Npys-cysteine at positions 4 and 5 showed relatively weak affinity only for μ receptors. When these S-activated opioid peptides were incubated with rat brain membrane preparations, it was found, by binding assay using radiolabelled and non-labelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin, that they label μ opioid receptors in a dose-dependent manner. The concentrations required to label half of the receptors were 0.2–2 μM for enkephalins and 10–30 μM for morphiceptins. These results suggested that the thiol group labelled by S-activated enkephalins and morphiceptins is present in the ligand binding site of receptor protein, but not in GTPase-binding protein.

INTRODUCTION

Several lines of evidence that indicate the existence of thiol group(s) in receptors of neurotransmitters and neuropeptides have been reported, although their functional roles have not yet been clarified. Larsen *et al.* [1] suggested that in opioid receptors there are at least two different types of thiol groups sensitive to N-ethylmaleimide. The first is the β-thiol of the cysteine residue in GTPase-binding protein (G_i). It has been suggested that the second is at or near the ligand binding site in the receptor protein. We have recently reported that [D-Ala^2,Leu(CH$_2$SNpys)^5]enkephalin (Npys is 3-nitro-2-pyridinesulphenyl) (**1**) can selectively label μ opioid receptors in the peripheral muscle tissues of guinea-pig ileum (GPI) and mouse vas deferens (MVD) [2]. The thiol group of G_i in these isolated smooth muscle preparations is considered to be not exposed, and thus the thiol labelled by **1** was assumed to be that in the receptor protein itself.

When membrane is prepared for purification of the receptor, the thiol groups of both receptor and G_i proteins are exposed and react with SH reagents. This inevitably requires examination of S-activated ligand peptides to determine whether they can bind discriminatively only to the receptors in such a membrane, especially if one intends to take advan-

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1: H-Tyr-D-Ala-Gly-Phe-Leu(CH_2SNpys)
2: H-Tyr-Cta(Npys)
3: H-Tyr-D-Ala-Cta(Npys)
4: H-Tyr-D-Ala-Gly-Cta(Npys)
5: H-Tyr-D-Ala-Gly-Phe-Cta(Npys)
6: H-Tyr-D-Ala-Gly-Phe-Leu-Cta(Npys)
7: H-Tyr-Pro-Cys(Npys)-Phe-NH_2
8: H-Tyr-Pro-Phe-Cys(Npys)-NH_2
9: H-Tyr-Pro-Phe-Pro-Cys(Npys)-NH_2

Fig. 1. Structures of Npys-containing enkephalins and morphiceptins.

tage of their ability to label the receptors for isolation and purification. S-activated receptor ligands should be much more specific for the receptor thiol than N-ethylmaleimide, which is a non-specific SH-alkylating reagent.

In the present study, in addition to peptide **1**, we have prepared two other series of S-activated peptides, namely enkephalin analogues (**2–6**) containing S-Npys-cysteamine [Cta(Npys)] and morphiceptin analogues (**7–9**) containing S-Npys-cysteine [Cys(Npys)] (Fig. 1). Although enkephalin can bind to both μ and δ opioid receptors, morphiceptin is highly selective for μ receptors. We herein describe the receptor-binding characteristics of these S-activated enkephalin and morphiceptin analogues.

EXPERIMENTAL

Materials

3-Nitro-2-pyridinesulphenyl chloride (Npys-Cl) was purchased from Kokusan Chemical Works (Tokyo, Japan), bacitracin from Sigma (St. Louis, MO, USA), bovine serum albumin from Janssen (Olen, Belgium) and digitonin from Wako (Osaka, Japan). The other reagents, of analytical grade, were purchased from Nakarai Tesque (Kyoto, Japan). [^{3}H][D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]DAGO) and [^{3}H][D-Ala^2,D-Leu^5]enkephalin ([^{3}H]DADLE) were purchased from New England Nuclear (Boston, MA, USA).

Peptides

All peptides were prepared by the conventional solution method. The synthesis of [D-Ala^2,Leu(CH_2SNpys)^5]enkephalin (**1**) has been reported previously [2]. A series of cysteamine-containing enkephalin analogues (**2–6**) were prepared from their respective cystamine-bridged dimer by reduction of the disulphide bond and subsequent treatment with Npys-Cl. Another series of Cys(Npys)-morphiceptins (**7–9**) were prepared from Cys(MBzl)-morphiceptins via direct substitution of the methoxybenzyl (MBzl) group with Npys by treatment with Npys-Cl. Purification was carried out by gel filtration on a Sephadex G-15 column. The synthesis of peptides will be reported elsewhere.

Receptor-binding assay

Radioligand receptor-binding assays using rat brain membrane preparations were carried out essentially as described previously [3]. [^{3}H]DAGO (1.4 TBq/mmol) and [^{3}H]DADLE (1.5 TBq/mmol) were used as tracers specific for μ and δ opioid receptors, respectively, at the final concentration of 0.25 n*M*. Incubations were carried out at 25°C for 60 min in 50 m*M* Tris–HCl buffer (pH 7.5) containing 0.1% bovine serum albumin. Bacitracin (100 μg/ml) was added to the buffer as an enzyme inhibitor.

Dose–response curves were constructed utilizing 7–10 doses. The results were analysed by the computer program ALLFIT [4] and the data were used to construct the least-squares estimates of the logistic curves relating the binding of labelled ligand [^{3}H]DAGO and [^{3}H]DADLE to concentrations of unlabelled ligands.

Affinity labelling

Rat brain membranes in 50 m*M* Tris buffer (pH 7.5) were incubated with Npys peptides (0.001–100 μ*M*) or without ligands (controls) in the presence of bacitracin (100 μg/ml) at 25°C for 30 min. Membranes were then centrifuged (39 100 *g*, 15 min) and resuspended in the same volume of buffer for 1–6 washes. Washed membranes were finally assayed for binding of DAGO using [^{3}H]DAGO as a tracer as described above.

RESULTS AND DISCUSSION

Table I shows the receptor-binding affinities of these S-activated Npys-containing enkephalin and morphiceptin analogues for the μ ([^{3}H]DAGO) and δ ([^{3}H]DADLE) receptors. Peptides **2**, **3**, **4** and **7** were almost completely inactive. In contrast, enkephalin analogues **5** and **6**, which have the N-termi-

TABLE I

RECEPTOR BINDING AFFINITY OF Npys-CONTAINING ENKEPHALINS AND MORPHICEPTINS

Compounds	Relative receptor affinity	
	[^{3}H]DAGO	[^{3}H]DADLE
1	100[a]	100[a]
2	0.40	0.11
3	0.56	0.11
4	0.80	0.25
5	105	100
6	61	140
7	0.64	Inactive
8	13	Inactive
9	4.3	Inactive

[a] The IC_{50} values of compound **1** were 0.019 μM ([^{3}H]DAGO) and 0.012 μM ([^{3}H]DADLE).

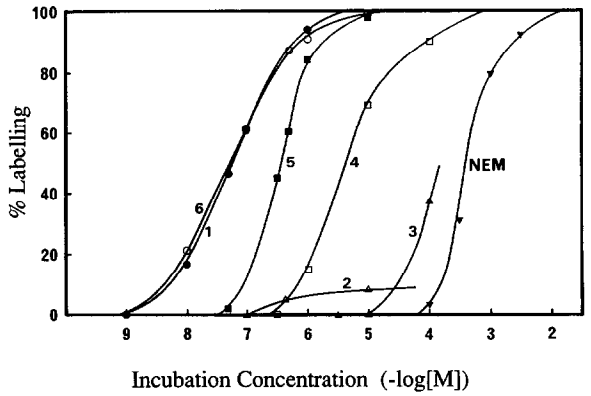

Fig. 2. Affinity labelling of μ opioid receptors by Npys-containing enkephalins. The percentage labelling was calculated by subtracting the total binding of DAGO with from that without preincubation with Npys-containing enkephalins. Numbers show the compounds indicated in Fig. 1 and NEM is N-ethylmaleimide.

nal Tyr-D-Ala-Gly-Phe sequence, were highly active for both μ and δ receptors. Morphiceptin analogues **8** and **9** were also active, though 7- to 23-fold less potent than enkephalin analogues. It should be noted that these morphiceptin analogues were inactive for the δ receptors, indicating that **8** and **9** are specific for the μ receptors.

The ability of enkephalin and morphiceptin analogues to cross-link the receptors can be examined by their preincubation with membranes and subsequent binding assays. When Npys-containing peptides are incubated with rat brain membranes, they will first bind to the ligand binding site of receptors. However, if there is a thiol group near the peptide bound in the receptor, the Npys group will react with this free thiol, resulting in the formation of a disulphide bond. Such affinity labelling of receptors would substantially reduce the number of receptors available for binding the ligand added afterwards. Thus, after preincubation of membranes with Npys peptides the ordinary receptor-binding assay would estimate the amount of receptors unlabelled and consequently the amount of labelled receptors.

In the present study, in order to estimate the total amount of free receptors, the amount of DAGO enkephalin that displaces radiolabelled [^{3}H]DAGO was measured. This binding assay evaluates the extent of affinity labelling of the μ receptors, since DAGO binds only to the μ receptors. For the complete removal of peptide molecules bound non-specifically to the membrane, at least four washings were needed (data not shown). When [D-Ala^2,Leu(CH_2S)Npys^5]enkephalin **1** was incubated with rat

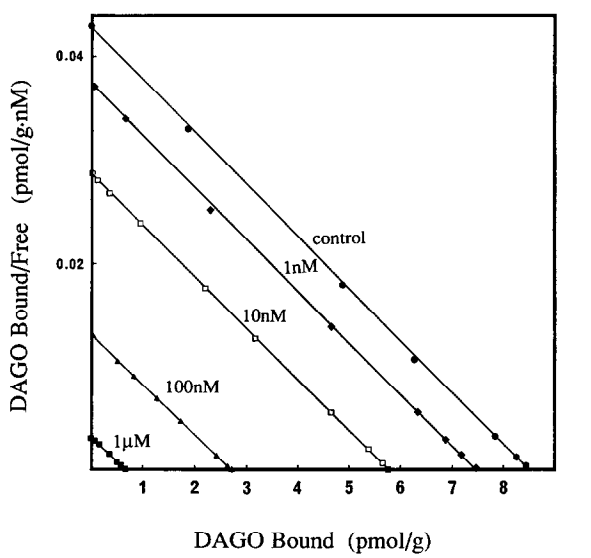

Fig. 3. Scatchard analyses of DAGO binding to μ receptors after preincubation with Npys-containing [D-Ala^2,Leu(CH_2SNpys)^5]enkephalin (**1**). Concentrations are the concentrations enkephalin **1** used for preincubation.

brain membranes, the amount of free receptors decreased sharply, depending on the concentration of **1** (Fig. 2). At the concentration equal to the dose which produces a 50% displacement of the tritiated ligand [^{3}H]DAGO (IC_{50}) (0.019 μM), **1** seems to occupy about 50% of the μ receptors (Fig. 2), indicating that almost all the peptides bound to receptors were trapped covalently by receptor thiols. This concentration is much (approximately 20 000 times) lower than the effective concentration (400 μM) of N-ethylmaleimide that abolished the half-maximal amount of enkephalins able to bind to the receptors [1]. From the Scatchard analyses (Fig. 3), it was found that the affinity constant of DAGO (*ca.* $7 \cdot 10^{10}$ M^{-1}) did not change despite the decrease in receptor population. These results indicate that the labelling of opioid receptors by S-activated enkephalins is exclusive and specific. It is thus very unlikely that labelling of G_i protein diminishes the binding, although it diminishes the binding of agonists [1].

[D-Ala^2]Enkephanyl-Cta(Npys)^6 (**6**) showed binding characteristics very similar to those of peptide **1**. On the other hand, concentrations about ten times higher were required for peptide **4** and **5** to label the same amount of receptors as **1** and **6** did (Fig. 2). The effective concentrations required to label the half-maximum number of receptors were about 0.3 and 2 μM for peptides **5** and **4**, respectively. By contrast, enkephalin analogues **2** and **3** labelled only to a smaller extent (10–30%), even at 100 μM. These results suggest that the thiol group in the binding site of receptors is present in the part where the enkephalin C-terminus binds.

When morphiceptin analogues **8** and **9** were incubated, peptide concentrations higher than 100 μM were needed to label all the receptors. This concentration is 100 times greater than that necessary for full labelling by enkephalin analogues **1** and **6** (Fig. 2). The concentration necessary to label half the receptors was 10–30 μM. The inefficiency of these morphiceptin analogues is presumably due to their poor receptor-binding ability as compared with enkephalin analogues. It should be noted, however, that peptides **8** and **9** may label only the μ receptors.

Enkephalin **1** has been tested also for the peripheral opioid receptors in GPI and MVD [2]. This analogue was found to label only the μ receptors by differential administration of receptor-selective ligands. The present results together with these obtained previously indicate that the opioid receptor protein contains a distinct free thiol group in the ligand binding site. Although a role for this thiol group in the molecular mechanism of receptor responses is not known, it might be useful to utilize such a functional group as a practical tool to purify the receptors.

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