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Purification and reconstitution of μ-opioid receptors in liposome

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ABSTRACT

Opioid receptors solubilized from rat brain membranes with digitonin were partially purified with a newly prepared affinity resin, AF-Amino Toyopearl, coupled with a μ-antagonist Tyr–Pro–Tyr–Tyr at the C-terminus of the peptide. The purified materials were reconstituted with an inhibitory GTP-binding protein (G_i) in liposome. From displacement analyses, two binding states, with a high and a low affinities for the μ-agonist [D-Ala^2, Me-Phe^4, Gly-ol^5]enkephalin, were observed in the reconstituted system with G_i; only a low-affinity state was observed in the reconstituted system without G_i. The results suggested that the purified materials contained the μ-opioid receptors and could functionally couple with G_i as observed in the cell membranes.

INTRODUCTION

The analgesic and euphoric responses to opiates are the results of cascading biological phenomena that are triggered by interaction of the opiates with specific receptors (opioid receptors). However, the molecular basis of the participation of opioid receptors in these phenomena is unknown.

Opioid receptors recognize not only exogenous alkaloids such as morphine, but also their endogenous ligands, namely opioid peptides such as enkephalins and endorphins. Although the precise number of opioid receptor types is still unknown, it is generally accepted that the opioid receptors can be subdivided into three major types, μ, δ and κ [1–3].

To identify opioid receptors, several approaches, including affinity labelling and affinity chromatography, have been tried [4,5]. However, little is known about the membrane-bound structural elements which allow each receptor to recognize selectively pharmacological ligands.

We have previously reported the purification of opioid-binding materials (δ-type receptors) using affinity resins with an enkephalin derivative [D-Ala^2, Leu^5]enkephalin as an affinity ligand [6]. The purified materials could be reconstituted with an inhibitory GTP-binding protein (G_i) in liposome [7]. We also have tried to cross-link the purified materials with [^{125}I]β-endorphin using a cross-linker [8].

In this study we used a new affinity resin, AF-Amino Toyopearl, coupled with Tyr–Pro–Tyr–Tyr (YPYY), to purify μ-type opioid receptors; the tetrapeptide YPYY was reported to be a weak antagonist for μ-opioid receptors [9]. To confirm whether the partially purified materials contained true receptors, the materials were reconstituted with G_i in liposome.

EXPERIMENTAL

Materials

The following materials were purchased as indicated: [^{3}H][D-Ala^2, D-Leu^5]enkephalin (43.9 Ci/mmol), [^{3}H][D-Ala^2, Me-Phe^4, Gly-ol^5]enkephalin (41.6 Ci/mmol), [^{3}H]naloxone (60.6 Ci/mmol), [^{3}H]ethylketocyclazocine (22.5 Ci/mmol) and Aquasol 2 (New England Nuclear, Boston, MA, USA); naloxone hydrochloride, soybean trypsin inhibitor, bacitracin, bovine serum albumin, brain extract, phosphatidylinositol, and low-molecular-

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mass standard mixture for gel electrophoresis (Sigma, St. Louis, MO, USA); AF-Amino Toyopearl (Tosoh, Tokyo, Japan); ultrafiltration membranes PM-30 and microconcentrator Centricon-30 (Grace Japan, Tokyo, Japan); nitrocellulose membrane BA-85 (Schleicher & Schüll, Dassel, Germany; silica gel 60 F_{254} precoated thin-layer plates (Merck, Darmstadt, Germany); Sephadex G-75 (Pharmacia LKB Biotechnology, Uppsala, Sweden); and Ag-Stain Daiichi (Daiichi Pure Chemicals, Tokyo, Japan).

The peptides were synthesized by the conventional solution method and the synthesis route is shown in Fig. 1. The final product, trifluoroacetyl-YPYY, was identified by fast atom bombardment mass spectrometry: *m/z* 701 ($[M + H]^+$). The homogeneity of the products was checked by thin-layer chromatography, using precoated thin-layer plates and the solvent systems dichloromethane–methanol (2:1) and *n*-butanol–acetic acid–water (4:1:2).

The affinity resin, AF-Amino Toyopearl with YPYY, was prepared as reported elsewhere [7]. Briefly, trifluoroacetyl-YPYY was coupled with AF-Amino Toyopearl using water-soluble carbodiimide, then the trifluoroacetyl group was removed by incubation of the modified resin in 0.1 *M* NaOH at room temperature for 24 h. Amino acid analysis of the hydrolysate of the resin obtained with 6 *M* HCl at 110°C for 24 h showed that 7–9 μmol of YPYY was present in 1 ml of the resin.

All other chemicals and chromatographic media were obtained from commercial sources and used as received.

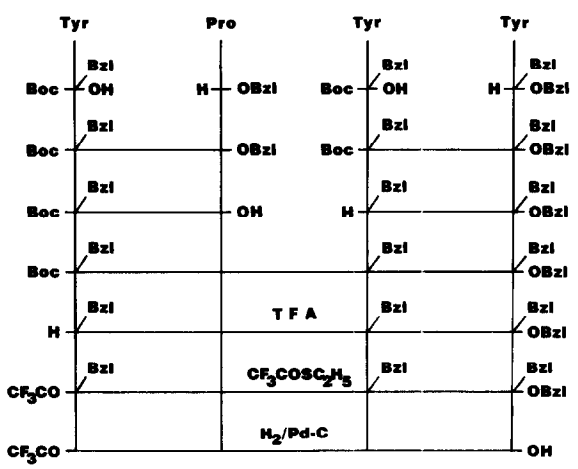

Fig. 1. Synthesis route for the peptide Tyr–Pro–Tyr–Tyr.

Purification of opioid-binding materials

The digitonin-solubilized opioid receptors were prepared from rat brain and the opioid-binding materials were purified by the batchwise method as described previously [6]. For radioreceptor binding assay, the partially purified materials were concentrated by ultrafiltration with an Amicon PM-30 membrane, and the concentrate was fractionated on a Sephadex G-75 column (25 × 1.6 cm I.D.), eluted with 10 m*M* N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid–KOH (TES–KOH) (pH 7.5) containing 0.1% of digitonin, 1 m*M* dithiothreitol and 10 m*M* $MgSO_4$.

Reconstitution of opioid-binding materials with G_i

G_i was purified from bovine brain by the reported method [7]. The purified opioid-binding materials and G_i were reconstituted essentially as reported previously [7] with minor modifications. Briefly, the brain extract (Folch Fraction I) and phosphatidylinositol were suspended in a buffer solution of 10 m*M* TES–KOH (pH 7.5) containing 100 m*M* NaCl, 0.1 m*M* EDTA and 0.3% of *n*-octyl-β-D-thioglucoside. The resulting suspension was sonicated in an atmosphere of nitrogen. This lipid mixture was added to the concentrated purified receptors and allowed to stand at room temperature for 10 min. The resulting mixture was gel filtered on Sephadex G-50 (coarse) and the void-volume fractions were collected. Next, G_i was added to the fractions and the mixture was allowed to stand on ice for 1 h followed by dilution with two volumes of 10 m*M* TES–KOH (pH 7.5) containing 100 m*M* NaCl and 0.1 m*M* EDTA. The suspension obtained was used as the reconstituted system.

Radioreceptor binding assay

The specific binding of ^{3}H-labelled ligands to the purified materials and to the reconstituted systems was assayed by the membrane filter method using nitrocellulose membranes (BA-85) as described previously [6,7].

Gel electrophoresis

The purified materials were identified by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) on 10% slab gel using the buffer system described by Laemmli [10]. After electrophoresis, the gel was stained with silver using Ag-Stain Daiichi.

Protein assay

Protein was determined by staining with Amido Black [11]. Bovine serum albumin was used as the standard.

RESULTS AND DISCUSSION

Purification of opioid-binding materials

In most attempts to purify opioid receptors by affinity chromatography, agonist-type ligands have been used for the affinity ligands of affinity resins [4,5]. However, the receptors coupled with GTP-binding proteins (G proteins) change their affinity states for agonists by association with or dissociation from the receptors [12]; dissociation of G proteins from the receptors lowers the affinity of the receptors for the agonists, but not for antagonists. Our previous study showed that active G proteins were lost during affinity chromatography [6]. Therefore YPYY, a weak antagonist for μ-opioid receptors [9], was used as an affinity ligand for purification of μ-opioid receptors.

The solubilized receptors were incubated with the affinity resin and then the opioid-binding materials were eluted from the resin with naloxone. After removing excess of free naloxone by gel filtration, the specific binding of [D-Ala^2, Me-Phe^4, Gly-ol^5]enkephalin (DAGO) to the purified opioid-binding materials was assayed. In the presence of 50 n*M* [^{3}H]naloxone, the purified materials showed a specific binding activity of *ca.* 180 pmol/mg protein, indicating a 300-fold increase over the corresponding value for the digitonin-solubilized receptors. Theoretical purification of more than 10 000-fold would have been needed to purify the μ-opioid receptors if they have a molecular mass of *ca.* 60 000 [4,5]. Several possibilities were considered as principal reasons for this discrepancy. First, the purified materials were a mixture of several proteins. SDS-PAGE of the purified sample showed, after silver staining, several protein bands corresponding to molecular masses in the following range: four major bands between 40 000 and 60 000 and more than five minor bands between 20 000 and 82 000 (Fig. 2).

The second possible explanation was that the environment of the receptors in the purified state differed from that in the cell membrane; the receptors are present in lipids in the cell membrane. The presence of acidic lipid is known to be essential for optimum opiate binding [13]. The solubilized materials reconstituted in liposome with G_i first showed a severalfold increase in the specific binding activity of the agonist compared with that of the original solubilized receptors in the detergent solution. Recently, Scheideler and Zukin [14] reported that the high specific agonist binding activity of the digitonin-solubilized receptors from NG108-15 cell membranes was recovered when the solubilized materials were reconstituted in liposome. Without reconstitution, they recovered less than 5% of the original binding activity. The solubilized and purified sample might have lost some lipid component, thus lowering the opioid binding of the sample. This would mean that some lipids play an important role in the opioid binding to the receptors.

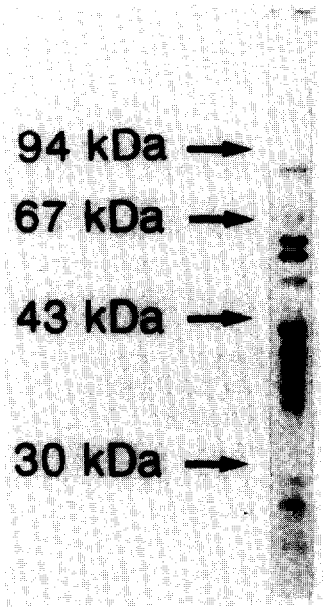

Fig. 2. SDS-PAGE of the purified materials. The purified materials were analysed by SDS-PAGE on 10% slab gel using the buffer system described by Laemmli [10]. After electrophoresis, the gel was stained with silver. The positions of molecular mass standards are shown by arrows (kDa = kilodalton).

Decoupling of the receptor from G_i causes a decrease in the specific binding activities to the agonists [15,16]. The reconstitution experiments supported this possibility. The DAGO-binding activities in the reconstituted systems with G_i increased several tenfold in comparison with those in the sys-

tems without G_i (see below). The same phenomenon of an increase in the agonist-binding activity has been observed in the reconstitution system of the μ-type of opioid receptors and G proteins [17].

Reconstitution of the purified materials in liposome

To confirm whether the purified materials contain the true receptors, the materials should be reconstituted with components which are believed to couple functionally with the opioid receptors in the original membranes.

In some opioid receptor systems, transformation of the signals is transduced via GTP-binding proteins. An inhibitory GTP-binding protein G_i links the activation of the receptors to the inhibition of adenylyl cyclase, thus lowering the concentration of cAMP [18–20]. A G protein of unknown function, G_o, and G_i may be involved in the functional coupling of the opioid receptors to the channels [21,22]. Therefore, we tried to reconstitute the purified materials with G proteins in liposome and clarified the functional coupling of the purified materials with them.

As the purified materials had little or no GTPase activity (data not shown), the materials contained little or no G_i, which has GTPase activity, and could not couple with G_i. We tried to reconstitute the purified materials with or without G_i in liposome and characterize the reconstituted system. In the absence of G_i, the system showed only a low affinity for the agonist DAGO; on the other hand, the addition of G_i to the system resulted in an increase in an apparently high-affinity state for the agonist (Fig. 3). Two states with different affinities were observed. The reconstituted systems with or without G_i did not show any high affinity state for the κ-agonist ethylketocyclazocine (Fig. 4), nor for the δ-agonist [D-Pen^2, D-Pen^5]enkephalin (data not shown). As reported elsewhere [15,16], opioid receptors are considered to be in a high-affinity state when they are coupled with G_i but in a low-affinity state when they are not.

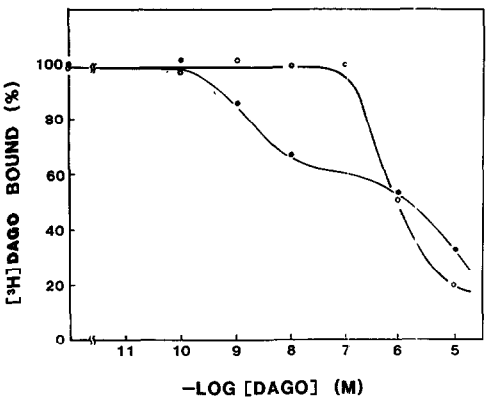

Fig. 3. Displacement of [^{3}H][D-Ala^2, Me-Phe^4, Gly-ol^5]enkephalin ([^{3}H]DAGO) binding by DAGO. The purified materials were reconstituted (●) with or (○) without G_i in liposome. The reconstituted systems were incubated with [^{3}H]DAGO in the absence or presence of various concentrations of DAGO at 30°C for 1 h. The fractions of protein-bound [^{3}H]DAGO were trapped with nitrocellulose membranes as described under Experimental. Each point is the average of triplicate determinations.

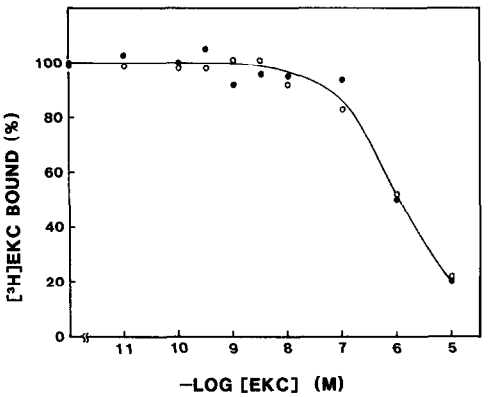

Fig. 4. Displacement of [^{3}H]ethylketocyclazocine ([^{3}H]EKC) binding by EKC. The purified materials were reconstituted (●) with or (○) without G_i in lipsome. The reconstituted systems were incubated with [^{3}H]EKC in the absence or presence of various concentrations of EKC. Experimental conditions as in Fig. 3.

DAGO showed a weak displacement in the reconstituted systems without G_i but a significant increase (35-fold) was observed in the system with G_i. This also supported the coupling of the purified receptors with G_i in the reconstituted system.

In conclusion, the opioid receptors partially purified with the affinity resin contained μ-type receptors and could functionally couple with G_i, as occurs in other transmembrane signalling pathways transduced with G_i [12].

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