Identification of Opioid-Binding Materials of Rat Brain[1)]

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Digitonin-solubilized opioid receptors from rat brain were purified with an affinity resin, AH-Sepharose coupled with [D-Ala^2, D-Leu^5]enkephalin (DADLE). Radioreceptor binding assay showed that the purified materials had specific opioid-binding activity of 310 pmol/mg protein on DADLE binding. Analyses by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) revealed that the materials were rich in two polypeptides; the major component had a molecular weight of 62000—64000. To establish the materials responsible for binding opiates, the purified materials were cross-linked with ^{125}I-labeled β-endorphin using bis[2-(succinimidooxycarbonyloxy)-ethyl]sulfone as a cross-linker. The molecular weight of 62000—64000, the major band of the purified materials on SDS-PAGE, agreed closely with that determined by the cross-linking experiment. The results suggested that the purified materials contained opioid-binding materials (opioid receptors).

Keywords opioid receptor; enkephalin derivative; affinity resin; affinity cross-linking; rat brain

In the process of signal transduction between receptors and catalytic units of adenylyl cyclase, a family of guanosine triphosphate (GTP)-binding proteins (G proteins[3)]) has been found to play an important role. A G protein links the activation of receptors to the regulation of adenylyl cyclase activity.[4)] In particular, the binding of a ligand to the opioid receptors causes the inhibition of adenylyl cyclase *via* signal transduction by an inhibitory G protein (G_i), thus lowering the concentration of cyclic adenosine monophosphate (cAMP).[5)]

Attempts to purify opioid receptors by affinity chromatography have been reported recently.[6)] We also have attempted to purify the opioid-binding materials using an affinity resin, AH-Sepharose with [D-Ala^2,Leu^5]enkephalin (DALE).[7)] Very recently, we prepared a new affinity resin, AH-Sepharose coupled with a δ-agonist, DADLE, for the purification of opioid-binding materials.[1)]

Affinity cross-linking is a useful method for the identification of hormone receptors; an exogenous cross-linker is added to a hormone-receptor complex to cause a covalent linkage.[8)] This technique was also successfully used to identify the opioid receptors in the membrane utilizing a radiolabeled ligand, [^{125}I]β-endorphin, and a cross-linker, BSCOES.[9)]

In this study, we purified the opioid-binding materials using the affinity resin, which has the δ-agonist DADLE as an affinity ligand, and attempted to cross-link the purified materials, which were reconstituted with G_i in liposome, with [^{125}I]β-endorphin using BSCOES as a cross-linker.

Materials and Methods

Materials The following compounds were purchased from the sources described: [^{3}H]DADLE (43.9 Ci/mmol), [^{3}H]naloxone (44.1 Ci/mmol), and Aquasol 2 (New England Nuclear Co.); (3-[^{125}I]iodotyrosyl^{27})-β-endorphin (human, 1825 Ci/mmol; Amersham); soybean trypsin inhibitor, bacitracin, bovine serum albumin, low-molecular-weight standard mixture for PAGE, brain extract, and phosphatidylinositol (Sigma Chemical Co.); AH-Sepharose (Pharmacia); BSCOES (Pierce Chemical Co.); X-ray film (Kodak); PM-30 ultrafiltration and Centricon-30 microconcentrators (Grace Co.). Enkephalin derivatives were synthesized as previously reported.[1,7b)]

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

Purification of Opioid-Binding Materials Neural membranes were prepared from rat brains without cerebella as described by Waterfield *et al.*[10)] and stored at −80 °C.

For solubilization and purification, the following buffer system was used: 10 mM Tes–KOH buffer (pH 7.5) containing 1 mM EGTA-K$^+$, 10 mM $MgSO_4$, 1 mM benzamidine–HCl, 0.01% bacitracin, 0.002% soybean trypsin inhibitor, 1 μM pepstatin, and 0.2 μM phenylmethanesulfonyl fluoride, referred to as "Mg buffer." The opioid receptors were solubilized from the membranes using digitonin as described previously.[7a)] Briefly, the stored membranes were thawed and resuspended at 0.5 mg protein per ml in ice-cold Mg buffer. The membranes were pelleted at 40000 × *g* (4 °C, 30 min), and resuspended in Mg buffer at 2—3 mg/ml. After incubation (30 °C, 1 h), the mixture was diluted 1 : 4 with Mg buffer and pelleted as described above. The obtained membranes were suspended in Mg buffer containing 2% digitonin at 2 : 1 detergent : protein ratio. This mixture was shaken gently (20 °C, 1 h) and the supernatant obtained from the centrifugation at 105000 × *g* (4 °C, 70 min) was collected.

The opioid-binding materials were purified by using AH-Sepharose coupled with DADLE as previously described.[1)] To minimize the nonspecific binding to the affinity resin, the solubilized fraction (7 ml) in Mg buffer (133 ml) containing 1 mM DTT was first pretreated with unmodified AH-Sepharose (10 ml; 0 °C, 15 min). After centrifugation, the supernatant was incubated with the affinity resin (10 ml; 30 °C, 1 h). The affinity resin bound opioid-binding materials were precipitated by centrifugation and washed with Mg buffer containing 1 mM DTT and 0.1% digitonin (50 ml × 2). The resin was then incubated in Mg buffer (100 ml) containing 0.1 mM DADLE, 1 mM DTT, and 0.1% digitonin (30 °C, 30 min). The mixture was centrifuged and the precipitated resin was washed with Mg buffer (50 ml × 2) containing 0.1 mM DADLE, 1 mM DTT, and 0.1% digitonin. The combined supernatants were concentrated by using an Amicon PM-30 to 0.5 ml. The concentrate was then fractionated on a Sephadex G-75 column (1.6 × 25 cm), eluted with 10 mM Tes–KOH (pH 7.5) containing 0.1% digitonin, 1 mM DTT, and 10 mM $MgSO_4$. The protein fraction was collected, concentrated with a Centricon-30, and stored in the presence of 30% glycerol at −80 °C until use.

Reconstitution of Opioid-Binding Materials with G_i G_i was purified from bovine brain by reported methods.[11)] The stored opioid-binding materials were thawed and gel-filtered on Sephadex G-50 to remove glycerol. The obtained opioid-binding materials and G_i were reconstituted by the method previously described.[12)] Briefly, the brain extract (Folch Fraction I) and phosphatidylinositol were suspended in a buffer solution, 10 mM Tes–KOH (pH 7.5) containing 10 mM $MgCl_2$, 0.3% *n*-octyl-β-D-thioglucoside. The resulting suspension was sonicated in a bath-type sonicator in an atmosphere of nitrogen. This lipid mixture was added to the concentrated purified receptors and allowed to stand at room temperature (15 min). The resulting mixture was gel filtered on Sephadex G-50 and void-volume fractions were collected. Next, G_i was added to the fractions and the mixture was allowed to stand on ice (1 h) followed by dilution with two volumes of 10 mM Tes–KOH (pH 7.5) containing 10 mM $MgCl_2$. The obtained suspension was used as a reconstituted system.

Cross-Linking Experiment The reconstituted materials were incubated with 1 nM [^{125}I]β-endorphin in 50 mM phosphate buffer (pH 7.5), containing 5% bacitracin, in the presence or absence of 10^{-5} M DADLE (25 °C, 1 h). After removal of free [^{125}I]β-endorphin by ultrafiltration (Centricon-

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30), covalent cross-linking was performed by the addition of BSCOES (final concentration, 1 mM) and incubation (0 °C, 15 min). After incubation, an excess of 1 M carbonate buffer was added to terminate the cross-linking. The mixture was then concentrated with a Centricon-30 and diluted with phosphate buffer; this washing procedure was repeated 3 times. Finally the obtained samples were analyzed by SDS-PAGE by the method of Laemmli.[13]

The gel was fixed, dried under vacuum and exposed to X-ray film (−70 °C, 20 d).

Radioreceptor Binding Assay The specific binding of [^{3}H]DADLE and [^{3}H]naloxone to the purified materials was assayed by the membrane filter method as previously described[7a] with a minor modification. The assay medium (0.44 ml) consisted of 10 mM Tes–KOH (pH 7.5) containing 10 mM $MgSO_4$, 0.5 mM DTT, 0.1% digitonin, radiolabeled ligand and the purified materials. Non-specific binding was measured in the presence of 10^{-5} M DADLE or naloxone. The mixtures were incubated (30 °C, 1 h) and then cooled on ice (10 min). They were filtered through a nitrocellulose membrane filter under vacuum and the filters were washed with ice-cold 10 mM Tes–KOH (pH 7.5) containing 10 mM $MgSO_4$ (3 ml × 3). The dried filters were counted in Aquasol-2 (10 ml) on a liquid scintillation counter.

Protein Assay Protein was determined by staining with Amido black.[14] Bovine serum albumin was used as the standard.

Results

Purification of Opioid-Binding Materials The solubilized receptors were incubated with the affinity resin and then the opioid-binding materials were eluted from the resin with DADLE. The fractions from gel filtration were subjected to radioreceptor binding assay (Fig. 1); the first protein fraction had the opioid-binding activity and the second was DADLE as identified by HPLC (data not shown).

The specific binding of [^{3}H]DADLE to the purified opioid-binding materials was assayed. Fraction Nos. 3—5 in Fig. 1 were combined and subjected to radioreceptor binding assay. Scatchard analysis gave a K_d of 30 nM and a B_{max} of 310 pmol/mg protein for the purified opioid-binding materials. The K_d value was almost identical with and the B_{max} was a little higher than the previously obtained values for the opioid-binding materials purified by using AH-Sepharose with DALE.[7a] The addition of glycerol during storage of the purified materials (at −80 °C) decreased the loss of the opioid-binding activity of the materials, which account for the increased B_{max} value compared with the previously obtained values.

Cross-Linking Experiment Because the purified materials showed two bands on SDS-PAGE,[1] we tried to cross-link the materials with iodinated β-endorphin using BSCOES to establish which band was responsible for binding opiates. Furthermore, some opioid receptors coupled with G_i in the membrane state, and the coupled receptors showed a high-affinity binding with agonists.[15] The purified materials showed little or no GTPase activity originating from G_i, so that the reconstituted materials with G_i were used in the cross-linking experiment to retain the binding activity of the receptors at a high-affinity state. The cross-linked materials were analyzed by SDS-PAGE and autoradiography; the material appeared at a position corresponding to a molecular weight of 67000—68000 (Fig. 2). This value agreed well with that obtained for the original purified materials on SDS-PAGE, if the combined molecular weight of β-endorphin and BSCOES is taken into account (*ca.* 4000). This band was diminished when the cross-linking experiment was undertaken in the presence of 10^{-5} M DADLE (data not shown).

Discussion

The materials purified by using an affinity resin with DADLE showed apparent molecular weights of 62000—64000 (the major band) and 39000—41000 (a minor band).[1] The major band molecular weight agreed well with that of the previously purified material.[7a] Also, it was close to the molecular weight of the material which was cross-linked with β-endorphin using BSCOES. The molecular weight of the minor band may correspond to that of the α-subunit of the G protein (41000 for G_i), but no direct evidence for this was obtained, since the purified materials showed little or no GTPase activity originating from the active α-subunit of G_i.

The purified materials had opioid-binding activity. Their specific binding activity was 310 pmol/mg protein, which is a little higher than that (200 pmol/mg protein) of the purified materials obtained previously.[7a] However, the specific binding activity was much lower than the activity of 16000 pmol/mg protein expected for a pure protein of

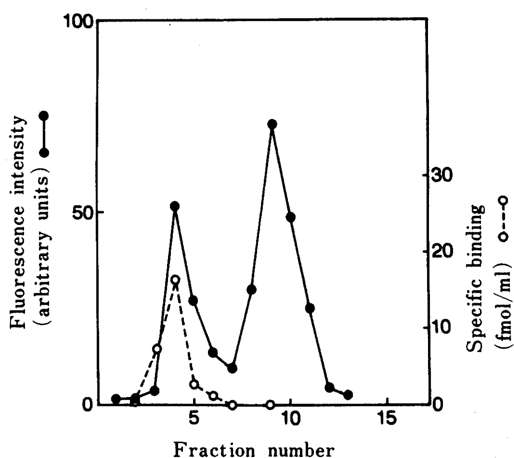

Fig. 1. Sephadex G-75 Gel Filtration

The materials eluted from the affinity resin by DADLE were concentrated to 0.5 ml with an Amicon PM-30 and applied to a Sephadex G-75 column (1 × 14 cm) equilibrated with 10 mM Tes–KOH buffer (pH 7.5) containing 0.1% digitonin and 10 mM $MgSO_4$ at 4 °C. One-milliliter aliquots were collected at a flow rate of 0.35 ml/min. Two peaks were obtained: the first was a protein fraction and the second was DADLE, which was identified by HPLC (data not shown). The specific activities were measured at 4 nM [^{3}H]DADLE. Non-specific binding was measured in the presence of 10^{-5} M DADLE.

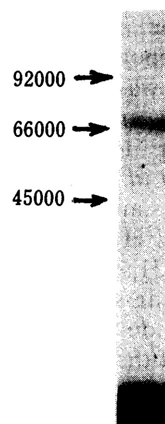

Fig. 2. Autoradiography of the Cross-Linked Materials

Affinity-purified materials with opioid-binding activity were reconstituted with G_i in liposome and cross-linked with [^{125}I]β-endorphin by BSCOES. The labeled materials were analyzed by SDS-PAGE 10% slab gel using the buffer system described by Laemmli.[13] The molecular weight standards are shown as 10^3 daltons.

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molecular weight 62000. Two possibilities were considered as principal reasons for this discrepancy. First, decoupling of the receptor-G_i interaction causes a decrease of the specific binding activity to agonists. As reported,[15] 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. 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. Consequently, we have to determine the binding activity of the antagonists for the purified materials. However, it was very difficult to obtain the specific antagonist (naloxone) binding activity because the nonspecific binding was too high, especially at high concentrations of the antagonist used; preliminarily, a K_d of 10 nM and a B_{max} of 1500 pmol/mg protein for [^{3}H]naloxone binding were obtained for the purified opioid-binding materials. Though these values were true, the level of [^{3}H]naloxone binding does not reach the expected activity of a purified protein of molecular weight 62000 (16000 pmol/mg protein). Therefore, we must consider another possibility to explain the discrepancy between the expected and the observed specific binding activities for the purified materials. The second possible explanation is that the environment of the receptors in the purified state differed from that in the cell membrane; the receptor in the cell membrane is embedded in lipid. The presence of acidic lipid is known to be essential for optimal opiate binding.[6c] The purified sample might have lost the lipid component, lowering the opiate binding of the samples. In support of this, when the purified materials in this study and G_i were reconstituted in liposomes, the specific binding activity to the agonist DADLE increased several tenfold.[12] The same phenomenon of an increase in the agonist-binding activity has been observed in the reconstitution system of μ-type opioid receptors and G proteins.[16]

Recently, determinations of the molecular weights of opioid receptors (and their subunit) have been reported through several different experimental approaches,[6d-g,9,17] suggesting that the receptors range in molecular weight from 110000 to 26000. For example, Simon's group reported that a polypeptide with a molecular weight of 65000 is a μ-type of opioid receptor and one with a molecular weight of 53000 is a δ-type, based on results from affinity chromatography and/or an affinity cross-linking technique.[6d,9] Cho *et al.* reported a μ-type with a molecular weight of 58000 which was purified by a combination of affinity chromatography and other techniques.[6e] On the other hand, according to reports by Newman and Barnard[17a] and Klee *et al.*,[17b] the ligand-binding sites of the μ- and δ-types have similar molecular weights of 58000 based on findings using [^{3}H][D-Ala^2, Leu^5]enkephalin chloromethyl ketone and [^{3}H]fentanyl isothiocyanate, respectively. In addition, Yeung showed that a 46000 protein is a binding protein common to all opioid receptor types.[17c] The molecular weight of the purified materials in this study is close to the value for the μ-site proposed by Simon *et al.*, but we could not determine from the molecular weight on SDS-PAGE whether our purified materials were of the μ- or δ-type.

We tried to reconstitute the purified materials with G_i in liposome and to characterize the reconstituted system. This system showed high-affinity binding of the agonist DADLE, but not when G_i was omitted from the reconstituted system.[12]

These data suggest that the purified materials contain opioid-binding materials (opioid receptors), which can interact with G_i as found in the cell membrane.[2]

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3) Abbreviations used follow the IUPAC-IUB tentative rules (*J. Biol. Chem.*, **247**, 977 (1972)). Additional abbreviations used are as follows: DALE, [D-Ala^2,Leu^5]enkephalin; DADLE, [D-Ala^2,D-Leu^5]enkephalin; Tes, *N*-tris[hydroxymethyl]methyl-2-aminomethanesulfonic acid; DTT, dithiothreitol; G proteins, guanine nucleotide-binding regulatory proteins; G_1, a G protein that inhibits adenylate cyclase; BSCOES, bis[2-(succinimidooxycarbonyloxy)-ethyl]sulfone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.
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