Affinity Resins for the Purification of Opioid-Binding Materials[1,2]

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Two new affinity resins for the purification of opioid-binding materials were prepared. One was AH-Sepharose coupled with [D-Ala^2, D-Leu^5]enkephalin and the other was AF-Amino Toyopearl with [D-Ala^2, Leu^5]enkephalin. Solubilized-opioid receptors from rat brain were treated with these affinity resins and the materials with opioid-binding activities were purified. On sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the purified materials showed one major band with a molecular weight of 62000—64000. The results suggested that the prepared resins are useful tools for the purification of opioid receptors.

Keywords opioid receptor; enkephalin derivative; affinity resin; radioreceptor binding assay

Recently, attempts to purify opioid receptors by affinity chromatography have been reported.[3] We also have shown that an affinity resin, AH-Sepharose with [D-Ala^2, Leu^5]-enkephalin (DALE), is a useful tool for purification of opioid-binding materials.[4] However, DALE is not completely specific for the opioid receptors,[5] so that a more specific ligand for the receptors should be used to discriminate between multiple receptor types.[6] We also need a new tool for the more efficient purification of the opioid-binding materials on a large scale, because the yield of the materials obtained with the previously prepared resin was too low to allow characterization of the materials in detail.

In this study, we prepared two new affinity resins for the purification of the opioid binding materials. One had a δ-agonist [D-Ala^2, D-Leu^5]enkephalin (DADLE) coupled to AH-Sepharose and the other had DALE coupled to AF-Amino Toyopearl. The ligand contents in the latter resin were sufficient to allow purification of the receptors in large quantities.

Experimental

Materials The following compounds were purchased from the sources described: [^{3}H]DADLE (43.9 Ci/mmol), Aquasol 2 (New England Nuclear, Co.); *S*-ethyl trifluoroacetate (Aldrich Chemical Company, Inc.); AH-Sepharose (Pharmacia). AF-Amino Toyopearl was a generous gift from Tosoh Co. All other chemicals were obtained from commercial sources and were of the highest purity obtainable.

Synthesis of the Peptides The enkephalin derivatives were synthesized by conventional solution methods employing fragment condensation as described in the previous study[1,4b] and identified by elemental analyses and/or fast atom bombardment mass spectrum (FAB-MS). Homogeneity of the products was checked on precoated thin layer chromatography (TLC) plates (Silica gel 60 F_{254}, Merck), using the following solvent systems: A, CH_2Cl_2–MeOH, 10:1; B, *n*-BuOH–AcOH–H_2O, 4:1:2. All the compounds described below showed homogeneous single spots on TLC (detection with ultraviolet (UV) and/or I_2).

Boc–Tyr(Bzl)–D-Ala–Gly–Phe–D-Leu–OBzl (1) Boc–Tyr(Bzl)–D-Ala–Gly and Phe–D-Leu–OBzl were coupled by the mixed carbonic anhydride method.[4b] Recrystallization of the product from AcOEt/petroleum ether gave compound **1**; mp 128—130 °C. Yield, 86%. $[\alpha]_D$ −4.5° (c=1.00, DMF). *Rf* 0.67 (A). *Anal.* Calcd for $C_{48}H_{59}N_5O_9$: C, 67.82; H, 7.00; N, 8.24. Found: C, 67.51; H, 7.07; N, 8.12. FAB-MS *m/z*: 850 ([M+H]$^+$).

CF_3CO–Tyr(Bzl)–D-Ala–Gly–Phe–D-Leu–OBzl (2) The Boc group of **1** was removed by treatment with TFA in the presence of anisole. Tyr(Bzl)–D-Ala–Gly–Phe–D-Leu–OBzl·TFA was obtained as an amorphous compound and trifluoroacetylated using *S*-ethyl trifluorothioacetate as reported.[4b] Compound **2** was recrystalized from AcOEt; mp 209.5—210 °C. Yield, 81%. $[\alpha]_D$ +3.40° (c=1.00, DMF). *Rf* 0.62 (A). *Anal.* Calcd for $C_{45}H_{50}F_3N_5O_8$: C, 63.89; H, 5.96; N, 8.28. Found: C, 63.81; H, 6.13; N, 8.32. FAB-MS *m/z*: 846 ([M+H]$^+$).

CF_3CO–Tyr–D-Ala–Gly–Phe–D-Leu (3) Hydrogenation of **2** gave quantitatively compound **3**, which was characterized as a dicyclohexylamine (DCHA) salt [mp 203—208 °C (dec.). *Anal.* Calcd for $C_{43}H_{61}F_3N_6O_8$: C, 60.98; H, 7.26; N, 9.92. Found: C, 61.00; H, 7.53; N, 9.95. FAB-MS *m/z*: 847 ([M+H]$^+$); 666 ([M+H−DCHA]$^+$)].

Preparation of Affinity Resins An affinity resin, AH-Sepharose coupled with DADLE (resin-1-DADLE) was prepared in the same way as previously reported for the preparation of AH-Sepharose coupled with DALE,[4b] except that DADLE was used instead of DALE. Amino acid analysis of the hydrolyzate of the resin with 6 N HCl (110 °C, 24 h) showed that *ca.* 0.4 μmol of DADLE was present in 1 ml of the resin. Another affinity resin, AF-Amino Toyopearl with DALE (resin-2-DALE), was prepared essentially according to the reported method[4b]; AF-Amino Toyopearl was used as an insoluble support instead of AH-Sepharose. Briefly, compound **3** (0.25 mmol) in DMF (5.8 ml) was added to the swollen AF-Amino Toyopearl (2.5 ml) in deionized water (7.2 ml). To this mixture, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide·HCl (5.75 mmol) was added and the pH was adjusted to 5.0. The mixture was gently shaken (room temperature, 24 h) and the gel was post-treated. Next, the gel was shaken in 0.1 M NaOH (room temperature, 24 h) to remove the CF_3CO-group from the peptide coupled to the gel and post-treated. Amino acid analysis of the hydrolyzate of the resin with 6 N HCl (110 °C, 24 h) showed that *ca.* 10—15 μmol of DALE is present in 1 ml of the resin.

Purification of Opioid-Binding Materials The digitonin-solubilized opioid receptors were prepared from rat brain and the opioid-binding materials were purified as previously described[4a] with minor modifications. 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% STI, 1 μM pepstatin, and 0.2 μM phenylmethanesulfonyl fluoride, referred to as "Mg buffer". To decrease the non-specific binding to the affinity resins, the solubilized fraction in Mg buffer containing 1 mM DTT was first incubated with AH-Sepharose or AF-Amino Toyopearl (0 °C, 15 min). The mixture was centrifuged and the supernatant was incubated with the affinity resin (30 °C, 1 h). The affinity resin bound opioid-binding materials was precipitated by centrifugation and washed with Mg buffer containing 1 mM DTT and 0.1% digitonin. The resin was then incubated in Mg buffer 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 containing 0.1 mM DADLE, 1 mM DTT, and 0.1% digitonin. The combined supernatants were concentrated by the use of an Amicon PM-30 membrane to 0.5 ml. The concentrate was then fractionated on a Sephadex G-75 column (1 × 14 cm) 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 and subjected to radioreceptor binding assay or concentrated with a Centricon-30 for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or protein determination.

Measurement of Specific Opioid-Binding Activities of the Purified Materials The specific binding of [^{3}H]DADLE (2—100 nM) to the purified materials was assayed by the membrane filter method.[4a] Non-specific binding was measured in the presence of 10^{-5} M DADLE. Protein was determined by staining with Amidoblack.[7]

Results

After solubilization of the active opioid receptors from rat brain with digitonin, the opioid binding materials were

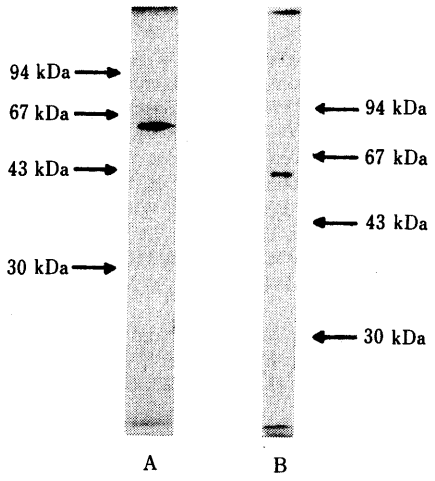

Fig. 1. SDS-PAGE of the Materials Purified with Affinity Resins

The purified materials were identified by electrophoresis on 10% slab gel using the buffer system described by Laemmli[11] and stained with silver. Lanes: A, the materials eluted from AF-Amino Toyopearl coupled with DALE; B, the materials eluted from AH-Sepharose coupled with DADLE. The molecular weight standards are shown as 10^3 daltons (kDa).

purified using the affinity resins. The solubilized receptors, pretreated with AH-Sepharose or AF-Amino Toyopearl to decrease the non-specific binding to the affinity resins, were incubated with the affinity resins for 1 h. Then the opioid-binding materials were eluted from the resins with DADLE. From the protein determination, the crude digitonin extract containing 15 mg of protein afforded 3 or 10 μg of the partially purified protein using AH-Sepharose with DADLE (resin-1-DADLE) or AF-Amino Toyopearl with DALE (resin-2-DALE), respectively.

The result of SDS-PAGE of the purified materials is shown in Fig. 1. The materials purified with resin-1-DADLE were analyzed in lane A. One major band with a molecular weight of 62000—64000 and a minor one with a molecular weight of 39000—41000 were observed. The pattern in lane B is of the materials purified with resin-2-DALE. One major band with a molecular weight 62000—64000 and several minor bands were observed.

These purified materials showed little or no GTPase activities (data not shown) originating from GTP binding proteins, especially an inhibitory GTP-binding protein (G_i), in opioid receptor systems.[8]

Discussion

In the present study, we purified opioid-binding materials from rat brain using two new affinity resins with enkephalin derivatives as ligands for opioid receptors. One resin (resin-1-DADLE) had a δ-agonist DADLE as an affinity ligand for opioid receptors. The other (resin-2-DALE) was composed of AF-Amino Toyopearl coupled with DALE. The content of DADLE in resin-1-DADLE (0.4 μmol/ml resin) was lower than that of DALE (1 μmol/ml resin),[4b] because of the solubility of the DADLE derivative in the solvent used in the coupling step to AH-Sepharose. The content of DALE in resin-2-DALE (10—15 μmol/ml resin) was much higher than in resins derived from AH-Sepharose.

From protein determination, the yield of the opioid-binding materials using resin-2-DALE was about 3 times higher than that using resin-1-DADLE or 2 times higher than that using AH-Sepharose with DALE (data not shown), due mainly to the ligand content in the resin. The purified materials showed specific opioid-binding of [^{3}H]DADLE. At saturation of [^{3}H]DADLE, specific binding activity of *ca.* 250 pmol/mg protein[9] or of *ca.* 150 pmol/mg protein[10] was observed for the materials purified with resin-1-DADLE or with resin-2-DALE, respectively. The difference in the specific activities may mainly reflect to the purity of the obtained materials, because several minor bands were observed in SDS-PAGE of the materials purified with resin-2-DALE, while only one minor band was seen in that of the materials purified with resin-1-DADLE. However, 5—10 time more crude solubilized receptors could be applied to resin-2-DALE than to resin-1-DADLE. Therefore, resin-2-DALE with a high content of the affinity ligand is suitable for large-scale purification of the receptors.

Analysis of the purified fractions by SDS-PAGE showed one major band with a molecular weight of 62000—64000. This value was identical with that of the previously purified materials.[4a] Moreover, the value agreed closely with that determined by crosslinking the purified materials by use of resin-1-DADLE with [^{125}I]β-endorphin.[9] Because various molecular weights of opioid receptors purified by affinity chromatography or identified by affinity labeling have been reported from several laboratories,[2] we cannot determine from the molecular weight on SDS-PAGE whether our purified materials are of the μ or δ type. The materials need to be characterized in more detail.

One promising approach for the characterization of the receptor systems is the reconstitution study of the purified receptors with other components which are known to be functionally coupled with the receptors in the original cell membrane. In some opioid receptors, an inhibitory GTP-binding protein G_i is functionally coupled to the receptors.[8] Therefore, we tried to reconstitute the purified materials with G_i.[10] The reconstituted system of the materials purified with resin-2-DALE and G_i showed a high binding activity and a low binding activity to the δ-agonist DADLE, but only a low activity was observed when G_i was omitted from the reconstituted system.

These results suggested that affinity resins (especially resin-2-DALE) are useful tools for the purification of the opioid receptors, and it appeared that the purified materials contain δ-type opioid receptors.

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References and Notes

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2) 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; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; OBzl, benzyl ester; DMF, dimethylformamide; TFA, trifluoroacetic acid; Tes, *N*-tris[hydroxymethyl]methyl-2-aminomethanesulfonic acid; DTT, di-

thiothreitol; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; STI, soybean trypsin inhibitory; G proteins, guanine nucleotide-binding regulatory proteins; G_i, a G protein that inhibits adenylate cyclase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GTPase; guanosine triphosphatase.

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