Affinity purification of the opioid receptor in NG 108-15 cells using an avidin-biotin system with a novel elution method

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Affinity purification of the opioid receptor in NG 108-15 cells was carried out using an affinity resin based on the avidin-biotin interactions, but a new elution method was employed with a radioligand of sub-micromolar concentration. A synthesized biotinyl derivative of leucine-enkephalin has a high affinity for the δ-receptor, but the affinity was lowered 10-fold in the presence of avidin. The new elution method is based on this affinity decrease and resulted in a 100-fold purification over the initial crude materials in the single step. SDS-polyacrylamide gel electrophoresis of the purified receptor revealed three polypeptides of 58, 65 and 71 kDa as possible components of the δ-receptor.

Opioid receptor *Affinity chromatography* *Enkephalin analog* *Avidin* *Biotin*

1. INTRODUCTION

The existence of several different types of opioid receptor – μ, δ and κ, at least – has been established. However, the molecular entity of the receptors is still an open question, although several attempts have made some progress in the studies of μ- as well as δ-receptors by solubilization/purification [1–4] and by affinity labeling [5–9]. Affinity chromatography is a technique of considerable importance in the isolation of the receptors concerned, since active receptor molecules are necessary to assess the functional reconstitution of the isolated receptors. The receptor-ligand interaction is strong in general, with nanomolar range of affinity: that is the case in the opioid receptor system. Therefore, a large excess ligand (more than micromolar concentration) was used to elute the opioid or opiate receptor from the affinity column [4,10], and the activity of the eluted receptor was measured only after extensive removal of the elution ligand by gel filtration or dialysis. We developed a new technique of affinity chromatography using an avidin-biotin system, in which a mild elution with radioligand of reasonable concentrations (sub-micromolar) makes it possible to perform the immediate binding assay after the elution. We report here an application of this method for a single-step purification of the opioid receptor in NG 108-15 cells.

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Abbreviations: biocytinamide, N^{ε}-biotinyllysine amide; Boc, *t*-butyloxycarbonyl; EDC HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HOBt, 1-hydroxy-benztriazole; TFA, trifluoroacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfate; DADLE, [D-Ala^2,D-Leu^5]-enkephalin

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2. MATERIALS AND METHODS

2.1. *Synthesis of [(leucine-enkephalinyl-hydrazido)succinyl]biocytinamide* **1**

The biotinyl analog of leucine-enkephalin **1** was synthesized according to the following procedures. Biocytinamide [11] was treated with succinic anhydride. The succinylated material was coupled with N^{α}-Boc-leucine-enkephalin hydrazide by EDC HCl and HOBt, followed by removal of the Boc group with TFA. Composition of the product was verified by amino acid analysis (Tyr/Gly/Phe/Leu/Lys = 1.00:2.00:1.05:1.00:1.04) and a single elution peak was observed by HPLC analysis.

2.2. *Solubilization of NG 108-15 cells and receptor binding assay*

NG 108-15 cells were grown and harvested as described [12,13]. The cell suspension in 0.3 M sucrose-25 mM Hepes-KOH (pH 7.5) was homogenized with a Polytron PT-10 [14] and solubilized in 10 mM CHAPS as described [1] with a slight modification. The solubilized receptor (1 ml) in 50 mM Hepes-KOH plus 10 mM $MgSO_4$ buffer (pH 7.5) [buffer A] containing 2 mM CHAPS and bacitracin (0.1 mg/ml) was incubated for 2 h at 37°C with 8 nM [^{3}H]DADLE and various concentrations of ligand **1**. When avidin was to be added, its molar ratio to **1** was maintained at 1:4 since one avidin molecule is able to bind four biotins. The incubation was terminated by a rapid filtration of the sample aliquots (in duplicate) through a nitrocellulose filter (25 mm diameter) under vacuum [15]. The filter was washed three times with 3 ml cold buffer A. When column fractions were assayed, the [^{3}H]DADLE concentration was 100 nM: it was added to the passed-through fractions while the fractions eluted with 100 nM [^{3}H]DADLE were assayed directly. Nonspecific binding of radioligand was measured in the presence of 100 μM [D-Ala^2]leucine-enkephalin.

3. RESULTS

3.1. *Binding characteristics of the biotinylated ligand*

The synthesized biotinyl analog of leucine-enkephalin **1** was tested in a receptor displacement assay against [^{3}H]DADLE, as shown in fig.1. Compound **1** had an IC_{50} value of 50 nM in the absence of avidin. Its K_d value was calculated to be 12 nM from the following equation:

$$IC_{50} = K_d(1 + [L]/K_L) \quad (1)$$

where IC_{50} = 50 nM, [L] represents the concentration of [^{3}H]DADLE used (8 nM) and K_L the dissociation constant of [^{3}H]DADLE (2.6 nM, obtained from a separate experiment, which agreed well with the reported value [16]). The K_d value obtained was in close agreement with that of leucine-enkephalin (5 nM [17]). In the presence of avidin, IC_{50} = 650 nM or K_d = 159 nM was obtained. These data show that the affinity of **1** was decreased 13-fold in the presence of avidin, as demonstrated by Koman and Terenius [18] in analogous compounds.

3.2. *Purification of opioid receptors from NG 108-15 cells*

The receptor-biotinyl ligand complex formed by incubating the solubilized receptor and **1** was separated by affinity chromatography with an avidin-conjugated agarose. A large excess of **1** (3 μM) over the receptor sites (2.2 nM) was used to facilitate rapid and complete complex formation. The incubation mixture was poured into an avidin-agarose column and circulated through the column. After crude receptor preparation passed through, the DADLE binding activity (31%) retained by the column was eluted in the presence of 100 nM [^{3}H]DADLE (fig.2). The overall recovery of activity was 2.3%, but the specific activity of fractions eluted with [^{3}H]DADLE was 27.2 pmol per mg of protein (table 1); a 100-fold purification over the initial solubilized preparation was achieved.

3.3. *SDS gel electrophoresis*

Samples at different purification steps were analyzed by SDS gel electrophoresis (fig.3). The gel pattern of the materials eluted with 100 nM [^{3}H]DADLE shows three bands, with molecular masses of 58, 65 and 71 kDa, which are observed in the solubilized preparation (starting material) but hardly in the passed-through fraction.

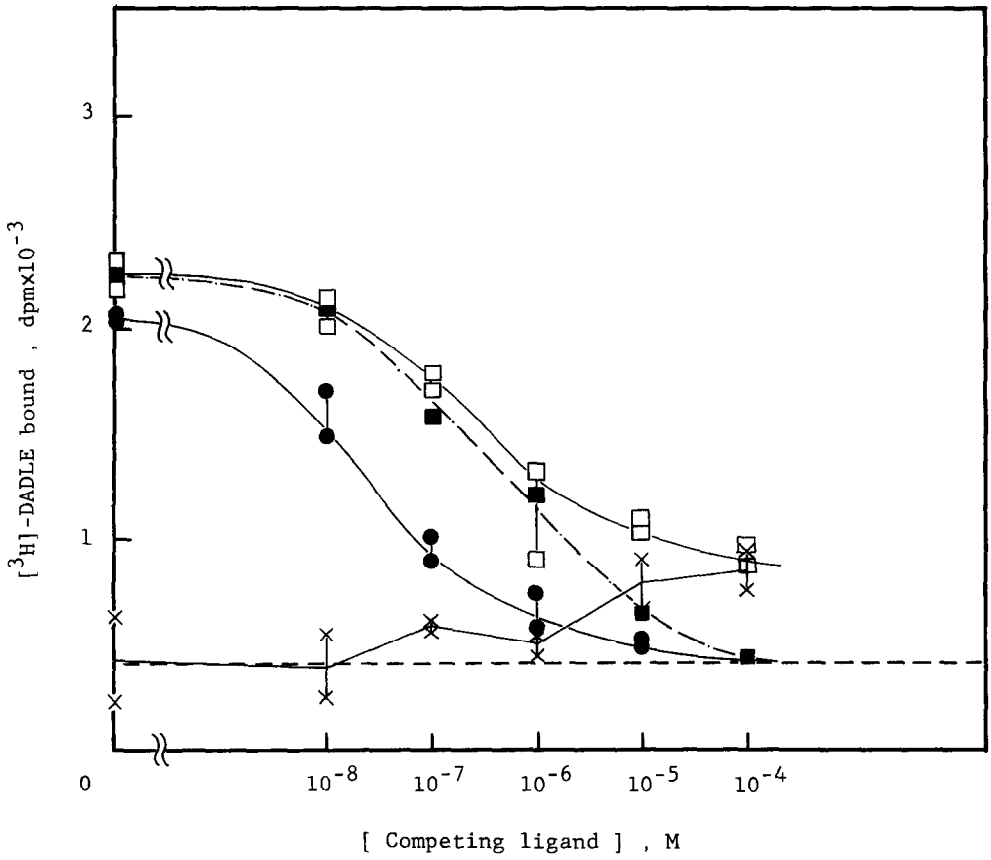


Fig.1. Displacement curves of the synthesized biotinyl analog of leucine-enkephalin **1** (●) and **1** + avidin 4:1 (□), using 8 nM [^{3}H]DADLE as radioligand. Nonspecific binding of [^{3}H]DADLE in the absence (– – –) and presence (×) of avidin is indicated. The nonspecific binding in the latter case showed dose-dependency of avidin and the corrected displacement curve for **1** + avidin (■) is also indicated after normalizing the nonspecific binding components (×—×) to the broken line level.

Table 1

Purification of DADLE-binding protein

	[^{3}H]DADLE bound[a] (pmol)	Protein (mg)	Specific activity[b]	% initial binding activity
Solubilized preparation	22.3	89.5	0.25	100
100 nM [^{3}H]-DADLE eluate	0.503	0.0185	27.2	2.3

[a] Binding activities were measured at 100 nM [^{3}H]DADLE
[b] Expressed as pmol of [^{3}H]DADLE bound per mg of protein

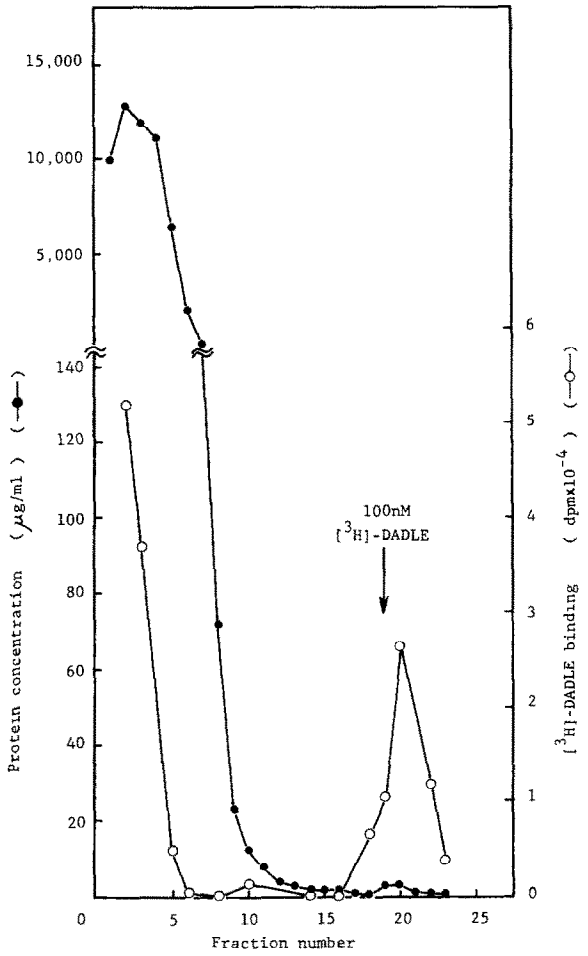


Fig.2. Elution profile from an affinity column. The solubilized crude receptor was incubated with 3 μM **1** for 3.5 h at 4°C, and the mixture was applied to an avidin-agarose column (2 ml) and circulated through the column for 2 h at 4°C. The column was washed with buffer A containing 2 mM CHAPS and three proteinase inhibitors (1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μM pepstatin A), and then eluted with the buffer containing 100 nM [^{3}H]DADLE. Buffer was changed where indicated and 2 ml fractions were collected.

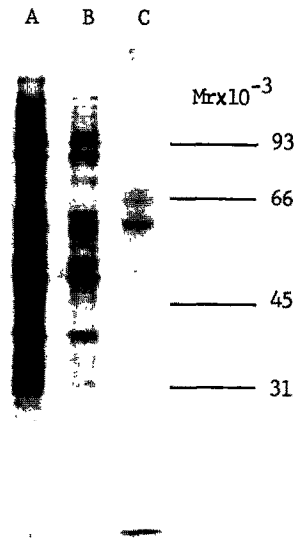
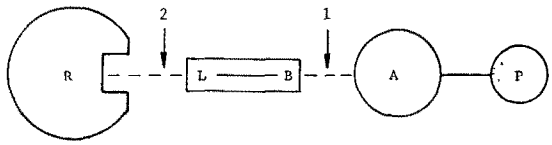


Fig.3. SDS-polyacrylamide gel (10%) electrophoresis of the column fractions in fig.2 under reducing conditions (50 mM dithiothreitol). Lanes: A, solubilized preparation that poured into the column (2.7 μg); B, fraction 6 (1.2 μg); C, fraction 19 and 20 eluted with 100 nM [^{3}H]DADLE (0.35 μg). The gel was silver-stained.



R.receptor , L-B biotinyl ligand , A-P avidin-agarose

Fig.4. A schematic illustration of the receptor-ligand interaction on the avidin-agarose.

4. DISCUSSION

The biotinylated enkephalin ligand **1** retains a sufficient affinity for the receptor (K_d = 12 nM), compared with that of leucine-enkephalin (K_d = 5 nM), the parent compound, although a substantial size of succinyl biocytinamide moiety was introduced. In the presence of avidin the affinity of **1** decreased 13-fold, probably due to a steric hindrance evoked on the two macromolecules, receptor and avidin, in which a rather small molecule **1** intervenes. This character of affinity decrease was utilized for the novel elution method presented here.

Fig.4 illustrates schematically the idea of this method. Applications of the avidin-biotin system to the affinity purification of hormone receptors have been pioneered by Hofmann and his colleagues [19,20]. Major problems seem to exist at the elution step where one tries to cleave the interaction at site 1 in fig.4, which is very strong in general ($K_d \simeq 10^{-15}$ M). The present method de-

rived from Hofmann's initial works is directed to cleave at site 2, where K_d is in the range of 10^{-9} M in most of the receptor systems and is expected to increase (affinity decrease), in the presence of avidin, as demonstrated above.

At elution with [^{3}H]DADLE, we assumed that a single ligand displacement from **1** to [^{3}H]DADLE occurs on the receptor molecule which was retained on the avidin-agarose. The concentration of [^{3}H]DADLE required to elute half of the receptor was calculated to be 52 nM using eqn 1, where K_d for [^{3}H]DADLE is 2.6 nM, [L] represents the concentration of **1** used (3 nM) and K_L the dissociation constant of **1** in the presence of avidin (159 nM). Using 100 nM [^{3}H]DADLE in this case, the opioid receptor was eluted and a 100-fold purification over the initial preparation was achieved.

The eluted materials contain three polypeptides of 58, 65 and 71 kDa as shown by SDS-PAGE. NG 108-15 cells are known to carry only a single type of opioid receptor, with δ-subtype characteristics [14], and the three polypeptides demonstrated here must be candidates for the δ-receptor components. The 58 kDa polypeptide is one of the most probable δ-receptor component(s) in NG 108-15 cells, as previously demonstrated by affinity labeling [6] and by photoaffinity labeling [9].

While affinity decrease of the biotinyl ligand for the receptor, which was brought about in the presence of avidin, is an advantage for eluting the receptor with ligands of low concentration, it is a defect in terms of receptor recovery. As shown in fig.2, a significant amount of receptor activity was not retained on the column. However, the elution with radioligand of reasonable concentrations followed by an immediate binding assay can compensate for the drawback, particularly in labile receptor systems. It could be expected that, in most receptor systems, the affinity of the receptor-biotinyl ligand complex will be decreased by avidin-agarose. This method may therefore be widely applicable to the purification of receptors.

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