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AFFINITY CHROMATOGRAPHY OF SOLUBILIZED OPIOID BINDING SITES USING CH-SEPHAROSE MODIFIED WITH A NEW NALTREXONE DERIVATIVE

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An affinity gel containing a newly synthesized derivative of naltrexone, β-naltrexyl-6-ethylenediamine (NED), was used to purify opioid binding sites by 300-450-fold. Binding sites solubilized from brains of toad, rat and cow using digitonin in the presence of 0.5 M NaCl are retained by the gel and can be eluted with naloxone (1-3 μM) in yields of 20-25%. The biospecificity of the interaction of opioid binding sites with modified beads is supported by the following: 1) unmodified beads do not retain the binding sites, 2) binding sites prebound with an opioid are not retained, 3) dilution of NED gels with unmodified Sepharose progressively reduced efficiency of retention and 4) specific receptor ligands elute binding sites retained on the NED gels.

We have reported successful extraction of active opioid binding sites from the brain membranes of toads and bullfrogs using the detergent digitonin (1) and from mammals by digitonin in the presence of high NaCl concentration (2). The soluble binding sites, which retain the biochemical characteristics of the membrane bound binding sites, were shown to be glycoproteins and were purified to some extent by their interaction with wheat germ agglutinin (WGA-agarose)(3).

To examine the physical and chemical properties and functions of a receptor on the molecular level, it is necessary to purify the protein to as near homogeneity as possible. Since the population of opioid receptor proteins, i.e., opioid binding sites, relative to the proportion of non-specific proteins is miniscule, it is most advantageous to utilize a method of purification, such as affinity chromatography, which exploits the specificity of ligand-receptor interactions. We report here the partial purification of solubilized opioid binding sites on an affinity matrix containing β-naltrexyl-6-ethylenediamine (NED), a new derivative we have synthesized by reductive amination of the antagonist naltrexone. The gel biospecifically retains digitonin-solubilized material from toad, rat and cow brain. Active binding sites

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can subsequently be eluted using high concentrations of naloxone and are substantially purified by this single step.

MATERIALS AND METHODS

[^{3}H]diprenorphine (27 Ci/mmol), unlabeled diprenorphine and naltrexone were generously supplied by Dr. Richard Hawks, National Institute on Drug Abuse. The following chemicals were also used: naloxone (Endo Laboratories), sodium cyanoborohydride (Sigma), 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (Fluka) and ethylenediamine (Fisher). CH-Sepharose 4B beads were purchased from Pharmacia. To increase water solubility, a 1% aqueous digitonin (ICN Nutritional Biochemicals) suspension was heated with stirring to boiling, allowed to sit overnight, filtered through Millipore HAWP and then lyophilized. The resulting powder readily dissolved in water. Toads were purchased from West Jersey Biological Supply, Wenonah, NJ, rats from Taconic Farms, Germantown, NY and fresh cow brains from Max Insel Cohen Co., Livingston, NJ.

Crude membrane fractions from the brains of toad (bufo marinus) and male Sprague-Dawley rats and bovine corpus striatum were prepared as described previously (4) and stored in .32 M sucrose (1:6 w/v)at -70°C until needed. Brain membrane fractions were solubilized with 0.5% digitonin and 0.75 M NaCl in 50 mM Tris, 1 mM K_2EDTA, 10 μM leupeptin, pH 7.4 (buffer A) by our previously published method (3).

Specific binding in digitonin-solubilized extracts and column fractions was assayed by incubation with [^{3}H]diprenorphine (0.5-0.75 nM) ± unlabeled diprenorphine (2 μM) for 60 min at 25°C. Where necessary, samples were diluted with Tris, EDTA buffer to give a detergent concentration $\leq$0.1%. For all samples, drug bound to solubilized material was separated from free ligand by a modification of the polyethylene glycol precipitation method used by us previously (2) and originally described by Cuatrecasas (5). Filters were counted in Hydrafluor (National Diagnostics, Somerville, NJ).

Protein concentrations were determined by a modified Coomassie blue assay (6) using a γ-globulin standard. Purified samples of low protein content were concentrated using Millipore CX-immersible filters.

To synthesize NED, ethylenediamine was attached to naltrexone via reductive amination following the protocol of Portoghese et al. (7). After purification, the synthesis produces β-NED in good yield. Purity of the product was determined by TLC and compared with controls: 1) $CHCl_3$:CH_3OH:NH_4OH (70:30:2) Rf's = 0.24 NED, 0.78 naltrexone, 0.69 β-naltrexol; 2) $CHCl_3$:CH_3OH:NH_4OH (90:10:2) Rf's = 0.11 NED, 0.60 naltrexone, 0.35 β-naltrexol; 3) BuOH:pyr:H_2O:CH_3COOH (40:20:20:20) Rf's = 0.50 NED, 0.74 naltrexone.

CH-Sepharose 4B was swollen in 0.5 M NaCl, washed with 0.5 M NaCl (100x dry weight) and distilled water (200x dry weight). The swollen gel was added to an aqueous solution of NED (0.2 mol/gm gel) and treated with water-soluble carbodiimide (1.2 mol/gm gel). The pH was adjusted to 5.0 and the mixture gently shaken overnight at 25°C. To remove unreacted ligand, the gel was washed exhaustively with 20% ethanol, 10 mM borate, 1 M NaCl, pH 8.0 and 100 mM acetate, 1 M NaCl, pH 5.5. Since a radioactive tracer had been used in the synthesis of the ligand, the concentration of bound ligand was estimated by counting a portion of the gel in Hydrafluor.

Before use, the gel was washed extensively with 50 mM Tris, 1 mM K_2EDTA, 10 μM leupeptin, 200 mM NaCl (buffer B). Retention of solubilized material and elution from NED-CH-Sepharose were performed in a batchwise manner. Gel and solubilized material (1 ml gel/4 ml solubilized preparation) were incubated with frequent shaking for 45 min at 25°C. Dilution of soluble material 1:1 or 1:5 with buffer B during incubation had no effect on retention. The "flow-through" was collected by pouring the incubated mixture into a column 1.5 cm x 30 cm and the gel in the column rinsed with 5-10 times the gel volume with buffer B containing 0.05% digitonin (buffer C). The retained binding sites were eluted by incubation with 1-3 μM naloxone in buffer C for 45 min at 25°C. After the eluate was collected, the gel was rinsed with 5 ml buffer C. Before binding activity

was determined, eluate and rinse fractions, as well as appropriate controls, were dialyzed (Spectrapor #1, MWCO 6,000-8,000) against buffer B containing 0.025% digitonin or treated with Biobeads SM2 (that had been treated with digitonin) to remove naloxone.

RESULTS

To permit coupling of naltrexone to a gel equipped with a spacer ending in a free carboxyl group (CH-Sepharose 4B, Pharmacia) ethylenediamine was attached via reductive amination of the ketone carbonyl in the 6 position. As suggested by the synthesis of similar compounds in the laboratories of Jiang et al. (8) and Portoghese et al. (7), the reaction produces β-NED, a compound which retains high affinity for the opioid binding site (IC_{50} for displacement of [^{3}H]naltrexone is 15 nM) and now contains an amino group. The concentration of NED incorporated into the gel by coupling via water-soluble carbodiimide is in the range of 2-4 μmoles/ml of packed gel.

A number of controls were carried out to determine the biospecificity of the interaction of the soluble opioid binding sites with the NED gel. The matrix itself, in the presence or absence of NaCl, does not retain opiate binding sites solubilized from toad brain membranes (Table 1) or from rat brain membranes (data not shown). When NED was coupled to the CH-Sepharose, the gel retained a high percentage (70-90%) of applied binding sites as measured by loss of binding activity in the flow-through and rinses from the column (Table 1). Furthermore, dilution of NED gels with unmodified Sepharose progressively reduces the efficiency of the gel in retaining solubilized binding sites.

The fact that the interaction is by affinity chromatography is confirmed by the inability of soluble binding sites prebound with [^{3}H]diprenorphine to be retained by the gel, i.e., 84% of applied binding sites appear in the flow-through. Similarly, sites prebound with an excess of cold naltrexone (10 μM) do not interact with the gel since binding activity is detected almost quantitatively in the flow-through after removal of cold drug (data not shown). The strongest evidence of biospecificity is the ability of the specific receptor ligand, naloxone (1-3 μM), to elute solubilized material retained by the affi-

Table 1. Interaction of solubilized opioid binding sites with affinity chromatography and control gels.

Matrix	Brain Extract	Retention[a] Percent	Elution[b] Percent
CH-Sepharose 4B	Toad	<1	-
CH-Sepharose 4B treated with water-soluble carbodiimide	Toad	<1	-
CH-Sepharose 4B coupled to ethanolamine	Toad	<1	-
NED-CH-Sepharose 4B	Toad	95	N.D.[c]
	Rat	73±5	22±3
	Cow	80±6	24±5
NED-CH-Sepharose 4B 1:5	Toad	72±5	25±6
	Rat	51±1	N.D.
	Toad-prelabeled with [^{3}H]diprenorphine(5 nm)	16	-
NED-CH-Sepharose 4B 1:10	Toad	70	-
1:20	Toad	55	-
1:50	Toad	35	-
1:100	Toad	18	-

[a] % is based upon [^{3}H]diprenorphine binding present in equivalent amount of solubilized extract.

[b] % is based upon the amount of material retained by the gel.

[c] N.D. - not detectable.

Results are the average of duplicate experiments except where averages ± SEM are indicated for toad (n=9), rat (n=5) and cow (n=3).

nity matrix. Table 1 indicates that 20-25% of the retained solubilized binding sites from the brains of the three species examined were eluted from the NED affinity gel.

Experiments with solubilized material from toad brain membranes indicate that 91±10 pmol (9 expts.) of [^{3}H]diprenorphine were bound per mg protein after elution from the NED gel compared to 0.24±0.02 (9 expts.) to crude solubilized fractions. Table 2 presents data from a typical experiment where toad brain extract was purified by elution from the NED gel approx. 450-fold compared to the applied material. Preliminary experiments with brain extracts from rat and cow indicate a similar degree of purification.

The eluted material was active after four days at 4°C, bound opiates stereospecifically and retained its ability to bind to WGA agarose columns. Preliminary results from electrophoresis on SDS-polyacrylamide gels of the NED purified fraction (toad) showed, after silver staining, six protein

Table 2. Data from a typical experiment with NED-CH Sepharose using solubilized material from toad brain membranes.

Sample	Total mg protein	Total pmol opioid bound	pmol bound mg protein	Percent Yield protein	Percent Yield binding activity
Crude solubilized material	39.6	10.8	0.27	100	100
Control--SM_2 treated extract	28	10.2	0.36	72	95
Flow-through	33.4	3.1	0.09	84	28
Rinse 1-20 ml	5.3	0.18	0.03	13	2
Rinse 2-20 ml	2.5	0.17	0.07	6	2
Rinse 3-20 ml	N.D.*	N.D.	N.D.	N.D.	N.D.
Elution	0.011	1.37	124 (470-fold enrichment)	0.04	13 (19% of retained)

*N.D.- not detectable

bands corresponding to molecular weights in the following range: four bands between 40K-65K and two bands of lesser intensity around 200K.

DISCUSSION

This paper presents results on the partial purification of solubilized opioid binding sites achieved by using a new affinity matrix that contains an ethylenediamine derivative of naltrexone. Our NED-CH-Sepharose 4B gel is stable, reusable, has very low if any nonspecific interactions and, most importantly, interacts efficiently and biospecifically with opioid binding sites in digitonin extracts of toad, rat and cow brain membranes. The eluted fraction from the affinity matrix is enriched over the crude solubilized preparation. This purification is in the range of 300-450 fold for a single pass through the column. While the purification of soluble opioid binding sites has been reported by three other laboratories (9,10,11), the specific activity (pmol opioid bound/mg protein) of the NED-purified fraction is significantly higher than any of the previously reported purified fractions.

It is of interest to note, that while undiluted NED beads do retain solubilized toad brain binding sites, no detectable binding sites can be eluted.

If soluble binding sites are retained on undiluted NED beads, rinsed, and the gel is diluted with unmodified beads, elution occurs. This result suggests that the local concentration of NED competes too effectively against μM naloxone to allow elution of retained toad brain binding sites. In contrast, soluble binding sites from both rat and cow brain are retained and eluted more efficiently from undiluted NED-CH-Sepharose. A possible explanation for the difference in the interaction between the gel and solubilized binding sites from the toad vs. mammalian brain tissue may reflect an inherent difference in the nature and types of opioid receptors present in the brain membrane homogenate. The types of opioid binding sites which are retained on and eluted from the gel, as well as the biochemical nature of the affinity purified material is under investigation. We hope to obtain an even greater degree of purification by combining affinity chromatography with other techniques such as sucrose gradients, and lectin affinity chromatography.

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