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Purification to homogeneity of an active opioid receptor from rat brain by affinity chromatography

(opioid antagonists/polyclonal antibodies/immunoaffinity chromatography)

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ABSTRACT **Active opioid binding proteins were solubilized from rat brain membranes in high yield with sodium deoxycholate in the presence of NaCl. Purification of opioid binding proteins was accomplished by opioid antagonist affinity chromatography. Chromatography using the δ-opioid antagonist *N,N*-diallyl-Tyr-D-Leu-Gly-Tyr-Leu attached to ω-aminododecyl-agarose (Affi-G) (procedure A) yielded a partially purified protein that binds selectively the δ-opioid agonist [^{3}H]Tyr-D-Ser-Gly-Phe-Leu-Thr ([^{3}H]DSLET), with a K_d of 19 ± 3 nM and a B_{max} of 5.1 ± 0.4 nmol/mg of protein. Subsequently, *Lens culinaris* agglutinin-Sepharose 4B chromatography of the Affi-G eluate resulted in isolation of an electrophoretically homogeneous protein of 58 kDa that binds selectively [^{3}H]DSLET with a K_d of 21 ± 3 nM and a B_{max} of 16.5 ± 1.0 nmol/mg of protein. Chromatography using the nonselective antagonist 6-aminonaloxone coupled to 6-aminohexanoic acid-Sepharose 4B (Affi-NAL) (procedure B) resulted in isolation of a protein that binds selectively [^{3}H]DSLET with a K_d of 32 ± 2 nM and a B_{max} of 12.4 ± 0.5 nmol/mg of protein, and NaDodSO$_4$/PAGE revealed a major band of apparent molecular mass 58 kDa. Polyclonal antibodies (Anti-R IgG) raised against the Affi-NAL protein inhibit the specific [^{3}H]DSLET binding to the Affi-NAL eluate and to the solubilized membranes. Moreover, the Anti-R IgG inhibits the specific binding of radiolabeled Tyr-D-Ala-Gly-*N-methyl*-Phe-Gly-ol (DAMGO; μ-agonist), DSLET (δ-agonist), and naloxone to homogenates of rat brain membranes with equal potency. Furthermore, immunoaffinity chromatography of solubilized membranes resulted in the retention of a major protein of apparent molecular mass 58 kDa. In addition, immunoblotting of solubilized membranes and purified proteins from the Affi-G and Affi-NAL matrices revealed that the Anti-R IgG interacts with a protein of 58 kDa.**

Pharmacological, biochemical, and behavioral studies provide evidence that the effects of opioid peptides and opiates on the central and peripheral nervous systems are mediated by three major types of receptors, μ, δ, and κ (1). Experiments aimed at isolation of the subtypes of the opioid receptor, reviewed recently, suggest that they represent different proteins with molecular sizes ranging from 110 to 33 kDa (2, 3). However, due to the heterogeneity of the opioid receptor, the lack of strict selectivity of most opioid ligands for the particular subtype, and the variety of tissues used, it is difficult to compare results from different laboratories. Molecular masses around 60 kDa have been reported for either δ (4–6) or μ (6–11) opioid receptor subtypes, depending on the source, the detergent for solubilization, the ligand, and the method used for purification.

Whether opioid receptor subtypes represent distinct gene products or whether signal specificity is encoded in post-translational modifications of a single protein structurally influenced by association with different membrane components is beginning to be resolved by cloning experiments. In recent publications, two groups reported the cloning of the δ-subtype opioid receptor from neuroblastoma × glioma hybrid cells (NG-108 15) (12, 13).

In the present study we report (*i*) purification to apparent homogeneity of an active δ-opioid binding protein (OBP) with an apparent molecular mass of 58 kDa from solubilized rat brain membranes by antagonist affinity chromatography, (*ii*) generation of polyclonal antibodies against the 58-kDa partially purified protein, and (*iii*) identification of a protein with molecular mass of 58 kDa from the solubilized membranes and partially purified active OBPs using immunoaffinity chromatography and immunoblotting.

MATERIALS AND METHODS

Chemicals. [^{3}H]Tyr-D-Ser-Gly-Phe-Leu-Thr ([^{3}H]DSLET; 41.5 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. ([^{3}H]Tyr-D-Ala-Gly-*N-methyl*-Phe-Gly-ol ([^{3}H]DAMGO; 47.7 Ci/mmol) and [^{3}H]naloxone (43 Ci/mmol) were from Amersham. Unlabeled enkephalin analogs, naloxone, ω-aminododecyl-agarose, 6-aminohexanoic acid-Sepharose 4B, lentil-lectin-Sepharose 4B [*Lens culinaris* agglutinin (LcH-Sepharose)], Amberlite XAD-2, sodium deoxycholate (DocNa), trypsin/chymotrypsin inhibitors, methyl α-D-mannopyranoside, phosphatidylinositol [soybean (PI)], Freund's adjuvant, goat anti-rabbit IgG alkaline phosphatase conjugate, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and protein markers were from Sigma. A microbicinchoninic acid protein assay kit was from Pierce. *N,N*-Diallyl-Tyr-D-Leu-Gly-Tyr-Leu (Diallyl-G) (14) and 6-aminonaloxone (15) were prepared as described.

Membrane Preparation and Solubilization. Brains without the cerebellum from male Wistar rats were homogenized in 10 volumes of 10 mM Tris·HCl, pH 7.4/0.32 M sucrose using 12 strokes of a Teflon glass homogenizer. The homogenate was centrifuged at 1500 × *g* for 10 min and the supernatant was centrifuged at 30,000 × *g* for 30 min. The pellet (P_2 fraction) was suspended in the original buffer (10 mg of protein per ml) and stored at −80°C. For solubilization, membranes were thawed and centrifuged at 30,000 × *g* at 4°C. A pellet (360 mg) was resuspended in 60 ml of 50 mM Tris·HCl, pH 7.4/100 mM NaCl containing 1% DocNa and 1 mg of trypsin/chymotrypsin inhibitors, stirred gently in an ice bath for 40 min, and

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Abbreviations: DSLET, Tyr-D-Ser-Gly-Phe-Leu-Thr; DTLET, Tyr-D-Thr-Gly-Phe-Leu-Thr; DAMGO, Tyr-D-Ala-Gly-*N-methyl*-Phe-Gly-ol; Diallyl-G, *N,N*-diallyl-Tyr-D-Leu-Gly-Tyr-Leu; Affi-G resin, Diallyl-G coupled to ω-aminododecyl-agarose; Affi-NAL resin, 6-aminonaloxone coupled to 6-aminohexanoic acid-Sepharose 4B; LcH, *Lens culinaris* agglutinin; DocNa, sodium deoxycholate; DMF, dimethylformamide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; PI, phosphatidylinositol; BSA, bovine serum albumin; OBP, opioid binding protein; NEM, *N*-ethylmaleimide.

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centrifuged at 108,000 × g for 1 hr at 4°C. Excess detergent was removed by addition of Amberlite XAD-2 resin (24 g) previously equilibrated with 50 mM Tris·HCl, pH 7.4/100 mM NaCl/0.05% DocNa (buffer A) under gentle stirring for 30 min at 4°C. After removal of the resin the filtrate was diluted with buffer A to a protein concentration of 1–2 mg/ml for further purification. For binding experiments of the solubilized proteins, the supernatant from the ultracentrifugation was dialyzed overnight against 10 mM Tris·HCl, pH 7.4/0.32 M sucrose/0.05% DocNa (buffer B) and diluted 20-fold with the same buffer.

Affinity Resins. *Procedure A.* The Affi-G matrix was prepared by coupling Dially-G peptide to preswollen ω-aminododecyl-agarose gel in the presence of dicyclohexylcarbodiimide and *N*-hydroxysuccinimide in dimethylformamide (DMF)/water. After filtration and washings, residual amino groups were blocked by acetylation with acetic acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).

Procedure B. The Affi-NAL resin was prepared by coupling preswollen 6-aminohexanoic acid-Sepharose 4B gel to 6-aminonaloxone in the presence of EDC. After filtration and washings with DMF/water, free carboxyl groups on the resin were allowed to react with ethanolamine in the presence of EDC.

Affi-G Chromatography. Prior to use the Affi-G resin was equilibrated with buffer A. The solubilized fraction (150 ml) was incubated with Affi-G gel (50-ml bed volume) under shaking for 1 hr at 25°C. The suspension was filtered through a coarse sintered funnel and the resin was washed extensively with buffer A (10-bed volumes) at 4°C until the absorption at 280 nm of the washings reached zero. The retained proteins were eluted by incubation with a solution of 2 μM Diallyl-G peptide (60 ml) in buffer A for 1 hr at 25°C. The filtrate was dialyzed overnight in the presence of PI for further purification and binding assays (see below).

LcH-Sepharose 4B Chromatography. A vortexed dispersion of 15 mg of PI in 10 ml of buffer A was added to the eluate from the Affi-G resin (20 ml), and the mixture was dialyzed overnight against 50 mM Tris·HCl (pH 7.4) containing 0.05%, DocNa. The dialysate (8 ml of dialysate per ml of gel) was incubated with LcH-Sepharose gel (preequilibrated in buffer A/1 mM $MnCl_2$) for 1 hr at 4°C. After filtration and washing with buffer A (10 bed volumes) containing 1 mM $MnCl_2$ the glycoproteins bound on the gel were eluted by incubation with 4 bed volumes of 10 mM Tris·HCl, pH 7.4/0.195 M sucrose/0.125 M methyl α-D-mannopyranoside/0.05% DocNa for 1 hr at 4°C. The eluate was diluted 25-fold with buffer B and used for binding experiments.

Affi-NAL Chromatography. A procedure similar to the one used with the Affi-G resin was followed. Briefly, solubilized preparation was incubated with Affi-NAL resin (1 ml of gel per 3 ml of solubilized material) under gentle shaking for 1 hr at 4°C. The suspension was filtered and washed with ice-cold buffer A (10 bed volume). The adsorbed proteins were eluted by incubation with 60 ml of 1 μM naloxone in buffer A for 1 hr at 25°C. The filtrate was dialyzed overnight at 4°C against 50 mM Tris·HCl, pH 7.4/0.05% DocNa in the presence of PI, diluted 40-fold with buffer B, and tested for binding activity.

Generation of Polyclonal Antibodies (Anti-R). Forty to 60 μg of the OBP obtained from the Affi-NAL resin, in 1 ml of phosphate-buffered saline (PBS, pH 7.4) containing 0.15% $NaDodSO_4$, was emulsified with 1 ml of complete Freund's adjuvant. Two rabbits were injected intradermally (20–30 μg of protein per rabbit). For the subsequent booster injections, at 2-week intervals, 15 μg of protein per rabbit emulsified in incomplete Freund's adjuvant was given intramuscularly at contralateral sites. The rabbits were bled fortnightly and the sera were separated. The immunoglobulins were purified by precipitation with 18% Na_2SO_4, redissolved in PBS, and dialyzed overnight against PBS. Aliquots were stored at −70°C. Protein was determined by the method of Bradford (22).

Immunoaffinity Chromatography. Anti-R affinity polyacrylamide beads were prepared as described (16). Briefly, 20 mg of Anti-R IgGs was added to glutaraldehyde-activated Bio-Gel P-2 beads (4 ml in 15 ml of phosphate buffer, pH 7.5), and the mixture was shaken gently overnight at room temperature. The beads were washed with the same buffer, 1 M NaCl, and 50 mM phosphate buffer (pH 7.5). Free aldehydic groups were blocked by reacting with 100 mM ethanolamine (pH 7.5) for 6 hr at room temperature. Finally, the beads were extensively washed with PBS and packed in a column. Solubilized preparation (75 ml) in buffer B was chromatographed through the column twice for a period of 2 hr. After extensive washings the proteins retained on the column were eluted with 10 ml of 0.1 M glycine-HCl (pH 2.8) containing 0.05% $NaDodSO_4$. The eluate was concentrated on an Amicon PM-10 membrane and analyzed by $NaDodSO_4$/PAGE.

Gel Electrophoresis and Staining. Ultrafiltrated protein samples were solubilized in sample buffer (4% $NaDodSO_4$/10% glycerol/0.001% bromphenol blue/62.5 mM Tris·HCl, pH 6.8/100 mM dithiothreitol) for 3 min at 95°C and electrophoresed in 0.8-mm-thick gels according to the method of Laemmli (17). The stacking gel contained 4% acrylamide and the running gel was composed of a linear gradient of 8–12% acrylamide. The proteins were visualized by the silver staining method (18). Molecular mass standards are as follows: bovine serum albumin (BSA), 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa; and α-lactalbumin, 14 kDa.

Western Blot. Samples of solubilized preparation and ultrafiltrated Affi-NAL and Affi-G eluates were solubilized in $NaDodSO_4$/PAGE sample buffer and run on a 10% polyacrylamide separating gel. The electrophoresed proteins were transferred to nitrocellulose membranes using a semidry method with the 2117 Multiphor II electrophoresis system (Pharmacia LKB). Following transfer, the membranes were rinsed in TBS (10 mM Tris/150 mM NaCl, pH 7.5) and blocked in TBS/3% BSA with gentle stirring for about 40 min. After washing, the Anti-R antibody in TBS/0.5% BSA was added and the membranes were incubated overnight at 4°C. A goat anti-rabbit IgG alkaline phosphatase conjugate in TBS/0.5% BSA (dilution 1:1000) was used to detect bound Anti-R antibody. The color development was performed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate in 0.1 M Tris·HCl, pH 9.5/100 mM NaCl/5 mM $MgCl_2$.

Opioid Binding. Aliquots (1 ml) of dialyzed eluates from the affinity resins or the solubilized preparation, suitably diluted in buffer B, were incubated for 1 hr at 25°C with tritiated opioid ligands in the presence and absence of unlabeled ligands in a final volume of 1.2 ml. After cooling on ice for 15 min, the samples were filtered by suction through Whatman GF/B glass filters previously soaked in 0.3% polyethyleneimine (19), washed three times with 2.5 ml of ice-cold 50 mM Tris·HCl (pH 7.4), and dried and the radioactivity was measured by liquid scintillation spectroscopy in 10 ml of Aquasol. All measurements were done in duplicate or triplicate samples and specific binding was defined as the displacable binding by 10 μM unlabeled ligand. Data from two or more experiments were analyzed using the LIGAND program (20) modified by McPherson for the "cold saturation" calculation method (21).

Protein Determination. Protein in P2 fractions and solubilized preparation was determined in triplicate samples by the Bradford method (22) using BSA as standard. Aliquots of eluates from the affinity resins were dialyzed against water and suitably concentrated to 0.5–20 μg of protein per ml for

protein determination by the microbicinchoninic acid protein assay kit (no. 23235) of Pierce (23).

RESULTS

Solubilization of Active OBPs from Rat Brain. Solubilization of opioid binding sites from rat brain synaptosomal membranes in high yield was achieved by treatment with 1% DocNa in the presence of 100 mM NaCl and protease inhibitors as described in *Materials and Methods*. The yield of the opioid binding sites of the solubilized extract was estimated by saturation experiments. Comparison of the binding parameters for [^{3}H]DSLET of synaptosomal membranes, K_d of 1.6 ± 0.2 nM and B_{max} of 200 ± 20 fmol/mg of protein, measured in 10 mM Tris·HCl, pH 7.4/0.32 M sucrose, with those of the solubilized extract (dialyzed and diluted in buffer B), K_d of 4.8 ± 0.5 nM and B_{max} of 120 ± 30 fmol/mg of protein (n = 5), showed that about 60% of opioid binding sites had been solubilized (data not shown). The solubilized proteins in buffer B were also shown to bind [^{3}H]naloxone with a K_d of 4.4 ± 0.3 nM and a B_{max} of 70 ± 10 fmol/mg of protein (n = 2), whereas the same preparation diluted in 50 mM Tris·HCl, pH 7.4/100 mM NaCl/0.05% DocNa yielded a K_d of 3.3 ± 0.7 nM and a B_{max} of 120 ± 25 fmol/mg of protein for [^{3}H]naloxone binding (n = 5).

Purification of Active OBPs. In this study we used two purification procedures (A and B) based on affinity chromatography of immobilized opioid antagonists: the δ-opioid antagonist Diallyl-G and the nonselective antagonist 6-aminonaloxone. The δ-opioid antagonistic properties of Diallyl- G, an analog of α-casein exorphins (24), have been reported (14). Moreover, this peptide displaces [^{3}H]DSLET from rat brain membranes with an IC_{50} of 0.7 ± 0.1 μM, whereas concentrations >10 μM are needed for half-maximal displacement of [^{3}H]DAMGO. In addition, it has been shown that Diallyl-G inhibits dose-dependently the low K_m GTPase activity of a pertussis toxin-sensitive GTP binding protein in rat brain membranes (25), suggesting that Diallyl-G, like ICI 174864 (26), may hinder the association between opioid receptors and G proteins. On the other hand, 6-aminonaloxone displaces [^{3}H]DSLET, [^{3}H]DAMGO, and [^{3}H]naloxone from rat brain membranes with almost equal potency—K_i values of 24 nM, 10 nM, and 15 nM, respectively (data not shown).

In procedure A, purification to homogeneity of active OBPs from solubilized membranes was accomplished in two steps: affinity chromatography using Diallyl-G coupled to ω-aminododecyl-agarose (Affi-G) followed by chromatography on LcH Sepharose 4B. The proteins isolated by Affi-G chromatography bind [^{3}H]DSLET specifically with a K_d of 19 ± 3 nM and a B_{max} of 5.1 ± 0.4 nmol/mg of protein (Fig. 1*A*) and the yield was about 0.10% of the protein used for purification. The silver-stained NaDodSO$_4$/PAGE pattern of these proteins reveals a predominant band of apparent molecular mass 58 kDa followed by four or five weaker bands of lower mass (Fig. 2, lane 3). LcH-Sepharose 4B chromatography of the Affi-G eluate resulted in isolation of a protein that selectively binds [^{3}H]DSLET with a K_d of 21 ± 3 nM and a B_{max} of 16.5 ± 1.0 nmol/mg of protein (Fig. 1*B*). The NaDodSO$_4$/PAGE pattern of an ultrafiltrated sample revealed, after silver staining, a single protein band of 58 kDa (Fig. 2, lane 4). The two-step sequential affinity chromatography of the solubilized rat brain membranes yielded a purified protein with high specific activity for [^{3}H]DSLET (16.5 nmol/mg of protein), which is close to the theoretical value (17.3 nmol/mg) assuming 1 mol of ligand per mol of receptor of 58 kDa. Purification of the proteins isolated from the Affi-G and LcH matrices accounts for 28% and 88% of the theoretical value, respectively.

In procedure B, partial purification was achieved using 6-aminonaloxone attached to 6-aminohexanoic acid-Sepharose 4B (Affi-NAL). The isolated proteins bind specifically [^{3}H]DSLET with a K_d of 32 ± 2 nM and a B_{max} of 12.4 ± 0.5 nmol/mg of protein (Fig. 3). The NaDodSO$_4$/PAGE pattern of these proteins shows a predominant band of 58 kDa followed by a few weaker bands of lower and higher mass (Fig. 2, lane 5). The yield of this preparation accounts for 0.5% of the protein used for purification and its purity for 60%.

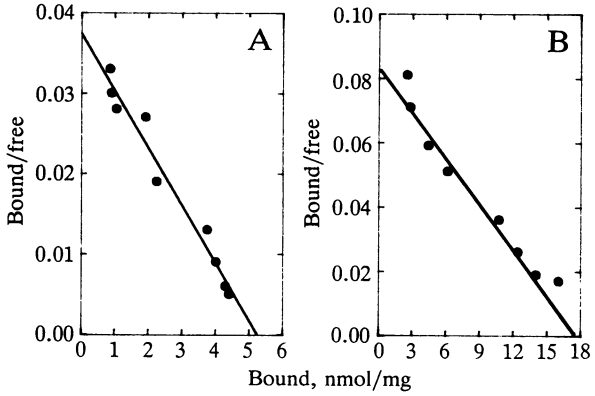

FIG. 1. Scatchard plots of [^{3}H]DSLET binding to proteins purified by the Affi-G and the LcH-Sepharose 4B chromatography. Experiments were performed in triplicate samples containing 0.14 ± 0.03 μg of the Affi-G eluate or 0.13 ± 0.02 μg of LcH-Sepharose eluate, [^{3}H]DSLET (7 nM), and increasing concentrations of unlabeled DSLET in a final volume of 1.2 ml. (*A*) The binding parameters of the Affi-G eluate from three experiments were K_d = 19 ± 3 nM and B_{max} = 5.1 ± 0.4 nmol/mg of protein. Nonspecific binding was 15–20% of the total binding. (*B*) The binding parameters of the LcH-Sepharose eluate from three different experiments were K_d = 21 ± 3 nM and B_{max} = 16.5 ± 1.1 nmol/mg of protein. Nonspecific binding was 8–10% of the total binding.

As shown in Fig. 4, opioid ligands, Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET; δ-agonist), Diallyl-G (δ-antagonist), and

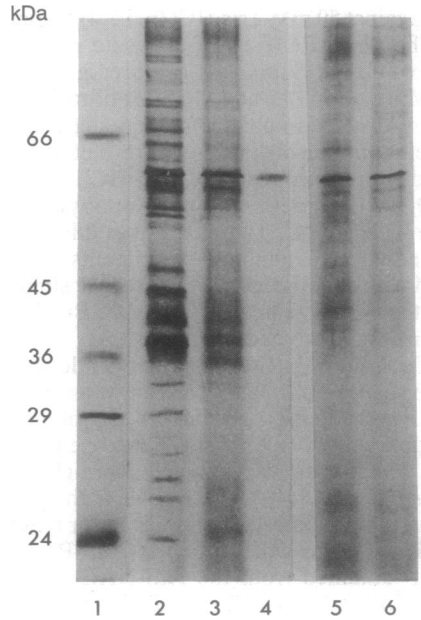

FIG. 2. NaDodSO$_4$/PAGE of OBPs isolated by affinity chromatography and immunoaffinity chromatography, visualized by silver staining. Lanes: 1, molecular mass standards; 2, DocNa (100 μg)-solubilized membranes; 3, Affi-G (40 ng)-purified proteins; 4, LcH-Sepharose 4B (15 ng) proteins; 5, Affi-NAL (50 ng)-purified proteins; 6, eluate (60 ng) of proteins specifically bound to the Anti-R IgG immunoaffinity column after chromatography of solubilized membranes.

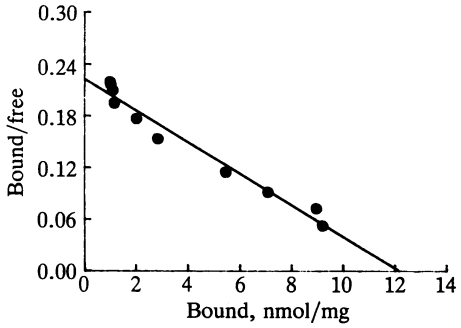

FIG. 3. Scatchard plot of [^{3}H]DSLET binding to the purified protein from the Affi-NAL matrix. Experiments were performed in triplicate samples containing 0.75 ± 0.15 μg of protein, [^{3}H]DSLET (7 nM), and increasing concentrations of unlabeled DSLET in a final volume of 1.2 ml. Data from three experiments yielded a K_d of 32 ± 2 nM and a B_{max} of 12.4 ± 0.5 nmol/mg of protein. Nonspecific binding was 12–15% of the total binding.

naloxone displace [^{3}H]DSLET bound to the Affi-NAL eluate with IC_{50} values of 42 ± 6 nM, 112 ± 35 nM, and 1000 ± 120 nM, respectively. Moreover, pretreatment of the Affi-NAL protein with increasing concentrations of *N*-ethylmaleimide (NEM) inhibited the specific binding of [^{3}H]DSLET in a saturable manner. Half-maximal inhibition was achieved at 5 μM NEM and specific binding was abolished at 60 μM NEM (data not shown). Sulfydryl reagents such as NEM are known to inhibit binding of opioid agonists to their receptors in neural membranes (ref. 27 and references therein).

Under the conditions of the binding assay (buffer B, either in the presence or absence of PI), the proteins isolated by procedures A and B do not bind [^{3}H]DAMGO or [^{3}H]naloxone at concentrations of radioligands between 5 and 10 nM. However, the addition of PI during dialysis stabilizes the preparations for several days, without affecting their binding parameters or their selectivity for opioid agonists.

Polyclonal Antibodies (Anti-R) Against the OBPs. Polyclonal antibodies were raised against the Affi-NAL partially purified proteins (see *Materials and Methods*). As shown in Fig. 5*A*, the Anti-R IgG inhibits the specific binding of [^{3}H]DSLET to the Affi-NAL-purified receptor and to the solubilized preparation with half-maximal concentrations of 1 μg and 8 μg of protein, respectively. In addition, increasing concentrations of Anti-R IgG displace [^{3}H]DSLET bound to the solubilized preparation with an IC_{50} value of around 8 μg (Fig. 5*B*). Moreover, preincubation of rat brain membranes with increasing concentrations of Anti-R IgG inhibited in a saturable way

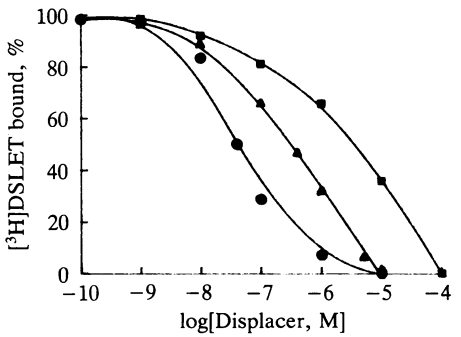

FIG. 4. Displacement of [^{3}H]DSLET binding to the protein purified from the Affi-NAL matrix by opioid ligands. Triplicate samples of the Affi-NAL eluate, containing 1.0 ± 0.2 μg of protein, [^{3}H]DSLET (7 nM), and increasing concentrations of unlabeled DTLET, Diallyl-G, and naloxone in a final volume of 1.2 ml were incubated for 60 min at 25°C. IC_{50} values (means of two different experiments) are as follows: DTLET (●), 42 ± 6 nM; Diallyl-G (▲), 112 ± 35 nM; naloxone (■), 1000 ± 120 nM.

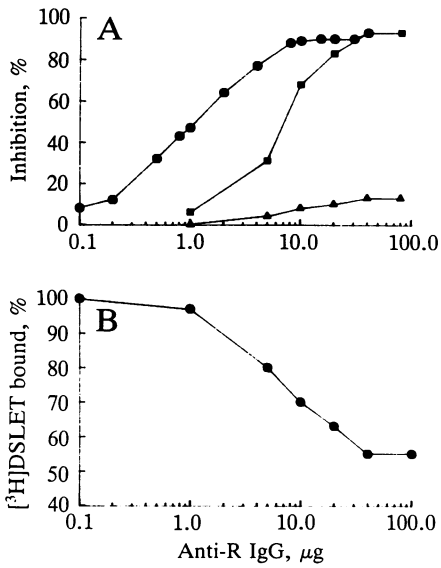

FIG. 5. Binding experiments were performed by the filtration method as described in *Materials and Methods*. Points are the mean of two different experiments in duplicate. (*A*) Inhibition of [^{3}H]-DSLET binding to the Affi-NAL-purified protein and to solubilized membranes by the Anti-R IgG. ●, Affi-NAL-purified protein samples (0.70 μg/ml) preincubated with increasing concentrations of Anti-R IgG for 30 min at 25°C. After addition of [^{3}H]DSLET (9 nM) in a final volume of 1.2 ml, incubation was continued for 45 min. ▲, Control experiments with the Affi-NAL protein (0.70 μg/ml) preincubated with increasing concentrations of preimmune serum and subsequently with [^{3}H]DSLET under the same conditions. ■, Samples of solubilized membranes (120 μg of protein per ml) preincubated with increasing amounts of Anti-R IgG for 30 min at 25°C. Incubation was continued for 45 min after addition of [^{3}H]DSLET (9 nM) in a final volume of 1.2 ml. (*B*) Displacement of bound [^{3}H]DSLET to solubilized membranes by the Anti-R IgG. Samples of solubilized membranes (120 μg of protein per ml) were incubated with [^{3}H]DSLET (5 nM) for 45 min at 25°C. Increasing amounts of Anti-R IgG were then added and incubation was continued for 45 min. Rabbit preimmune IgG (40 μg) used as control displaced only 10% ± 3% of [^{3}H]DSLET.

the specific binding of [^{3}H]DSLET, [^{3}H]DAMGO, and [^{3}H]naloxone (2 nM) with almost equal potency (20–25 μg for half-maximal inhibition), whereas 80 μg of preimmune serum inhibited binding only by 10% (data not shown).

Immunoaffinity Chromatography and Western Blot. Dialyzed solubilized membrane preparation was chromatographed through an immunoaffinity column, prepared by coupling Anti-R IgG to activated polyacrylamide beads. $NaDodSO_4$/PAGE of the ultrafiltrated eluate revealed a predominant band of 58 kDa and a few bands of lower molecular mass (Fig. 2, lane 6). Moreover, $NaDodSO_4$/PAGE of solubilized rat brain membranes, and proteins purified by the Affi-G and Affi-NAL matrices and subsequent immunoblotting, revealed that the Anti-R IgGs interact with a protein of 58 kDa (Fig. 6). The additional minor band of 45 kDa appearing on the electrophoretogram of the solubilized membranes (Fig. 6, lane 1) may possibly represent a degradation product or a subunit of the opioid receptor(s).

DISCUSSION

In this work we present evidence that affinity chromatography of solubilized rat brain membranes using two different types of opioid antagonists coupled to resins, the Affi-G and the Affi-NAL matrices, resulted in isolation of proteins of 58 kDa that bind specifically the δ-agonist DSLET with similar affinity. Subsequently, lectin chromatography of the Affi-G eluate yielded an electrophoretically homogeneous protein of 58 kDa

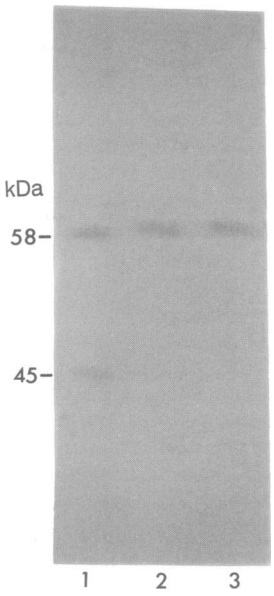

FIG. 6. Immunoblots on nitrocellulose of solubilized rat brain membranes and of proteins purified from the Affi-G and Affi-NAL resins fractionated on 10% NaDodSO$_4$/PAGE. Protein markers are indicated on the left. Lanes: 1, solubilized rat brain membranes (100 μg); 2, Affi-G protein (15 μg); 3, Affi-NAL protein (8 μg).

that has the expected specific activity for [^{3}H]DSLET of a pure receptor.

Under the conditions of the binding assay (buffer B), the K_d values for DSLET binding of all three preparations were increased by one order of magnitude (20–30 nM) as compared to those of membranes and solubilized preparations (2–5 nM). On the other hand, the B_{max} values were highly increased and the total binding activity of the isolated proteins increased significantly, on the order of 10- to 100-fold, depending on the purification procedure. These findings could suggest that important membrane components such as lipids, G proteins, and other constituents have been removed during the purification through the antagonist-affinity matrices. In addition, we should mention that dialysis and composition of the buffer (buffer B) used for dilution are critical for the binding of [^{3}H]DSLET to these preparations. Interestingly, the isolated proteins are unable to bind [^{3}H]DAMGO or [^{3}H]naloxone regardless of the presence or absence of PI. Their high specific activity for the δ-agonist, DSLET, could possibly be due to a protein–detergent micellar conformation in favor of the δ-agonist, suggesting that these proteins may represent a *δ-opioid receptor subtype*. However, at present it would be premature to claim that these proteins fulfill the criteria of the δ-opioid receptor. On the other hand, in competition binding experiments, we have shown that [^{3}H]DSLET bound to the Affi-NAL protein can be displaced by DTLET (δ-agonist), by Diallyl-G (δ-antagonist), and, poorly, by naloxone. In addition, pretreatment of the Affi-NAL protein with low concentrations of NEM affects dramatically the binding of [^{3}H]-DSLET. These findings partly support the δ-opioid features of the protein.

To further characterize the isolated putative δ-type opioid receptor, polyclonal antibodies (Anti-R immunoglobulins) were generated to the Affi-NAL proteins. These antibodies (*i*) interact with determinants for DSLET binding of the Affi-NAL protein and protein(s) contained in the solubilized membranes, (*ii*) inhibit binding of [^{3}H]DSLET, [^{3}H]DAMGO, and [^{3}H]naloxone to synaptosomal membranes with equal potency, implying that μ- and δ-opioid subtypes share a common region, and (*iii*) recognize a 58-kDa protein in the solubilized preparations and the Affi-G and Affi-NAL proteins.

Proteins of apparent molecular mass around 58 kDa for purified and partially purified μ- and δ-subtypes of the opioid receptor have been reported by other investigators (4, 5, 8–10). A δ-opioid receptor of 58 kDa covalently linked to the δ-selective ligand Super-FIT was isolated from NG108-15 hybrid cells (4). A μ-subtype protein of 58 kDa was isolated from rat brain by succinylmorphine affinity chromatography (8). Recently, a δ-opioid receptor of 58 kDa has been isolated from CHAPS-solubilized bovine frontal cortex and NG 108-15 cells by succinylmorphine affinity chromatography (28). Although numerous reports and recent cloning experiments (29) provide evidence for the existence of μ- and δ-opioid receptor subtypes, as separate entities, pharmacological and biochemical studies aiming to unravel the physiological function of μ- and δ-opioid receptor support the notion that in neuronal membranes μ- and δ-subtypes may exist associated in a complex with allosterically interacting μ and δ sites (ref. 30 and references therein). Further experiments are necessary to better characterize the opioid selectivity of the isolated protein and study its interactions with phospholipids, G proteins, and effector systems.

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