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Comparison of [^{125}I] β-Endorphin Binding to Rat Brain and NG108-15 Cells Using a Monoclonal Antibody Directed Against the Opioid Receptor

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SUMMARY

The properties of [^{125}I]β_h-endorphin-binding sites from rat brain membranes and membranes from the NG108-15 cell line were compared using a monoclonal antibody directed against the opioid receptor and opioid peptides as probes. The binding of [^{125}I] β_h-endorphin to both rat brain and NG108-15 membranes yielded linear Scatchard plots with K_d values of 1.2 nM and 1.5 nM, respectively, and B_{max} values of 865 fmol/mg rat brain membrane protein and 1077 fmol/mg NG108-15 membrane protein. A monoclonal antibody, OR-689.2.4, capable of inhibiting μ and δ binding but not κ binding to rat brain membranes, noncompetitively inhibited the binding of 1 nM [^{125}I]β_h-endorphin to rat brain and NG108-15 membranes with an IC_{50} value of 405 nM for rat brain membranes and 543 nM for NG108-15 membranes. The monoclonal antibody also inhibited the binding of 3 nM [^{3}H][D-penicillamine^2, D-penicillamine^5] enkephalin to NG108-15 membranes with an IC_{50} value of 370 nM. In addition to blocking the binding of [^{125}I]β_h-endorphin to brain membranes, the antibody also displaced [^{125}I]β_h-endorphin from membranes. Site-specific opioid peptides had large variations in their IC_{50} values depending on whether they were inhibiting [^{125}I]β_h-endorphin binding to rat brain or the NG108-15 membranes. When the peptides were tested with the monoclonal antibody for their combined ability to inhibit [^{125}I]β_h-endorphin binding to both membrane preparations, the peptides and antibody blocked binding as though they were acting at allosterically coupled sites, not two totally independent sites. These studies suggest that μ-, δ-, and β-endorphin-binding sites share some sequence homology with the 35,000-dalton protein that the antibody is directed against.

Of the known endogenous opioid peptides, β-endorphin, a 31-amino acid peptide, has been found to be the most potent endogenous analgesic agent (1). β-Endorphin-binding sites have been identified in a wide variety of species and tissues including rat brain (2, 3) and the neuroblastoma × glioma hybrid, NG108-15 (4). β-Endorphin has almost equal affinity for the μ and δ opioid receptors, while having 100-fold less affinity for the κ site (5). β-Endorphin has also been shown to bind with high affinity to μ_1 opioid receptors (6). A distinct β-endorphin receptor, the ϵ-receptor, has been proposed based on bioassays with the rat vas deferens (7) and brain membrane binding assays using the benzomorphans (8). Using [^{125}I]β_h-endorphin (Tyr^{27} radiolabeled), the properties of β-endorphin-binding sites in rat cortex (9), whole rat brain (10), and human striatum (11) have been characterized.

The neuroblastoma × glioma hybrid cell line, NG108-15, contains only δ opioid receptors (12). However, the binding of [^{3}H]β_h-endorphin to NG108-15 membranes is not completely blocked by opioids, suggesting that β_h-endorphin may be binding to a non-opioid receptor in this cell line, in addition to binding to the δ opioid receptor (4). In this cell line, β-endorphin has shown inhibitory modulation of adenylate cyclase activity (13, 14), which is mediated through only one type of opioid receptor (14). The study reported here compares the properties of β-endorphin-binding sites in rat brain and NG108-15 cells. A monoclonal antibody, directed against the opioid receptor, and opioid peptides were used to probe the multiple sites.

A monoclonal antibody, OR-689.2.4, capable of inhibiting μ and δ opioid binding but not κ binding to rat brain membranes has been obtained by immunizing a mouse with a partially purified receptor complex (15). The monoclonal antibody is of the IgM class, as determined by Ouchterlony analysis using antiserum specific for μ chains, γ chains, and α chains (16). The monoclonal IgM is directed against a 35,000-dalton protein as determined by immunoprecipitation (16). The specificity of this antibody for the opioid receptor has been demonstrated by its inability to inhibit β-adrenergic, nicotine, diazepam, or

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ABBREVIATIONS: [^{125}I]β_h-endorphin, (3-[^{125}I]iodotyrosyl^{27})β-endorphin (human); DAGO, [D-Ala^2,(Me)Phe^4,Gly(ol)^5]enkephalin; DPDPE, [D-penicillamine^2,D-penicillamine^5]enkephalin; DADLE, [D-Ala^2,D-Leu^5]enkephalin; KIU, kallikrein-inhibiting unit; BSA, bovine serum albumin.

muscarinic cholinergic binding to rat brain membranes (16). In addition, other mouse immunoglobulins and a control mouse monoclonal IgM were not capable of blocking opioid binding to membranes (16). Fab fragments, with a molecular weight of 48,000, have been prepared from the OR-689.2.4 IgM (17). These Fab fragments have been shown to be noncompetitive inhibitors of μ and δ binding to rat and guinea pig brain membranes (18). In contrast, they did not inhibit κ binding to rat or guinea pig brain membranes or guinea pig cerebellum (18).

By using the OR-689.2.4 monoclonal antibody and specific opioid peptides to investigate the multiple opioid-binding sites, similarities and differences in the binding of [^{125}I]β_h-endorphin to rat brain and NG108-15 membranes were determined.

Materials and Methods

Generating the OR-689.2.4 monoclonal antibody. The IgM, OR-689.2.4, was produced by immunizing a BALB/c mouse with a partially purified opioid receptor complex as previously described (19, 20). The IgM was obtained by culturing the OR-689.2.4 cell line in Ex-cell 300, a low protein serum-free medium. The culture supernatant was concentrated on Millipore's Minitan with a 100,000 molecular weight cutoff membrane. The concentrated IgM, 1–4 mg/ml, was dialyzed at a 1:100 ratio (volume IgM/volume 50 mM Tris-HCl, pH 7.5) against 50 mM Tris-HCl, pH 7.5. The dialyzed IgM was filtered through a 0.2-μm filter and stored at −70°.

Binding of [^{125}I]β_h-endorphin to rat brain and NG108-15 membranes. Rat brain membranes, excluding cerebellar tissue, were prepared from male Sprague-Dawley rats and washed at 37° for 30 min as previously described (21). The neuroblastoma × glioma cell line, NG108-15, was cultured in Ex-cell 300 medium containing 5% fetal bovine serum, 1 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine. At confluence, the cells were centrifuged at 200 × *g* for 10 min at 4°. The pellets were homogenized in 50 volumes (w/v) of 50 mM Tris-HCl, pH 7.5, using a Polytron (Brinkmann) homogenizer. After centrifugation at 40,000 × *g* for 30 min, the pellet was resuspended in 10 volumes of 50 mM Tris-HCl, pH 7.5, and aliquots of the NG108-15 membranes were stored at −70° until use. Protein concentration was determined by the method of Bradford (22).

For [^{125}I]β_h-endorphin binding assays, 40 μg of rat brain or NG108-15 membrane protein were added to polypropylene tubes in a final volume of 0.5 ml of 50 mM Tris-HCl, pH 7.5, containing 0.20% BSA and 0.01% bacitracin. When opioid peptides were tested for their ability to inhibit binding, they were incubated with membranes at 25° for 60 min prior to the addition of the [^{125}I]β_h-endorphin. Varying concentrations of the OR-689.2.4 IgM were incubated at 25° with the membranes for 60 min prior to the addition of [^{125}I]β_h-endorphin. To determine if the OR-689.2.4 IgM could displace bound [^{125}I]β_h-endorphin, rat brain membranes were incubated with 1 nM [^{125}I]β_h-endorphin for 60 min at 25°, followed by the addition of varying concentrations of the IgM for an additional 60-min incubation prior to filtration. When opioid peptides and the IgM were tested in combination, membranes were incubated with the peptides for 30 min prior to the addition of the IgM, then for an additional 60-min incubation. [^{125}I]β_h-Endorphin, obtained lyophilized, was reconstituted according to the manufacturer's recommendations in 0.25% BSA, 5% lactose, 0.2% L-cysteine hydrochloride, 10 mM citric acid, and 800 KIU/ml aprotinin. The reconstituted [^{125}I]β_h-endorphin was aliquoted in 4-ml Nunc polypropylene tubes at 1 μCi in 10 μl/tube, and stored at −20° until use. One tube (1 μCi) was used for each assay, consisting of 8–12 samples assayed in triplicate for a total of 24–36 separate tubes. Just before use, unlabeled β_h-endorphin in 50 mM Tris-HCl, pH 7.5, 0.20% BSA, and 0.01% bacitracin was added to the 1 μCi of [^{125}I]β_h-endorphin to reach the desired final concentration. The samples were incubated with the [^{125}I]β_h-endorphin at 25° for 60 min. Nonspecific binding was measured by the inclusion of 1 μM β_h-endorphin. Binding assays were terminated by filtering the samples through Whatman GF/B glass fiber filters using a Brandel 48-well cell harvester. The GF/B filters were soaked in 0.25% polyethylenimine for at least 60 min before filtration. After filtration, the filters were washed three times with 4 ml of ice-cold 50 mM Tris-HCl, pH 7.5. Each filter was placed in a 12 mm × 75 mm polystyrene tube and counted in a gamma counter.

Binding of [^{3}H]DPDPE to NG108-15 membranes. The binding of [^{3}H]DPDPE to NG108-15 membranes was measured in a similar manner. In a final volume of 1 ml, 100 μg of NG108-15 membrane protein were incubated in 50 mM Tris-HCl, pH 7.5, containing 0.2% BSA, 0.01% bacitracin, and [^{3}H]DPDPE in polypropylene tubes for 3 hr at 25° prior to filtration through Whatman GF/B filters. A 3-hr incubation period was used because of the slow association rate of [^{3}H]DPDPE with the δ receptor (23). Nonspecific binding was measured by the inclusion of 10 μM DADLE. When the OR-689.2.4 IgM was tested for its ability to inhibit [^{3}H]DPDPE binding, NG108-15 membranes were incubated with the IgM at 25° for 60 min before the addition of [^{3}H]DPDPE to a final volume of 0.5 ml. After filtration of the samples, the GF/B filters were washed as described for the [^{125}I]β_h-endorphin binding assays. The filters were added to 7 ml of Liquiscint scintillation fluid, and radioactivity was measured by liquid scintillation spectrometry at a counting efficiency of 35–50%.

Materials. (3-[^{125}I]Iodotyrosyl^{27})β-endorphin (human) (2000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). [^{3}H]DPDPE (Mosberg's δ agonist) (29 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All opioid peptides were obtained from Peninsula Laboratories (Belmont, CA). Ex-cell 300 medium was obtained from JR Scientific (Woodland, CA). Male Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Boston, MA). The NG108-15 cell hybrid was obtained from Dr. W. Klee (National Institute of Mental Health). The Brandel 48-well cell harvester was purchased from Brandel, Inc. (Gaithersburg, MD). Polyethylenimine and bacitracin were purchased from Sigma Chemical Co. (St. Louis, MO). Liquiscint scintillation fluid was purchased from National Diagnostics (Somerville, NJ).

Results

Binding of [^{125}I]β_h-endorphin to rat brain and NG108-15 membranes. The binding of [^{125}I]β_h-endorphin to rat brain and NG108-15 membranes was measured over the concentration range of 0.1 nM–12.8 nM [^{125}I]β_h-endorphin. As can be seen in Fig 1A, the binding of [^{125}I]β_h-endorphin to rat brain membranes yielded a linear Scatchard plot. A K_d value of 1.2 ± 0.49 nM and a B_{max} value of 865 ± 47 fmol/mg of membrane protein were obtained with rat brain membranes. A linear Scatchard plot was also generated for the binding of [^{125}I]β_h-endorphin to the NG108-15 membranes, as can be seen in Fig. 1B. A K_d value of 1.5 ± 0.42 nM and a B_{max} value of 1077 ± 124 fmol/mg of NG108-15 membrane protein were recorded. Whereas the K_d values are essentially identical, the NG108-15 cell membranes have approximately 20% more [^{125}I]β_h-endorphin-binding sites per mg of membrane protein than do rat brain membranes.

The binding of [^{3}H]DPDPE to NG108-15 membranes was measured over the concentration range of 0.25 nM–8.0 nM. A linear Scatchard plot was also obtained for the binding of this δ-selective peptide, with an apparent K_d value of 2.3 ± 0.03 nM and a B_{max} value of 648 ± 91 fmol/mg of NG108-15 membrane protein. The B_{max} value for [^{3}H]DPDPE binding to NG108-15 membranes is about 60% of the value obtained for [^{125}I]β_h-endorphin, suggesting that there are β-endorphin-binding sites in NG108-15 membranes that are not δ opioid-binding sites.

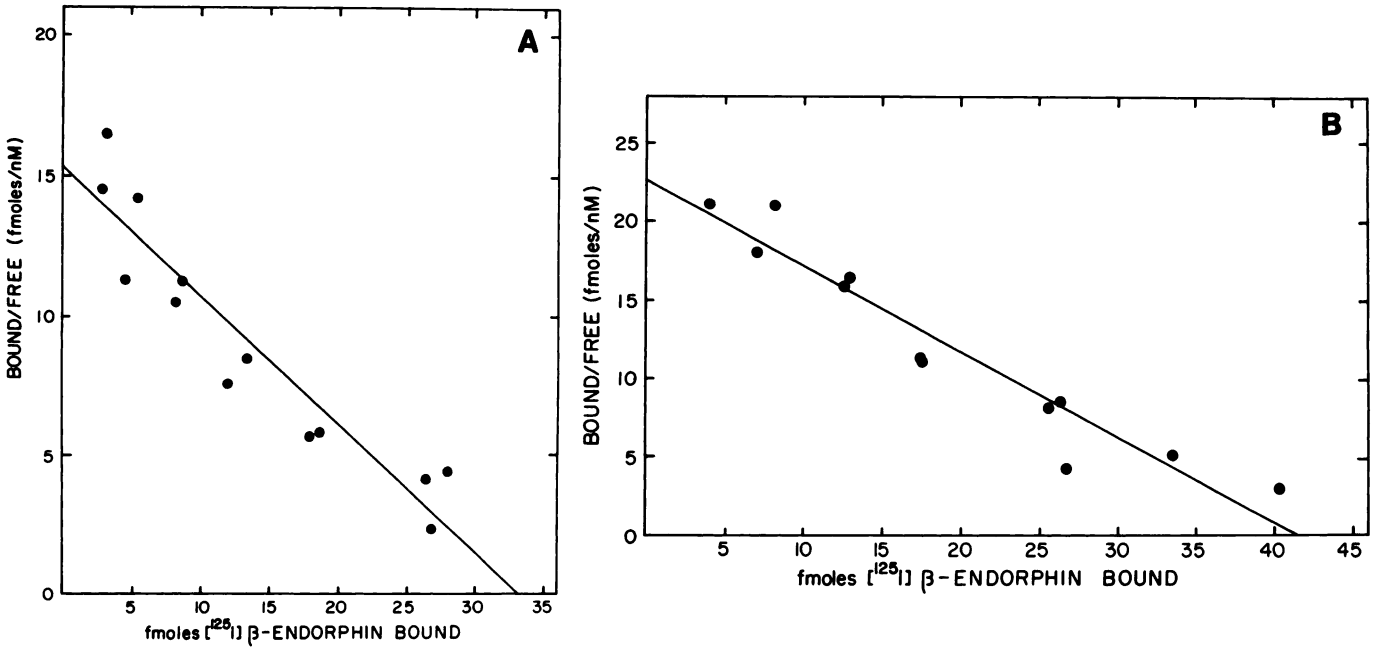

Fig. 1. Scatchard analysis of the binding of [^{125}I]β_h-endorphin to rat brain membranes and NG108-15 membranes. In a final volume of 0.5 ml, 40 μg of rat brain membrane protein (A) or NG108-15 membrane protein (B) were incubated for 60 min at 25° with 0.1–12.8 nM [^{125}I]β_h-endorphin in 50 mM Tris-HCl, pH 7.5, 0.20% BSA, and 0.01% bacitracin. Nonspecific binding was measured by the inclusion of 1 μM β_h-endorphin. Scatchard analysis of specific binding data yielded a K_d value of 2.2 nM and a B_{max} value of 33.1 fmol/40μg of membrane protein for rat brain membranes (A). A K_d value of 1.8 nM and a B_{max} value of 41.3 fmol/40 μg of membrane protein were obtained with NG108-15 membranes (B). The best fit of the experimental data was consistent with a one-site model for both rat brain and NG108-15 membranes. Each point is the average of triplicate determinations. The experiment was replicated three times with comparable results.

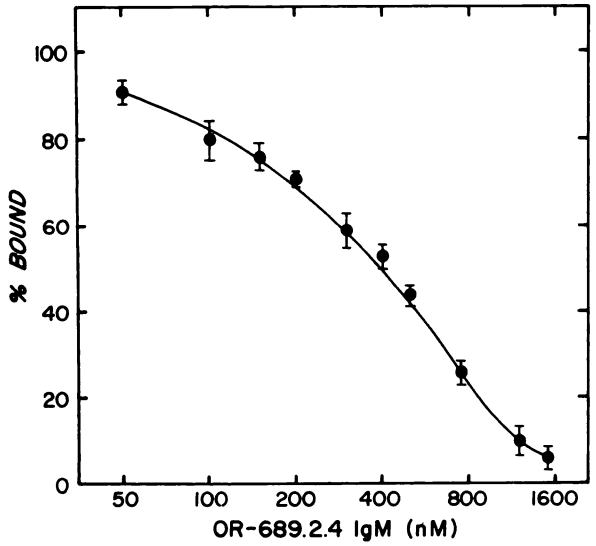

Fig. 2. Titration of OR-689.2.4 IgM in inhibiting the binding of 1 nM [^{125}I] β_h-endorphin binding to rat brain membranes. In a final volume of 0.5 ml, 40 μg of rat brain membrane protein were incubated at 25° for 60 min with varying concentrations of the OR-689.2.4 IgM in 50 mM Tris-HCl, pH 7.5, 0.20% BSA, and 0.01% bacitracin. After this preincubation, 1 nM [^{125}I]β_h-endorphin was added for an additional 60-min incubation at 25° prior to filtration. Points represent the mean percentage bound ± standard error from three separate experiments performed in triplicate.

Whether these sites are opioid or non-opioid binding sites remains to be determined. In another study examining the binding of [^{3}H-Tyr^{27}]β_h-endorphin to NG108-15 membranes, neither morphine, naloxone, nor Leu-enkephalin suppressed more than 70% of the binding (4).

Ability of OR-689.2.4 IgM to inhibit [^{125}I]β_h-endorphin binding to rat brain and NG108-15 membranes. The monoclonal antibody was analyzed for its ability to inhibit [^{125}I]β_h-endorphin binding to rat brain and NG108-15 membranes. The IgM was also tested for its ability to block [^{3}H] DPDPE binding to NG108-15 membranes. As can be seen in Fig. 2, the OR-689.2.4 IgM inhibited the binding of 1 nM [^{125}I] β_h-endorphin to rat brain membranes in a concentration-dependent manner. With a 60-min incubation of membranes with the IgM prior to the addition of [^{125}I]β_h-endorphin, an IC_{50} value of 405 ± 35 nM was obtained with the OR-689.2.4 IgM.

The monoclonal antibody also inhibited the binding of 1 nM [^{125}I]β_h-endorphin and 3 nM [^{3}H]DPDPE to NG108-15 membranes, as shown in Fig. 3. With a 60-min preincubation of membranes with the IgM, an IC_{50} value of 543 ± 60 nM was obtained for the inhibition of 1 nM [^{125}I]β_h-endorphin binding to the NG108-15 membranes. An IC_{50} value of 370 ± 45 nM was obtained for the inhibition of 3 nM [^{3}H]DPDPE by the antibody. The antibody has been previously shown to inhibit the binding of [^{3}H]DPDPE to rat brain membranes with similar affinity (18).

To determine whether the monoclonal antibody could displace bound [^{125}I]β_h-endorphin from membranes, rat brain membranes were preincubated with 1 nM [^{125}I]β_h-endorphin for 60 min prior to the addition of varying concentrations of the IgM for an additional 60-min incubation. As depicted in Fig. 4, in addition to blocking the binding of [^{125}I]β_h-endorphin to brain membranes, the OR-689.2.4 IgM also displaced bound [^{125}I]β_h-endorphin from membranes. When the IgM was incubated with membranes prior to the addition of [^{125}I]β_h-endorphin, 530 nM IgM resulted in a 50% inhibition of [^{125}I]β_h-endorphin binding to membranes. A concentration of 1600 nM IgM was necessary to displace 50% of the bound [^{125}I]β_h-endorphin from membranes.

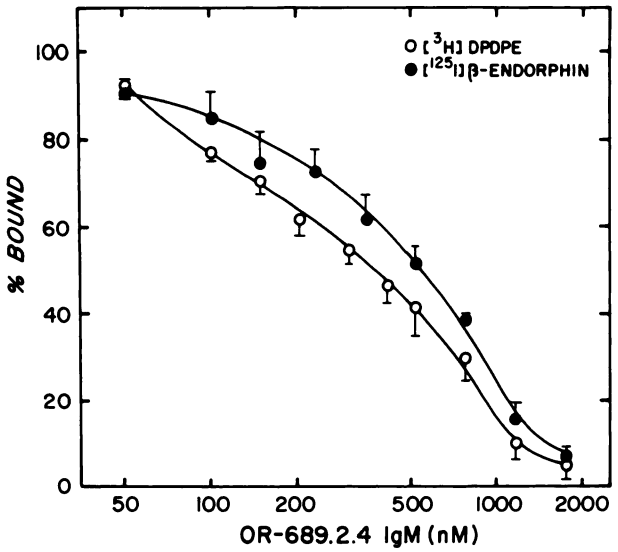

Fig. 3. Titration of OR-689.2.4 IgM in inhibiting the binding of 1 nM [^{125}I] β_h-endorphin or 3 nM [^{3}H]DPDPE to NG108-15 membranes. Varying concentrations of OR-689.2.4 IgM were incubated for 60 min at 25° with 40 μg of NG108-15 membrane protein for [^{125}I]β_h-endorphin experiments or 100 μg of membrane protein for [^{3}H]DPDPE experiments. [^{125}I]β_h-endorphin experiments were performed as described in the legend to Fig. 2. For [^{3}H]DPDPE experiments, OR-689.2.4 IgM and NG108-15 membranes were incubated at 25° for 60 min in 50 mM Tris-HCl, pH 7.5, containing 0.20% BSA and 0.01% bacitracin in a final volume of 0.5 ml. After this preincubation, 3 nM [^{3}H]DPDPE was added for an additional 3-hr incubation at 25°. Points depict the mean percentage bound ± standard error from three separate experiments performed in triplicate.

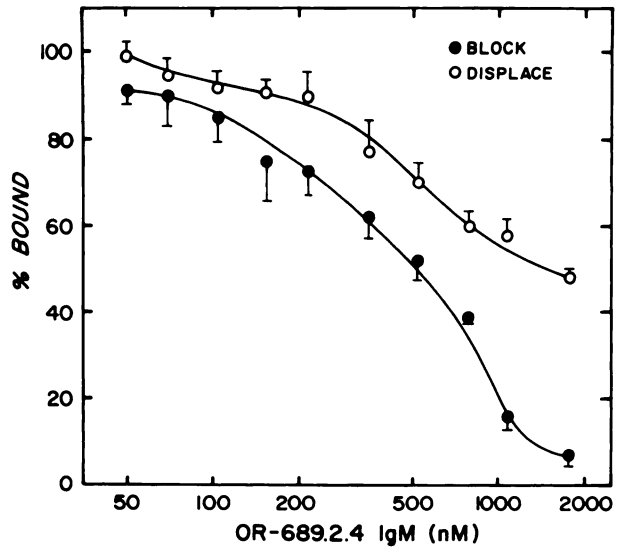

Fig. 4. Ability of OR-689.2.4 IgM to block and displace [^{125}I]β_h-endorphin from rat brain membranes. Under displacing conditions, 40 μg of rat brain membrane protein were incubated with 0.4 nM [^{125}I]β_h-endorphin in a final volume of 0.5 ml in 50 mM Tris-HCl, pH 7.5, 0.20% BSA, and 0.01% bacitracin for 60 min at 25°. Varying concentrations (50–2000 nM) of OR-689.2.4 IgM were added for an additional 60-min incubation prior to filtration. Under blocking conditions, membranes were first incubated with OR-689.2.4 IgM for 60 min. [^{125}I]β_h-Endorphin was then added for an additional 60-min incubation. Points represent the mean percentage bound ± standard error from three separate experiments performed in triplicate.

Determination of whether the OR-689.2.4 IgM is a competitive or noncompetitive inhibitor of [^{125}I]β_h-endorphin binding to rat brain and NG108-15 membranes. To determine whether the IgM was a competitive or noncompetitive inhibitor of [^{125}I]β_h-endorphin binding to rat brain and NG108-15 membranes, 400 nM OR-689.2.4 IgM was incubated with membranes for 60 min at 25°, followed by the addition of [^{125}I]β_h-endorphin at concentrations ranging from 0.01 nM to 1.6 nM. As depicted in the double reciprocal plot in Fig. 5, the OR-689.2.4 IgM was a noncompetitive inhibitor of [^{125}I]β_h-endorphin binding to rat brain and NG108-15 membranes. Measuring [^{125}I]β_h-endorphin binding to brain membranes (Fig. 5A), the control sample, without antibody, yielded an apparent K_d value of 0.80 nM and a B_{max} value of 29.8 fmol/40 μg of membrane protein. In the presence of 400 nM IgM, an apparent K_d value of 0.66 nM was obtained, whereas the B_{max} value was decreased to 10.4 fmol/40 μg of protein. With very low concentrations of [^{125}I]β_h-endorphin, 0.01–0.10 nM, the antibody acted as though it were a competitive inhibitor (data not shown). However, at concentrations of [^{125}I]β_h-endorphin greater than one-tenth the K_d value, the OR-689.2.4 IgM was a noncompetitive inhibitor. Whether the competitive inhibition seen at very low concentrations of [^{125}I]β_h-endorphin is the result of selective interaction of the antibody with the receptor or is merely the result of having a small number of bound cpm is not known. Since this observation was not seen at concentrations of [^{125}I]β_h-endorphin greater than 0.1 nM, the observation is not relevant to most of the β_h-endorphin-binding sites.

The antibody was also a noncompetitive inhibitor of [^{125}I]β_h-endorphin binding to NG108-15 membranes, as shown in Fig. 5B. In the absence of antibody, an apparent K_d value of 0.75 nM was obtained, whereas in the presence of OR-689.2.4 IgM, an apparent K_d value of 0.93 nM was obtained. The apparent K_d value did not change significantly in the presence of the antibody. However, the maximal number of binding sites was significantly reduced by the IgM. In the absence of antibody, a B_{max} value of 38.7 fmol/40 μg of protein was obtained. A B_{max} value of 20.9 fmol/40 μg of protein was obtained in the presence of 400 nM OR-689.2.4 IgM. Even at [^{125}I]β_h-endorphin concentrations below 0.1 nM, no competitive inhibition was seen with the antibody using NG108-15 membranes. Similar to the effect of sodium on the binding of agonists, the monoclonal antibody is a noncompetitive inhibitor of opioid binding to brain and NG108-15 membranes.

Determination of the ability of opioid peptides to inhibit [^{125}I]β_h-endorphin binding to rat brain and NG108-15 membranes. The μ-selective peptide DAGO, the δ-selective peptide DPDPE, and DADLE, which binds to μ and δ sites, were tested for their ability to inhibit the binding of 0.8 nM [^{125}I]β_h-endorphin to rat brain and the NG108-15 membranes, as shown in the displacement curves in Fig. 6. Both DAGO and DADLE were very potent in their ability to block [^{125}I]β_h-endorphin binding to rat brain membranes (Fig. 6A). In contrast, DPDPE was a weak inhibitor of [^{125}I]β_h-endorphin binding to rat brain, having an IC_{50} value of 4080 ± 810 nM (Fig. 6A). Nearly opposite results were obtained with the NG108-15 membranes, as is shown in Fig. 6B. DPDPE was a very potent inhibitor of 0.8 nM [^{125}I]β_h-endorphin binding to the NG108-15 membranes, having an IC_{50} value of 1.4 ± 0.16 nM. DADLE was almost as potent as DPDPE, whereas DAGO was less effective. DAGO had an IC_{50} value of 470 ± 10 nM for its

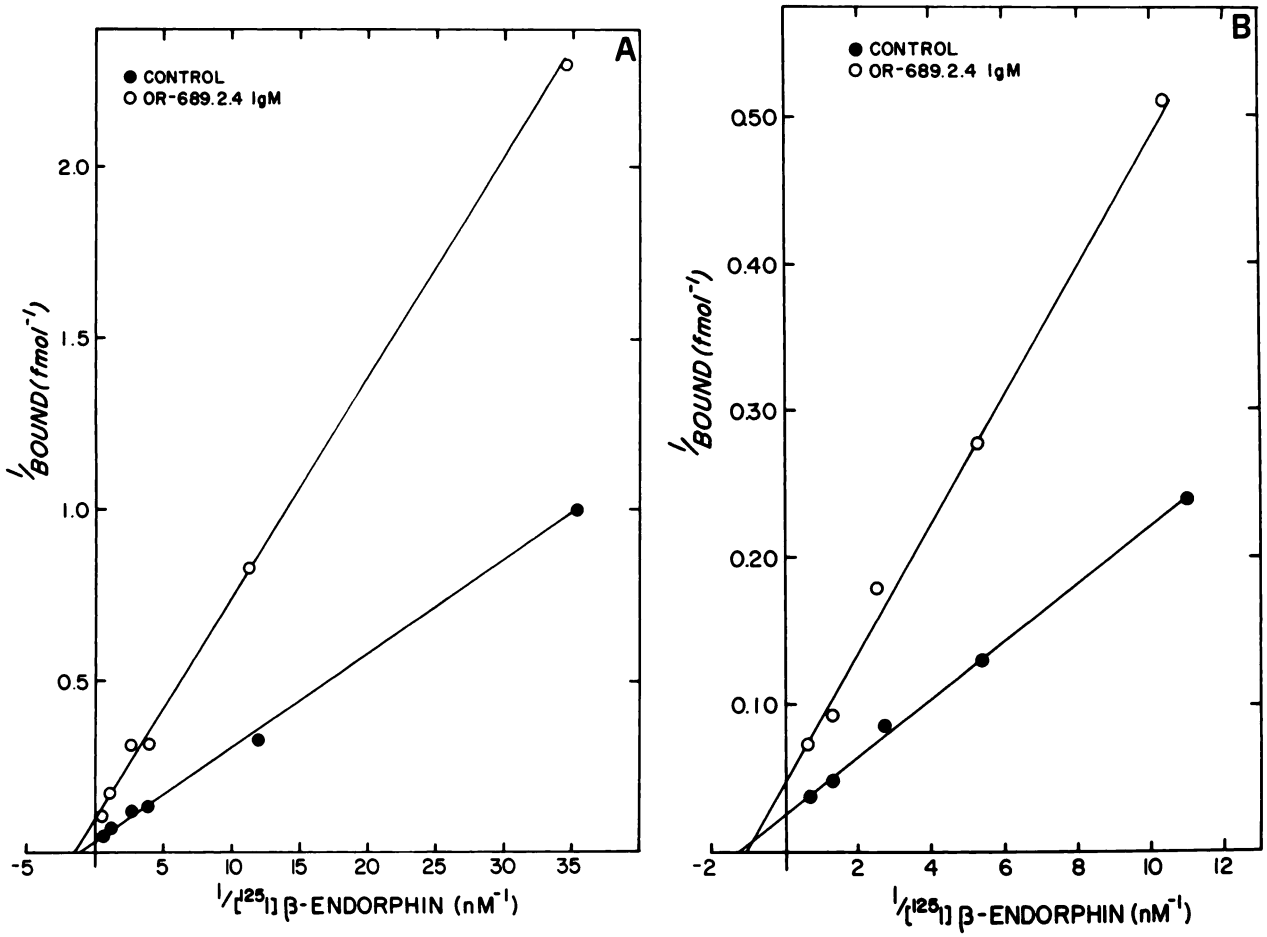

Fig. 5. Double reciprocal plot of the binding of [^{125}I]β_h-endorphin to rat brain membranes and NG108-15 membranes in the absence and presence of OR-689.2.4 IgM. Rat brain membranes (A) or NG108-15 membranes (B) were incubated for 60 min at 25° in the absence or presence of 400 nM OR-689.2.4 IgM as described in the legends to Figs. 2 and 3. [^{125}I]β_h-Endorphin (0.01–1.6 nM) was added for 60 min at 25° prior to filtration. With rat brain membranes (A), an apparent K_d value of 0.80 nM was obtained in control samples and an apparent K_d value of 0.66 nM was recorded in samples with 400 nM OR-689.2.4 IgM. The B_{max} value for the control samples was 29.8 fmol/40μg of membrane protein. In the IgM-containing samples, the B_{max} value was 10.4 fmol/40μg of protein. In NG108-15 membranes (B), an apparent K_d value of 0.75 nM was obtained in control samples and an apparent K_d value of 0.93 nM was found in samples with OR-689.2.4 IgM. The B_{max} value in the control sample was 38.7 fmol/40μg of protein and 20.9 fmol/40μg in samples containing 400 nM OR-689.2.4 IgM. The experiment was repeated five times with similar results. The OR-689.2.4 IgM was a noncompetitive inhibitor of [^{125}I]β_h-endorphin binding to rat brain and NG108-15 membranes.

inhibition of 0.8 nM [^{125}I]β_h-endorphin binding to NG108-15 membranes. Since there are 40% more β_h-endorphin-binding sites than DPDPE-binding sites in NG108-15 membranes, the inhibition seen with DAGO may suggest the presence of some μ opioid-binding sites. Alternatively, at higher DAGO concentrations, this peptide may be binding to δ sites.

Having observed the marked differences in the inhibitory effects of selective opioid peptides on [^{125}I]β_h-endorphin binding to rat brain and NG108-15 membranes, several endogenous opioid peptides were likewise examined for their ability to inhibit binding of 0.8 nM [^{125}I]β_h-endorphin to both membrane preparations. The IC_{50} values obtained for the different peptides are reported in Table 1. β_h-Endorphin 1–31 was equipotent in inhibiting the binding of 0.8 nM [^{125}I]β_h-endorphin to rat brain and NG108-15 membranes. β_h-Endorphin 1–26 and β_h-endorphin 1–27 were 3–4 times more potent in inhibiting [^{125}I]β_h-endorphin binding to the NG108-15 membranes than rat brain. The peptide γ-endorphin was 8 times more potent in the NG108-15 membranes than in the rat brain membranes. Of the endogenous peptides tested, α-neo-endorphin exhibited the greatest divergence in its ability to inhibit [^{125}I]β_h-endorphin binding to membranes. It had 15 times greater affinity for the β_h-endorphin-binding site in NG108-15 membranes than in rat brain. As shown in Fig. 6, the synthetic peptides, DAGO and DPDPE, had the greatest divergence in their ability to block [^{125}I]β_h-endorphin binding, depending on whether rat brain or NG108-15 membranes were tested.

Determination of whether opioid peptides and the monoclonal antibody bind to allosterically coupled sites or totally independent sites. Table 2 shows the ability of the opioid peptides and the OR-689.2.4 monoclonal antibody to inhibit 0.8 nM [^{125}I]β_h-endorphin binding to rat brain membranes. Samples were incubated with opioid peptides alone, the monoclonal antibody alone, or opioid peptides in combination with the monoclonal antibody. With a 60-min preincubation, 200 nM OR-689.2.4 IgM inhibited the binding of 0.8 nM [^{125}I]β_h-endorphin to rat brain membranes by 29%. A percentage inhibition was recorded for each opioid peptide tested alone against 0.8 nM [^{125}I]β_h-endorphin. If the antibody and opioid peptides were acting at two totally independent sites, one would expect an additive effect when the peptide and antibody were tested together. However, if they were acting at allosterically

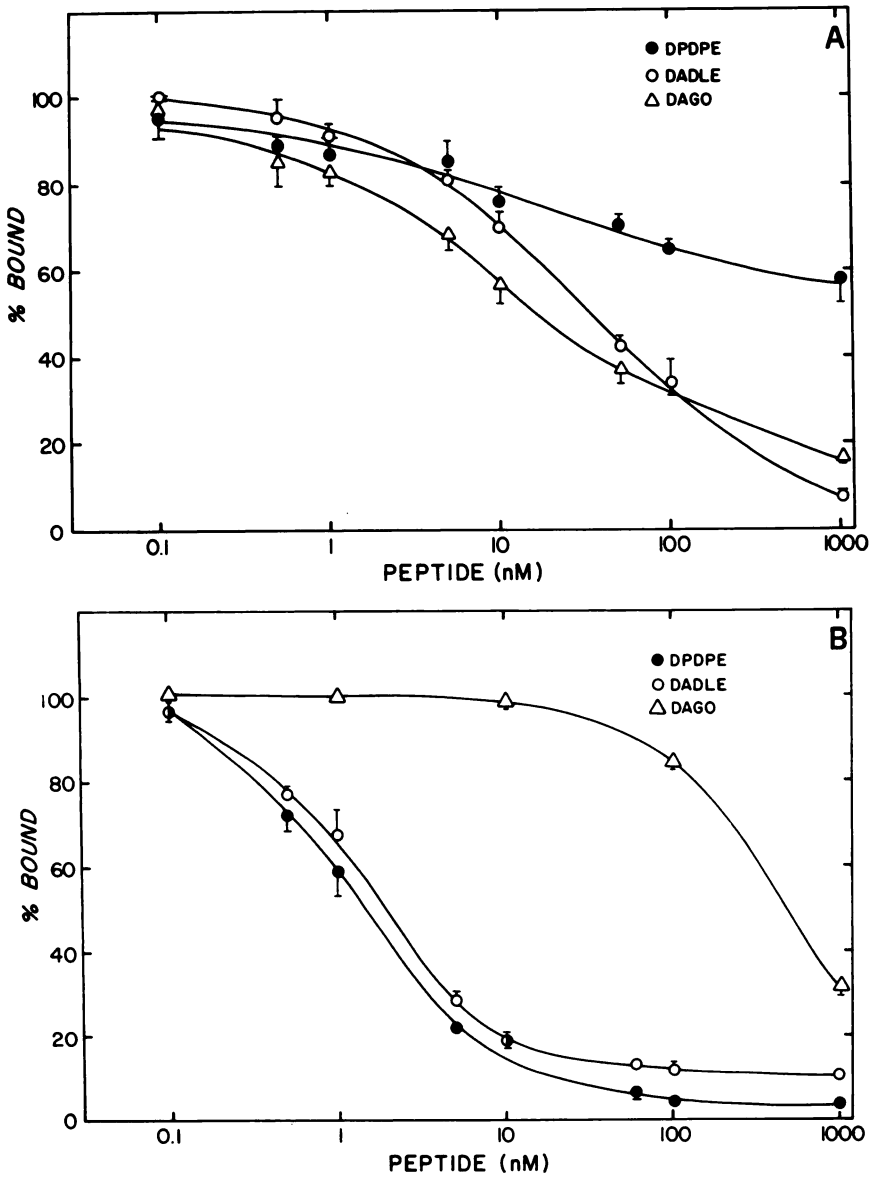

Fig. 6. Competition of DAGO, DPDPE, and DADLE for the binding of 0.8 nM [^{125}I]β_h-endorphin to rat brain (A) and NG108-15 membranes (B). Rat brain or NG108-15 membrane protein at a protein concentration of 80μg/ml was incubated for 60 min at 25° with varying concentrations of DAGO, DPDPE, and DADLE in 50 mM Tris-HCl, pH 7.5, 0.20% BSA, and 0.01% bacitracin. After this preincubation, 0.8 nM [^{125}I] β_h-endorphin was incubated with the membranes for 60 min at 25°. Nonspecific binding was measured by the inclusion of 1 μM β_h-endorphin. Points depict the mean percentage bound ± standard error from three separate experiments performed in triplicate.

TABLE 1

Competition of opioid peptides for [^{125}I]β_h-endorphin-binding sites on rat brain and NG108-15 membranes

Rat brain membrane protein or NG108-15 membrane protein, 40 μg, was incubated with varying concentrations (0.1 nM–100,000 nM) of the opioid peptides for 60 min prior to the addition of 0.8 nM [^{125}I]β_h-endorphin to a final volume of 0.5 ml in 50 mM Tris-HCl, pH 7.5, 0.20% BSA, and 0.01% bacitracin. After a 60-min incubation at 25°, the samples were filtered through Whatman GF/B glass fiber filters that had been soaked in 0.25% polyethylenimine. Data are presented as the mean IC_{50} values ± standard error obtained from three separate experiments, performed in triplicate.

Peptide	IC_{50} values	
	Rat brain	NG108-15
	nM	
β_h-Endorphin 1-31	1.1 ± 0.18	1.1 ± 0.26
β_h-Endorphin 1-27	6.2 ± 0.20	1.8 ± 0.11
β_h-Endorphin 1-26	7.7 ± 1.4	1.9 ± 0.44
α-Neo-Endorphin	30.0 ± 4.0	2.1 ± 0.25
γ-Endorphin	64.0 ± 4.9	7.8 ± 1.6
DAGO	16.3 ± 2.4	470.0 ± 10
DADLE	34.3 ± 5.7	2.0 ± 0.36
DPDPE	4080.0 ± 810	1.4 ± 0.16

coupled sites, a percentage inhibition that is less than the additive effect would be anticipated. For example, 200 nM OR-689.2.4 IgM inhibited the binding of [^{125}I]β_h-endorphin by 29% and 5 nM DAGO inhibited the binding by 43%. If the peptide and IgM were acting at two independent sites, when they were combined, an inhibition of 72% would be expected. However, if they were acting at allosterically coupled sites, an inhibition of 61% would be anticipated. As seen in Table 2, DAGO and the other opioid peptides appeared to be binding to sites that are allosterically coupled with the binding site for the monoclonal antibody. When DAGO and DPDPE were tested together, an additive effect was observed, indicative of each peptide binding at a site that did not conformationally alter the other site.

Similar results were obtained when the monoclonal antibody and opioid peptides were tested for their ability to inhibit 0.8 nM [^{125}I]β_h-endorphin binding to NG108-15 membranes as, shown in Table 3. The inhibition seen with the combination of IgM and opioid peptides indicated that the opioid peptides and the IgM were acting at allosterically coupled sites in the NG108-

TABLE 2

Ability of opioid peptides and OR-689.2.4 IgM to inhibit the binding of 0.8 nM [^{125}I]β_h-endorphin to rat brain membranes

Rat brain membrane protein, 40 μg, was incubated with the opioid peptides for 30 min at 25° in a final volume of 0.5 ml. Then, 200 nM OR-689.2.4 IgM was incubated with the membranes for 60 min prior to the addition of 0.8 nM [^{125}I]β_h-endorphin for an additional 60-min incubation. Data are presented as the mean percentage inhibition ± standard error from at least three separate experiments.

	% Inhibition		
	Peptide alone	Peptide + OR-689.2.4 IgM	Expected value if allosterically coupled sites
200 nM OR-689.2.4 IgM alone	29 ± 4		
5 nM DAGO	43 ± 4	57 ± 2	61 ± 3
300 nM DPDPE	34 ± 7	53 ± 4	54 ± 4
5 nM DADLE	32 ± 4	53 ± 6	50 ± 5
40 nM γ-Endorphin	46 ± 2	54 ± 5	60 ± 2
3 nM α-Neo-Endorphin	32 ± 4	47 ± 1	48 ± 4
2 nM β_h-Endorphin 1-27	42 ± 1	56 ± 1	59 ± 1
5 nM DAGO + 300 nM DPDPE	72 ± 6		56 ± 3

TABLE 3

Ability of opioid peptides and OR-689.2.4 IgM to inhibit the binding of 0.8 nM [^{125}I]β_h-endorphin to NG108-15 membranes

NG108-15 membrane protein, 40 μg, was incubated with the opioid peptides for 30 min at 25° in a final volume of 0.5 ml. Subsequently, 400 nM OR-689.2.4 IgM was incubated with the membranes for 60 min prior to the addition of 0.8 nM [^{125}I] β_h-endorphin for an additional 60-min incubation. Data are presented as the mean percentage inhibition ± standard error from at least three separate experiments.

	% Inhibition		
	Peptide alone	Peptide + OR-689.2.4 IgM	Expected value if allosterically coupled sites
400 nM OR-689.2.4 alone	35 ± 3		
1 nM DPDPE	41 ± 3	61 ± 1	59 ± 1
0.6 nM DADLE	39 ± 4	60 ± 3	58 ± 2
4 nM γ-Endorphin	43 ± 3	62 ± 2	61 ± 5
0.4 nM α-Neo-Endorphin	44 ± 2	56 ± 1	62 ± 3
1 nM β_h-Endorphin 1-26	42 ± 4	62 ± 2	63 ± 2
1 nM β_h-Endorphin 1-27	42 ± 3	54 ± 3	60 ± 2

15 membranes as well as rat brain. These studies suggest that β_h-endorphin binds to μ and δ opioid receptors and that these receptors share some sequence homology that is recognized by the monoclonal antibody.

Discussion

The monoclonal antibody blocked and displaced [^{125}I]β_h-endorphin binding to both rat brain and NG108-15 membranes in a manner similar to that previously exhibited for the binding of [^{3}H]DAGO and [^{3}H]DPDPE to rat brain membranes (18). Also, the ability of the antibody to inhibit [^{3}H]DPDPE binding to NG108-15 membranes and rat brain was comparable (18). As seen with the μ and δ ligands, the antibody was a noncompetitive inhibitor of [^{125}I]β_h-endorphin binding to both rat brain and NG108-15 membranes. Like sodium, the antibody seems to be binding to a site on the receptor, distinct from the ligand-binding site. The binding of the antibody to the receptor probably results in a conformational change in the receptor, rendering it unable to bind opioids. The IgM and Fab fragments from the IgM have been shown to inhibit binding to μ and δ sites in rat and guinea pig brain membranes about equally well (18). However, the antibody is ineffective at blocking binding to κ sites in rat brain or guinea pig cerebellum (18). Since β_h-endorphin has been shown to bind to μ and δ sites with about equal affinity, one would expect that an antibody capable of inhibiting binding to μ and δ sites would probably be able to inhibit the binding of β_h-endorphin. The data presented here support this conclusion, suggesting that μ and δ opioid receptors share sequence homology with the 35,000-dalton protein that the antibody is directed against, as a common structural component. The OR-689.2.4 IgM has been shown to recognize only nondenatured receptor (15). As a consequence, it has not been possible to separate neural membrane protein by electrophoresis in the presence of sodium dodecyl sulfate, transblot them to nitrocellulose, and look for reactivity of the monoclonal antibody with all membrane proteins. Therefore, it has not been feasible to determine whether this 35,000-dalton protein is a proteolytic fragment from a larger protein or whether the antigenic determinants recognized by the OR-689.2.4 IgM are also common to other proteins.

Additional support for the concept of shared structural homology between μ and δ opioid receptors comes from crosslinking studies using [^{125}I]β_h-endorphin. Such studies using [^{125}I] β_h-endorphin crosslinked to membranes have found multiple proteins specifically labeled (10, 11, 24–26). In guinea pig whole brain, proteins with molecular weights of 65,000, 53,000, 41,000, and 38,000 were specifically crosslinked with [^{125}I]β_h-endorphin, such that the incorporation of [^{125}I]β_h-endorphin into these four proteins was completely abolished by the inclusion of 1 μM bremazocine (10, 24). The labeling of the 65,000-dalton protein was reduced with DAGO but not with the δ-selective peptide, DPDPE, whereas the labeling of the 53,000-dalton protein was reduced with DPDPE and not DAGO. The labeling of the 41,000- and 38,000-dalton proteins was specifically reduced with both DAGO and DPDPE (10, 24). These data suggest that there are proteins that are common to both μ and δ opioid receptors. Crosslinking studies using human caudate and putamen showed proteins with molecular weights of 68,000, 40,000, 30,000, and 25,000 specifically crosslinked with [^{125}I-monoiodo-Tyr^{27},Leu^5]-β_h-endorphin (11). The labeling of all these proteins was eliminated by the inclusion of 1 μM morphiceptin or 1 μM naloxone. Similar studies using rat brain membranes have found specifically crosslinked proteins with molecular weights of 67,000, 58,000, 45,000, and 33,000 (26). Crosslinking studies have recently been performed using the NG108-15 cells, which express only δ opioid receptors, and the human neuroblastoma cell line SK-N-SH, which expresses only μ opioid receptors (10, 11, 24, 25). Specifically crosslinked proteins in NG108-15 membranes had molecular weights of 92,000, 56,000, 38,000, and 23,000 (11). Another study found proteins labeled in the NG108-15 membranes with molecular weights of 53,000 and 25,000 (10, 25). Studies using the SK-N-SH cells have found 64,000-, 25,000-, and 18,000-dalton pro-

teins crosslinked with [^{125}I]β_h-endorphin (11) and proteins with molecular weights of 92,000, 65,000, and 25,000 specifically labeled (25). The labeling of the 25,000-dalton protein from the NG108-15 cells and SK-N-SH cells was eliminated with both DAGO and DPDPE (25). Although there is not a clear consensus as to which [^{125}I]β_h-endorphin-crosslinked proteins are opioid receptor related, proteins have been specifically crosslinked with [^{125}I]β_h-endorphin. The labeling of some of these proteins is eliminated by the inclusion of either the μ-selective peptide, DAGO, or the δ-selective peptide, DPDPE. These experiments suggest that μ and δ opioid receptors share some structural homology.

The possibility that μ and δ opioid receptors share some common sequence homology is not surprising when one looks at the sequence homology of receptors that have been cloned. For example, the β-adrenergic receptor (27) and rhodopsin (28) display 22% sequence identity. The M_1 muscarinic acetylcholine receptor shares 30% sequence identity with the β-adrenergic receptor and 23% identity with rhodopsin (29). The progesterone receptor (30) shares extensive sequence homology with the glucocorticoid (31) and estrogen receptors (32) and the *v-erbA* oncogene protein (33). The complementary DNA sequence of the human insulin receptor (34) has greater than 20% sequence homology with the epidermal growth factor receptor (35) and products of the *src* family of oncogenes. In view of the large shared sequence homology among quite different receptors, it would probably be more surprising if μ and δ opioid receptors do not share any common sequences than if they do. The previous experiments (16, 18) and the data presented here support the concept that the 35,000-dalton protein or a fragment of this protein is common to both μ and δ opioid receptors. The definitive answer will come with the cloning of these two types of opioid receptor.

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