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Identification of Endogenous Opioid Receptor Components in Rat Brain Using a Monoclonal Antibody

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SUMMARY

A monoclonal antibody generated against the tertiary structure of a partially purified opioid binding protein was used to probe the structure of the dynorphin and β-endorphin receptors. The Fab fragment 3B4F11 inhibited completely the binding of ^{125}I-β-endorphin and [^{3}H]dynorphin to rat brain P_2 membranes with IC_{50} values of 26 ng/ml and 40 ng/ml, respectively. To explore further the interaction of 3B4F11 with the β-endorphin receptor, the effect of the Fab fragment on ^{125}I-β-endorphin cross-linking to rat brain membranes was examined. ^{125}I-β-endorphin was covalently bound to three major species of approximate molecular weights 108,000, 73,000, and 49,000. The δ-selective ligand D-Pen^2, D-pen^{5}enkephalin was least effective at inhibiting the cross-linking of β-endorphin, whereas the μ-selective ligand Tyr-D-Ala-Gly-*N*Me-Phe-Gly-ol and κ-selective ligand U50488 inhibited β-endorphin cross-linking to the 108,000 and 73,000 Da species. Both 3B4F11 and β-endorphin prevented the covalent binding of ^{125}I-β-endorphin to all three labeled species. These findings suggest that μ and κ receptor types might have some structural similarities, whereas the δ receptor type might differ in molecular size. In addition, the μ, κ, and δ ligands might have different primary sequences, whereas their tertiary structures might share regions of molecular homology with all three receptor constituents labeled by ^{125}I-β-endorphin. 3B4F11 will be a valuable tool for the purification and isolation of the several components of the β-endorphin receptor complex.

The existence of multiple opioid receptor types is supported by considerable pharmacological and biochemical evidence (1, 2). At least three receptor types can be distinguished, as follows: μ preferring morphine and similar ligands; δ, preferring enkephalins; and κ, interacting with benzomorphans. In an attempt to elucidate the functional significance of each of these types, some researchers have suggested that each is specific for a particular endogenous ligand, namely, β-endorphin for μ receptors, enkephalins for δ receptors, and dynorphin for κ receptors.

However, the relationship between endogenous opioids and opioid receptor types is complex. β-Endorphin actually binds to μ receptors with about equal affinity as that for δ receptors (2). Enkephalins, although preferring δ receptors, also have appreciable affinity for μ receptors, especially receptors of the μ_1 subtype (3). In addition, dynorphin has significant affinity for μ and δ, as well as κ binding sites (4, 5). Dynorphin also has unique pharmacological properties, quite distinguishable from those of κ or other opioid agonists (6, 7).

The relationship between different receptor types is equally complex and poorly understood because the different receptor subtypes have not been isolated and characterized. One strategy used to isolate opioid receptors has been to covalently crosslink a relatively nonselective endogenous opioid, β-endorphin, to its receptor in brain membranes (8, 9). Potential binding site proteins of several different molecular weights have been identified and the species detected differ with the tissue examined, correlating with the proportions of μ, δ, and κ receptor types present. Also, cross-linking of β-endorphin to certain proteins was inhibited differentially by pretreating the membranes with selective opioid ligands, suggesting that the different opioid receptor types do not have an identical structure (10).

Monoclonal antibodies have also been used to probe the structure of a variety of receptors and recently this strategy has been applied to study the structure of opioid receptors. Monoclonal antibodies have been raised to partially purified opioid receptor preparations (11, 12). The Fab fragments from these antibodies inhibit the binding of opioid ligands to rat brain membranes and can precipitate opioid binding sites from solubilized membrane preparations. The structure of the binding sites for the endogenous opioid ligands has not yet been probed with these antibodies.

The present study uses the monoclonal antibody generated

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ABBREVIATIONS: DPDPE, [D-Pen^2, D-pen^5]enkephalin; BSOCOES, bis [2-succinimidooxycarboxyloxyOethyl] sulfone; DAGO, Tyr-D-Ala-Gly-NM$_e$-Phe-Gly-ol; DMSO, dimethylsulfoxide; DTSSP, 3,3′ dithiobis (sulfosoccinimidylpropionate); EKC, ethylketocyclazocine; MBS, *m*-maleimidobenzoyl-*N*-hydroxy-succinimide ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

against the tertiary structure of the opioid binding protein in our laboratory (11) to 1) examine the relationship of endogenous ligands to their binding site and 2) probe the structure of different opioid receptor types. The antibody specifically inhibits its binding of opioid ligands but does not differentiate among the opioid receptor types. First, we determine whether the monoclonal antibody inhibits the binding of β-endorphin and dynorphin to rat brain membranes and, second, we test the ability of the antibody to prevent the crosslinking of β-endorphin to membrane proteins.

Experimental Procedures

Materials. Male Sprague-Dawley rats (200–250 g) were obtained from Bantin and Kingman (Freemont, CA). ^{125}I-27-β-endorphin (2000 Ci/mmol) was obtained from Amersham Corp. (Arlington, Heights, IL) and [^{3}H]dynorphin (20 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The unlabeled opioid ligands used were β-endorphin, dynorphin, DPDPE, DAGO (all from Peninsula Laboratories, Inc., San Carlos, CA), U50488H (Upjohn Co. Kalamazoo MI), etorphine (National Institute on Drug Abuse, Bethesda, MD), and EKC (Sterling-Winthrop Research Institute, Albany, NY). All electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, CA) except for pre-stained molecular weight standards, which were from Sigma Chemical Corp. (St. Louis, MO). KCl was purchased from Fisher Scientific (Pittsburgh, PA) and all other reagents including bovine serum albumin, HEPES, bacitracin, and NaCl were from Sigma. Crosslinking reagents were purchased from Pierce Chemical Company (Rockford, IL): MBS, DTSSP, and BSOCOES. Autoradiography supplies were purchased from Merry X-Ray (San Francisco, CA).

Generation of monoclonal antibodies to the purified opioid receptor. The opioid binding protein was purified from bovine brain as described by Cho *et al.* (13). Female BALB/C mice, age 4–6 weeks, were immunized with 3 μg per injection of partially purified opioid binding protein in complete Freund's Adjuvant. Booster doses were given subcutaneously every week for 3 weeks in incomplete adjuvant. The titer and specificity of the immune response were determined using a modified enzyme-linked immunosorbent assay (14). Spleen cells from mice that showed high immunoreactivity against the purified protein were fused with the NS-1 myeloma cell line, according to the procedures described by Galfre and Milstein (15). Culture supernatants from wells with positive growth were initially screened for their ability to bind the purified μ opioid receptor using the enzyme-linked immunosorbent assay technique. Positive antibody secreting hybridomas were cloned by limiting dilution and expanded. Ascites fluid was generated by injecting 5×10^6 hybridoma cells per pristane-primed BALB/C mice. The immunoglobulins from the ascites or from culture supernatant were purified by 18% Na_2SO_4 precipitation, followed by DEAE-Sephadex column chromatography. The purified immunoglobulins were then used in opioid binding assays.

Preparation of Fab fragments. The isotypic characterization of the monoclonal antibody was carried out using a sandwich enzyme-linked immunosorbent assay technique. To prepare Fab fragments, the immunoglobulin was adjusted to a final concentration of 20 mg/ml in phosphate buffer 0.05M. Cysteine and EDTA were added to a final concentration of 0.01 M and 0.002 M, respectively. The solution was incubated at 37° for 4 h with 0.2 mg of papain. The digest was dialyzed against 0.005 M phosphate buffer (pH 8). The dialysate was applied onto a DEAE-Sephadex column and fractionated with a linear gradient of increasing ionic strength of phosphate buffer (0.005 M to 0.3 M, pH 8.0, phosphate). The peak corresponding to the Fab fragments was pooled and concentrated on an Amicon Corp. (Lexington, MA) PM-30 membrane. The purity of the Fab fragment was determined on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis after reduction with 5.0 mM β-mercaptoethanol.

Preparation of rat brain or liver membranes. P_2 brain membranes were prepared according to the method of Cho *et al.* (13). The whole brain, minus cerebellum, was the source of the membrane. Protein determinations were made using the method of Lowry *et al.* (16). Liver was washed thoroughly in 0.32 M sucrose, 25 mM HEPES (pH 7.4) to remove blood, and membranes were prepared in the same manner as brain membranes.

^{125}I-β-Endorphin binding assay. HEPES buffer (25 mM, pH 7.4), bacitracin (0.005%), rat P_2 membrane (0.58 mg of protein), and the Fab fragment 3B4F11 (7.8–500 ng of protein) were incubated in a final volume of 1 ml in polypropylene tubes for 1 hr at 25°. Control samples were incubated with the same amounts of a Fab fragment generated from a nonspecific hybridoma cell line. ^{125}I-β-endorphin (1.0 nM) was added and the tubes were incubated for another hour at 25°. In addition, unlabeled β-endorphin (1 μM) was added to some tubes to determine nonspecific binding. The reaction mixture was filtered under vacuum through Whatman GF-B filters and the filters were rinsed three times with 0.005% bacitracin and 0.4% bovine serum albumin in ice cold HEPES buffer (25 mM). To reduce sticking of β-endorphin to the filters, the filters were soaked for 30 min before use in 0.01% polylysine, 0.4% bovine serum albumin, and 0.005% bacitracin in 25 mM HEPES buffer. The filters were counted in a γ-counter.

[^{3}H]Dynorphin binding assay. The dynorphin binding assay was a modification of the method of Young *et al.* (17). To remove endogenous dynorphin from the brain tissue, the P_2 membranes were incubated at 37° for 30 min, centrifuged at 14,000 rpm for 20 min, and resuspended in 0.2% bovine serum albumin, 25 mM HEPES, pH 7.4. The washed P_2 rat membrane (0.58 mg), 0.005% bacitracin, 25 mM HEPES buffer, and Fab fragment 3B4F11 (7.8–500 ng of protein) were incubated in polypropylene tubes in a final volume of 500 μl for 1 hr at 25°. [^{3}H]Dynorphin (3 nM) was added and the samples were incubated on ice for another 90 min. Unlabeled dynorphin (1 μM) was added to some tubes to determine nonspecific binding. Samples were filtered through Whatman GF-B filters presoaked in 0.1% bovine serum albumin, 0.1% polylysine, and 100 mM choline chloride in 25 mM HEPES buffer. The filters were washed three times with ice-cold HEPES buffer (25 mM) containing 0.4% bovine serum albumin and 0.1% polylysine.

^{125}I-β-Endorphin cross-linking. ^{125}I-β-Endorphin (2 nM), 0.005% bacitracin, HEPES buffer (25 mM, pH 7.4), KCl (100 mM), P_2 membrane (0.29 mg), plus unlabeled ligands were incubated in a final volume of 500 μl in polypropylene microfuge tubes at 25°. When the effect of Fab 3B4F11 was tested, the samples were preincubated with Fab 3B4F11 or control Fab for 1 hr at 25° before the addition of ^{125}I-β-endorphin. The tubes were then centrifuged for 5 min in the cold in a Fisher microfuge and the supernatant was decanted. The pellet was washed briefly with 1 ml of 0.005% bacitracin in 25 mM HEPES buffer and centrifuged again and the supernatant was decanted. The pellet was resuspended in 900 μl of 0.005% bacitracin in 25 mM HEPES buffer, 100 μl of cross-linking reagent (1 mM) or vehicle was added, and the mixture was incubated for 15 min on ice. BSOCOES and DTSSP were dissolved in DMSO, whereas MBS was dissolved in distilled water. The tubes were centrifuged for 5 min in the cold to terminate the reaction. The pellets were resuspended in 50 mM NaCl and shaken for 30 min at 25° to remove ^{125}I-β-endorphin that was not covalently cross-linked to the membrane. After a final centrifugation for 5 min, the pellets were either solubilized in treatment buffer (0.125 M Tris, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol) or the pellets were sliced off and counted in a γ counter.

Polyacrylamide gel electrophoresis. The cross-linked samples were examined by polyacrylamide gel electrophoresis under denaturing conditions using a 10% polyacrylamide running gel and a 4% polyacrylamide stacking gel according to the method of Laemmli (18). The same amount of protein was applied to each lane in a single experiment and varied from 43.5 to 72.5 μg between experiments. Molecular weights were determined by generating a plot of log M_r versus R_F using Sigma prestained molecular weight standards: α_2 macroglobulin (M_r = 180,000), β-galactosidase (M_r = 116,000), fructose-6-phosphate kinase (M_r = 84,000), pyruvate kinase (M_r = 58,000), fumarase (M_r = 48,500),

and lactic dehydrogenase (M_r = 36,500). The gels were dried using a Hoefer Scientific Instruments San Francisco, CA slab gel dryer (Drygel Jr.) and exposed to Eastman Kodak Co. (Rochester, New York) X-Omat AF film with intensifying screens (Cronex Lightening Plus) for 2 weeks at −80°C.

Data Analysis. The data for the binding assays are presented as the mean and standard error of three experiments performed in triplicate. The autoradiographs were analyzed using a Joyce-Loebl scanning densitometer (Ephortec) with Chromoscan 3.Rev5.1 software at a wavelength of 626 nm. The integral of each ^{125}I-labeled band was used to determine the per cent inhibition in band intensity induced by opioid ligands or Fab.

Results

Effect of 3B4F11 on the binding of endogenous opioid peptides to rat brain P_2 membrane. The Fab fragment 3B4F11 inhibited the binding of both ^{125}I-β-endorphin (1 nM) and [^{3}H]dynorphin (3 nM) to rat brain P_2 membranes (Fig. 1) with IC_{50} values of 40 ng/ml and 26 ng/ml, respectively. Some inhibition of the binding of both ligands was observed with the control Fab fragment, but maximum inhibition was only 19% for dynorphin and 38% for β-endorphin.

The β-endorphin binding assay was also performed with varying amounts of ^{125}I-β-endorphin (0.1–10 nM) in the presence of 50 ng of 3B4F11 or control Fab. The K_d and V_{max} for ^{125}I-β-endorphin binding in the presence of 3B4F11 were 5.7 nM and 352 fmol/mg, respectively, and 6.6 nM and 690 fmol/mg in the presence of control Fab.

Cross-linking of ^{125}I-β-endorphin to rat brain membrane. Preliminary experiments were performed to optimize conditions necessary for covalent labeling of the β-endorphin receptor with ^{125}I-β-endorphin. We found that 1) K^+ in the incubation medium is essential for the reduction of nonspecific cross-linking of β-endorphin and 2) the final NaCl wash is necessary to remove free ^{125}I-β-endorphin that has not been covalently bound. Of the three cross-linking reagents tested, BSOCOES produced the most efficient cross-linking. DTSSP produced a smear of radiolabeled bands along the entire molecular weight range examined, whereas MBS did not produce any clearly labeled bands (data not shown). In addition, varying the concentration of BSOCOES from 0.5 to 1.5 mM did not significantly alter the intensity or number of labeled bands.

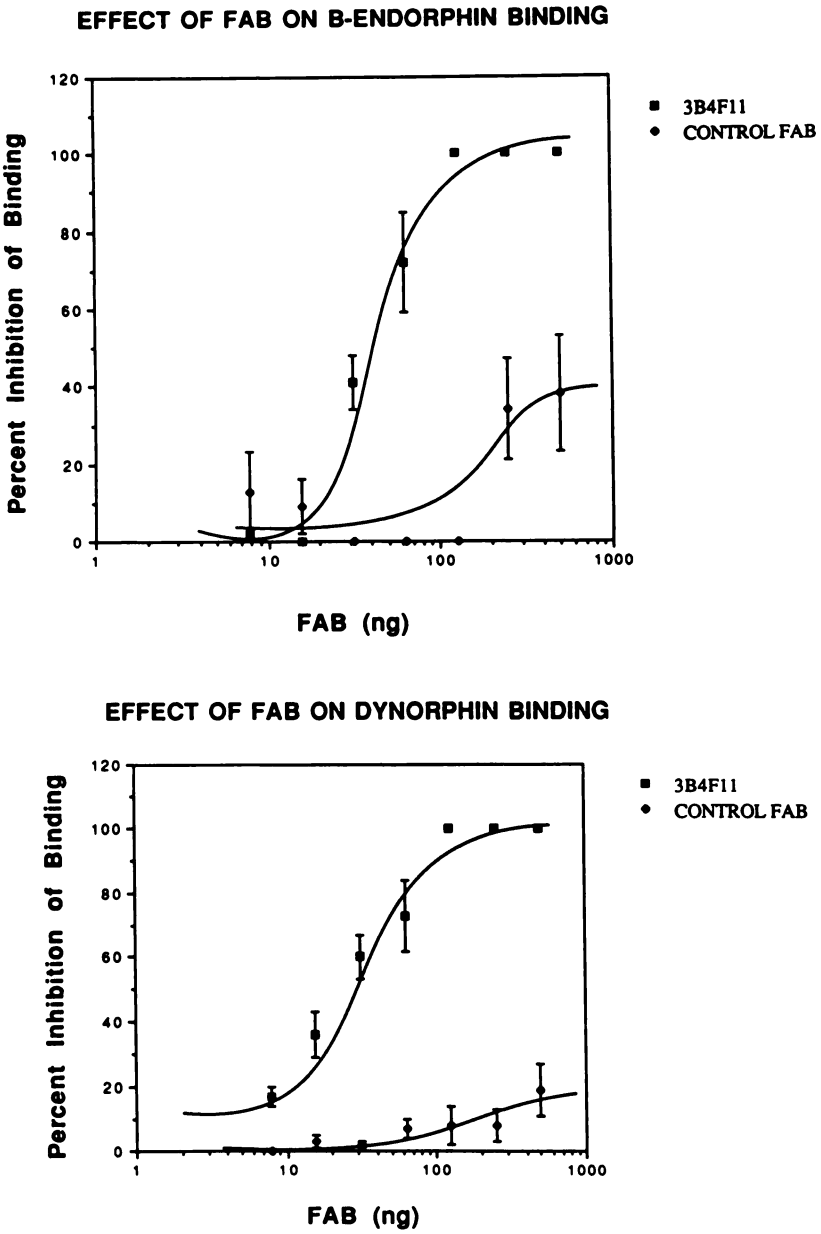

Fig. 1. Rat brain P_2 membranes (0.58 mg of protein) were incubated with 7.8–500 ng of protein of Fab 3B4F11 (□) or control Fab (♦) for 1 hr before the addition of ^{125}I-β-endorphin (1.0 nM) (*top*) or [^{3}H]dynorphin (3.0 nM) (*bottom*). Specific binding was determined in the presence of 1 μM β-endorphin and 1 μM dynorphin, respectively. Per cent inhibition of specific binding was calculated by dividing the specific binding at each Fab concentration by the specific binding determined in the absence of Fab. The data are presented as the mean ± standard error of three experiments performed in triplicate.

^{125}I-β-Endorphin was incubated with brain membranes, then cross-linked with 1 mM BSOCOES as detailed in *Experimental Procedures*. ^{125}I-β-Endorphin was cross-linked to three major species of approximate molecular weights 108,000; 73,000, and 49,000 (Fig. 2, *lanes 2* and *1A*). No cross-linking was observed when the samples were incubated in the presence of DMSO only (Fig. 2, *lanes 1* and *2A*). In addition, no higher molecular weight bands were observed when ^{125}I-β-endorphin alone was run on the gel (Fig. 2, *lane 3A*) or when the reaction supernatant, containing BSOCOES and free ^{125}I-β-endorphin, was tested (data not shown).

All three labeled species appear to be associated with the β-endorphin receptor because the addition of β-endorphin to the binding assay inhibited cross-linking (Fig. 3, *lane 3*). The covalent linkage of β-endorphin to the 108,000- and 73,000-Da bands was inhibited by up to 96%. In contrast, no specific covalent labeling was detected in rat liver (data not shown). The lack of β-endorphin cross-linking corresponds with the finding that little ^{125}I-β-endorphin-specific binding was detected in rat liver (5 fmol/mg of protein) compared with rat brain (711 fmol/mg of protein).

Table 1 summarizes the maximal effects of different opioid ligands on the cross-linking of β-endorphin to P_2 rat brain membrane. β-Endorphin produced the greatest inhibition of cross-linking to all three labeled species, whereas DPDPE was the least effective inhibitor. All of the ligands tested, except DPDPE, inhibited the labeling of the 108,000-Da and 73,000-Da bands more than the 49,000-Da band.

Fig. 3 shows a typical gel and scan of the effect of Fab 3B4F11 and control Fab on the cross-linking of β-endorphin to rat brain membrane. Fab 3B4F11 inhibited the cross-linking of β-endorphin to all three bands in a dose-dependent manner, whereas the control Fab did not affect the cross-linking. The scanning densitometer data from three averaged dose-response curves are summarized in Table 2. Like β-endorphin, Fab 3B4F11 inhibited ^{125}I-β-endorphin cross-linking to the 108,000-Da and 73,000-Da species more effectively than to the 49,000-Da band.

Discussion

The present study demonstrates that the antibody raised to a purified opioid binding protein in our laboratory 1) inhibits the binding of the endogenous ligands β-endorphin and dynorphin to rat brain membranes; and 2) inhibits cross-linking of ^{125}I-β-endorphin to brain proteins in the same pattern as β-endorphin, but not synthetic opioid ligands. These findings suggest that Fab 3B4F11 might prove more useful for the characterization and isolation of the macromolecular complex than the ligands presently available.

The Fab inhibits the binding of endogenous ligands much more potently than synthetic ligand binding. In contrast to IC_{50} values of 20–40 ng/ml for Fab inhibition of β-endorphin and dynorphin binding, the IC_{50} values for the opiate ligands [^{3}H] DAGO, [^{3}H]D-ALA-D-LEU-enkephalin, and [^{3}H]EKC ranged from 200 to 600 ng/ml (11). These data, combined with the

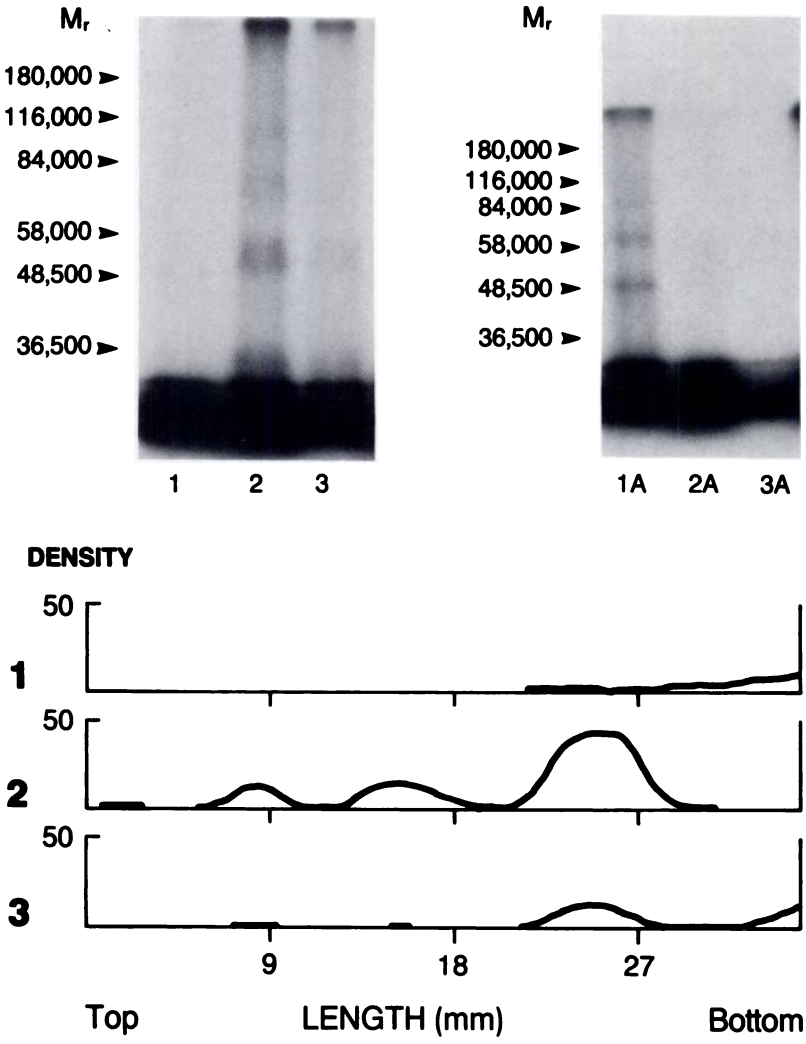

Fig. 2. Rat brain P_2 membranes were bound with 2 nM ^{125}I-β-endorphin and cross-linked with 1 mM BSOCOES (*lanes 2* and *1A*) or DMSO (*lanes 1* and *2A*). Equal amounts of denatured protein were then examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 10% running gel and a 4% stacking gel. In *lane 3*, β-endorphin (1 μM) was added to the binding assay before cross-linking. *Lane 3A* represents ^{125}I-β-endorphin alone, equal, in cpm, to the cross-linked samples, applied to the gel. The densitometer scans from *lane 1* (DMSO), *lane 2* (BSOCOES), and *lane 3* (BSOCOES plus β-endorphin) are also shown. The molecular weight markers for each gel are indicated.

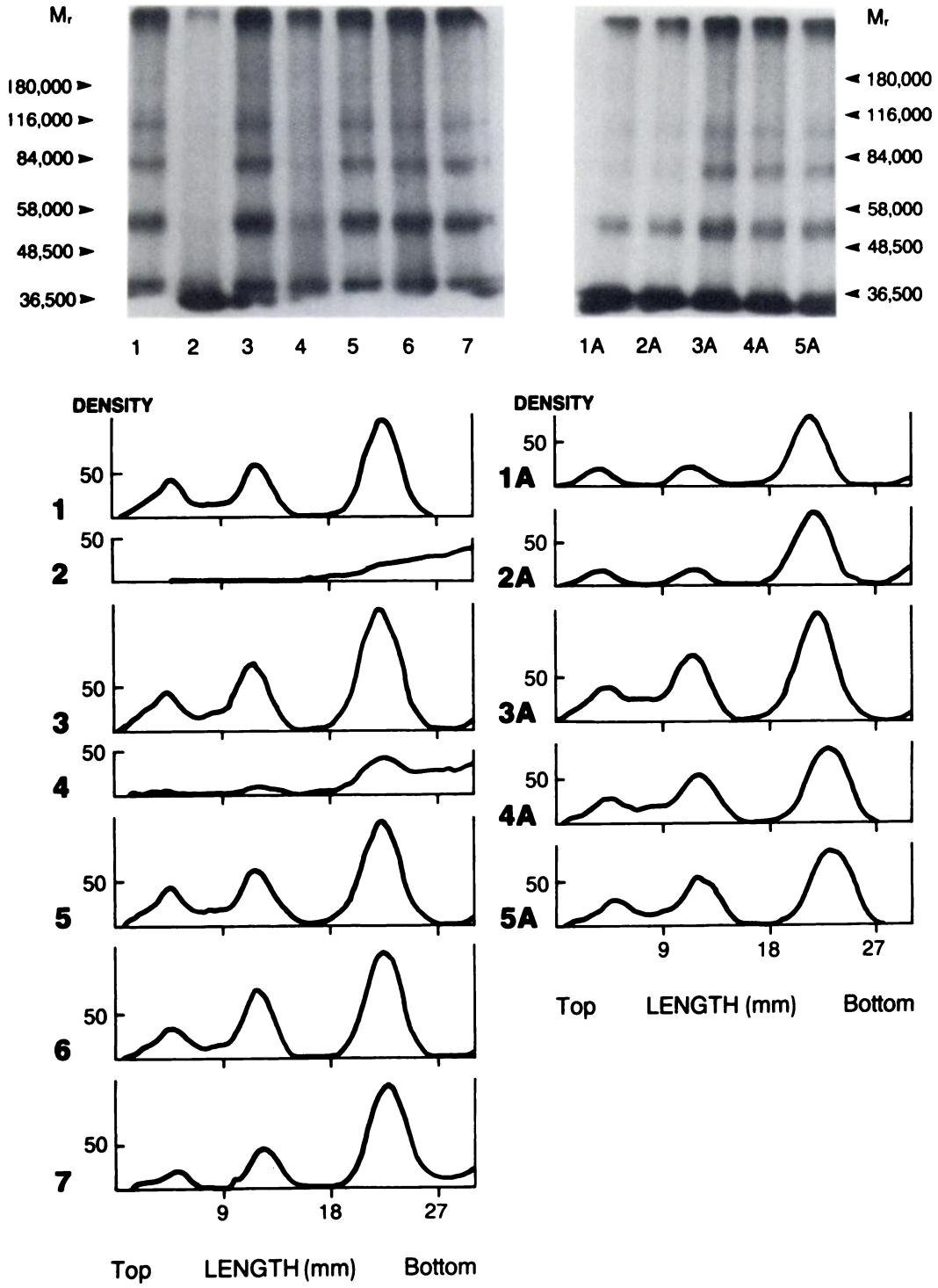

Fig. 3. Control Fab or 3B4F11 were added to the ^{125}I-β-endorphin binding assay before cross-linking with BSOCOES. *Lanes 1* and *1A*, BSOCOES alone, no competing ligand or Fab fragment added; *lane 2*, plus 200 ng of 3B4F11; *lane 3*, plus 200 ng of control Fab; *lane 4*, plus 100 ng of 3B4F11; *lane 5*, plus 100 ng of control Fab; *lane 6*, plus 50 ng of 3B4F11; *lane 7*, plus 50 ng of control Fab; *lane 2A*, plus 25 ng of 3B4F11; *lane 3A*, plus 25 ng of control Fab; *lane 4A*, plus 12.5 ng of 3B4F11; *lane 5A*, plus 12.5 ng control Fab. The densitometer scans corresponding to each lane and molecular weight markers are shown.

finding that 3B4F11 acts as a noncompetitive inhibitor of ligand binding, suggest that the Fab fragment 3B4F11 is not directed against the binding site itself but is directed against a portion of the receptor that is particularly important for the binding of the endogenous opioid peptides.

To further explore the interaction of 3B4F11 with the receptor for the endogenous opioids, we covalently labeled receptor complexes in rat brain membrane with ^{125}I-β-endorphin. The method used was similar to that of Howard *et al.* (8) with two important modifications. First, we included 100 mM KCl in the incubation medium during the binding with ^{125}I-β-endorphin. We found that this step greatly reduced the amount of nondisplaceable or background binding that was subsequently cross-linked to the membranes. Second, we washed the membranes with 100 mM NaCl after cross-linking. This step facilitated removal of ligand that was not covalently bound to the membrane. In the absence of this wash, a very large amount of free ^{125}I-β-endorphin was observed on the gels, and some of the cross-linked bands were obscured.

The addition of KCl was particularly critical, for we found that if we eliminated it, it was difficult to observe a difference in cross-linking between samples incubated with and without

TABLE 1

Effect of Opioid Ligands on Cross-Linking of ^{125}I-β-Endorphin to Rat Brain Membranes

The integral of each band was determined using a scanning densitometer. The per cent decrease in integral was calculated by dividing the integral of the band formed in the presence of opioid ligand plus BSOCOES by the integral of the band formed in the presence of BSOCOES alone. Data are presented as the mean and standard error of *N* experiments. In a single experiment and gel, each BSOCOES lane and BSOCOES plus ligand lane was run in duplicate.

Ligand (1 μM)	Decrease in Integral		
	108,000 Da	73,000 Da	49,000 Da
		%	
β-Endorphin	75 ± 4 (4)	78 ± 7 (4)	47 ± 8 (4)
Etorphine	36 ± 11 (4)	59 ± 13 (4)	17 ± 12 (4)
DPDPE	27 ± 5 (3)	22 ± 11 (3)	22 ± 1 (3)
DAGO	35 ± 9 (3)	51 ± 12 (3)	0 ± 0 (3)
EKC	34 ± 15 (3)	41 ± 15 (3)	7 ± 3 (3)
U50488H	27 ± 10 (3)	41 ± 19 (3)	2 ± 2 (3)
DPDPE + DAGO + U50488H	55 ± 10 (4)	30 ± 15 (4)	38 ± 13 (4)

TABLE 2

Effect of Fab on Crosslinking of ^{125}I-β-Endorphin to Rat Brain Membranes

The integral of each band was determined using a scanning densitometer. The percent decrease in integral was calculated by dividing the integral of the band formed in the presence of Fab plus BSOCOES by the integral of the band formed in the presence of BSOCOES alone. Data are presented as the mean and standard error of *N* experiments.

Fab	Decrease in Integral		
	108,000 Da	73,000 Da	49,000 Da
ng		%	
3B4F11			
12.5	0 ± 0 (3)	0 ± 0 (3)	2 ± 0 (3)
25	0 ± 0 (3)	7 ± 3 (3)	2 ± 1 (3)
50	35 ± 14 (3)	18 ± 8 (3)	0 ± 0 (3)
100	55 ± 17 (3)	70 ± 6 (3)	36 ± 10 (3)
200	82 ± 4 (3)	96 ± 2 (3)	41 ± 9 (3)
Control			
12.5	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)
25	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)
50	0 ± 0 (3)	0 ± 0 (3)	1 ± 0.7 (3)
100	0 ± 0 (3)	0 ± 0 (3)	6 ± 6 (3)
200	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)

unlabeled β-endorphin. We believe this may be due to a difference in the efficiency of cross-linking of specific and nonspecific binding and the ability of K^+ to reduce nonspecific binding. Although K^+ decreases specific binding (19), its effect on nonspecific binding appears to be greater. Our data (not shown), as well as those of others (8), indicate that a substantial percentage of radioactive ligand originally bound to the membrane (more than 50%) is not cross-linked. If this percentage is greater than that of the nonspecific binding not cross-linked, then the ratio of the two will be reduced relative to that observed before cross-linking. The smaller the ratio between specific and nonspecific binding, the more difficult it becomes to detect a significant difference between the two.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by autoradiography, revealed that three species with molecular weights of 108,000, 73,000 and 49,000 were covalently labeled by ^{125}I-β-endorphin. We cannot exclude the possibility that more than one protein is present in each species. Recent experiments in which β-endorphin-cross-linked samples are electrophoresed on larger gels indicate that the 73,000-Da band can indeed be resolved into two labeled constituents (data not shown). Furthermore, our previous studies demonstrate that the polyclonal antibody raised to the purified opioid receptor preparation binds selectively to a 58,000-Da species in rat brain membrane (20). Thus, the 73,000-Da species labeled with ^{125}I-β-endorphin might contain this 58,000-Da component. Our findings are in general agreement with a number of other groups reporting the isolation of three to four β-endorphin receptor components with molecular weights ranging from 94,000 to 23,000. The lack of agreement in reported apparent molecular weights might result from the differences in the sources of tissue and cell lines or in purification methods used, affinity chromatography (21, 22), irreversible affinity labeling (23), or cross-linking (8, 9, 24). All of these studies clearly indicate that the endogenous opioid receptor consists of several distinct protein components.

To determine whether each of the labeled species could represent a μ, δ, or κ subunit of the opioid receptor, the ability of various selective ligands to inhibit cross-linking was tested. The amount of each ligand that produced maximal inhibition was used as determined in dose-response curves. The δ selective ligand DPDPE was least effective in inhibiting the cross-linking of β-endorphin to any band, suggesting that β-endorphin was not preferentially linked to δ receptors in the whole rat brain. DAGO, U50488, and EKC prevented β-endorphin labeling of the 108,000-Da and 73,000-Da bands, but not the 49,000-Da band, suggesting that the two higher molecular weight components might be associated with the μ and κ opioid receptor complexes. These findings offer support for the postulate that μ and δ receptor components differ in molecular size. Using DPDPE and the μ-selective ligand Tyr-D-Ala-Gly-(*N*-Me)Phe-Gly-ol, Howard *et al.* (10) has identified ^{125}I-β-endorphin-labeled species that are selectively inhibited by each ligand. Our results differ from those of this group in that we were not able to demonstrate 100% inhibition of ^{125}I-β-endorphin cross-linking with any of the synthetic ligands or even the combination of DPDPE, DAGO, and U50488. Although the synthetic ligands are used typically to characterize opioid receptors, the β-endorphin receptor complex might contain proteins that do not interact with these ligands.

More interesting, however, was the finding that Fab 3B4F11 and β-endorphin were the most efficacious of all the inhibitors tested at preventing cross-linking of ^{125}I-β-endorphin to its receptor. Both the Fab fragment and β-endorphin might bind portions of the endogenous opioid receptor complex that do not bind μ and κ ligands but do bind, or are physically near, the β-endorphin binding site. The finding that the Fab fragment inhibits the labeling of all the cross-linked proteins strengthens the possibility that these proteins are associated with the opioid receptor. However, the ability of the antibody to inhibit well characterized opioid-mediated physiological actions, such as analgesia, must be confirmed.

Interestingly, Fab 3B4F11 does not inhibit the covalent linkage of β-endorphin to its receptor with as great a potency as it inhibits ^{125}I-β-endorphin binding to rat P_2 membranes. The IC_{50} for 3B4F11 inhibition of ^{125}I-β-endorphin (1.0 nM) binding is 40 ng/ml, whereas the IC_{50} for 3B4F11 inhibition of ^{125}I-β-endorphin (2 nM) cross-linking is 120 ng/ml. One possible explanation for this discrepancy is that filtering is used to separate bound from free ligand for the routine assay of β-endorphin binding, whereas the initial binding step of the cross-linking procedure used centrifugation to separate bound from

free ligand. Despite the higher concentrations needed, both β-endorphin and Fab 3B4F11 were able to selectively inhibit β-endorphin cross-linking to the membrane.

It is well accepted that the opioid receptor is a macromolecular complex consisting of multiple proteins and even lipids (25). The present study suggests that μ, κ, and δ ligands might bind only a portion of the endogenous opioid receptor complex (the 108,000- and 73,000-Da species), whereas Fab 3B4F11 might interact with the same receptor constituents as β-endorphin (the 108,000-, 73,000-, and 49,000-Da species). The monoclonal antibody is directed against the tertiary structure of the opioid receptor. Therefore, these different molecular weight species could represent 1) distinct proteins with antigenically similar regions in their tertiary structure or 2) multiple proteins with homologous regions and similar tertiary structure. Future efforts will focus on utilizing the Fab 3B4F11 to further purify and isolate these several components of the β-endorphin receptor complex.

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