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DIFFERENT MOLECULAR WEIGHT FORMS OF OPIOID RECEPTORS REVEALED BY POLYCLONAL ANTIBODIES

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SUMMARY: Polyclonal antibodies were raised against a purified opioid receptor from bovine brain (Cho, et. al., 1986), and shown to inhibit ^{3}H-diprenorphine binding to this receptor in a dose-dependent fashion. These antibodies were then used to characterize opioid-binding material present in rat brain and in NG108-15 neuroblastoma-glioma hybrid cells. Western blot analysis revealed that the antibodies reacted with a single species of 58,000 molecular weight in rat brain membranes; this closely corresponds in size to the bovine opioid receptor used to raise the antibodies. In contrast, the polyclonal antibodies reacted with a 45,000 molecular weight species in NG108-15 neuroblastoma-glioma hybrid cells; moreover, this band was specifically reduced in NG108-15 cells in which opioid receptors had been down-regulated by incubation with D-ala^{2}-D-leu^{5}-enkephalin for 24 hours. Thus at least two distinct opioid receptor molecules have been identified, which have antigenic similarities.

Several laboratories have reported solubilization and partial or complete purification of opioid receptors (Bidlack, et. al., 1981; Gioannini, et. al., 1985; Simonds, et. al., 1985; Maneckjee, et. al., 1985). We recently reported purifying to homogeneity a 58 kD protein from rat brain that binds opioid ligands with high affinity (Cho, et. al., 1986). In this paper we report the generation of polyclonal antibodies specific to this opioid binding protein, and their use to characterize opioid receptors in rat brain and in NG108-15 hybrid cells.

MATERIALS AND METHODS

Materials. ^{3}H-Diprenorphine (38ci/mMol) was purchased from Amersham.

Purification of opioid binding protein. The opioid binding protein was purified from bovine brain as described by Cho et. al. (1986). Briefly, P_2

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membranes were isolated and solubilized with Triton X-100. After centrifuging to remove particulate material, the Triton-solubilized supernatant was fractionated successively by 6-succinyl morphine affinity chromatography, Ultrogel filtration, wheat germ agglutinin affinity chromatography, and preparative isoelectric focussing. SDS gel electrophoresis revealed a single 58 kD band, in agreement with Cho, et. al. (1986).

Immunization protocol. Ten micrograms of the purified opioid binding protein in 0.5 ml of phosphate buffered saline pH 7.0 were emulsified with 0.5 ml of Freund's complete adjuvant (Grand Island Biologicals). Rabbits were injected intradermally at multiple sites. For the subsequent booster injections, the purified receptor in incomplete Freund's adjuvant was given intramuscularly at contralateral sites. The rabbits were bled fortnightly and the sera separated and stored at -20°C.

Enzyme-linked immunosorbent assay (ELISA). The titer and the specificity of the antibodies were determined using the ELISA technique according to the procedures described in Roy and Michaelis (1984). The purified receptor protein was linked to the wells of polystyrene plates by coating the plates with poly-L-lysine and fixing the protein using 0.05% (vol/vol) gluteraldehyde.

Immunoprecipitation. Rat P_2 membranes were prepared as described by Cho et. al. (1983). The rat P_2 membranes were first solubilized with 1% Triton-X-100 and the solubilized receptor partially purified by passage over a wheat germ lectin column. The wheat germ eluate was incubated with saturating concentrations (1:10 dilution) of anti-receptor antiserum for 1 hr. at room temperature. S. aureus suspended in 0.05M tris buffer (pH 7.4) (25 mg/50 ul) were added to the antigen-antibody mixture and incubated at room temperature for 30 minutes. The samples were centrifuged for 1 minute in a microfuge, the supernatant removed and assayed for ^{3}H-diprenorphine binding.

Binding assays. Opioid binding determinations were carried out according to the method of Tovey et. al. (1974), with slight modifications. Samples containing 0.2 ug of purified receptor protein were first reconstituted with 40 ug/0.1 ml of phosphatidyl inositol. The reconstituted protein was preincubated with varying concentrations of dialysed culture supernatant or purified antibody for 1 hour at 37°C. ^{3}H-labelled ligand was added, the mixture incubated at 25° C for 30 minutes with constant shaking, and cooled in an ice bath for 20 minutes. Unbound ligand was removed by the addition of 0.1 ml of tris buffer containing 2.6% charcoal and 2% bovine serum albumin. The mixture was centrifuged for 4 minutes in an Eppendorf 5413 Centrifuge. 0.5 ml of the supernatant was transferred to a cocktail solution (Scintiverse II) and the radioactivity measured by liquid scintillation spectrophotometry. Specific binding was defined as that displaced by 10^{-5} M of unlabelled ligand.

Western blot. Western blots were done essentially as described by Towbin et. al. (1979). P_2 membranes and membranes from NG108-15 hybrid cells were prepared and solubilized with SDS, run on 1.5 mm polyacrylamide gels (10% acrylamide) in SDS (SDS-PAGE), and transferred to nitrocellulose filters for 45 minutes at room temperature. Following transfer, the filters were rinsed in Phosphate buffered saline (pH 7.4) (PBS) and incubated in PBS plus 5% bovine serum albumin and 1% gelatin. Proteins reacting with the anti-opioid receptor antiserum were detected by incubating the filters in 20 ml of TBS, 1% gelatin containing 20 ul of anti-receptor antibody, overnight at 4°C. Bound first antibody was subsequently detected using goat anti-rabbit IgG horse radish peroxidase conjugate and horse radish peroxidase color development reagent.

RESULTS

Antibody response against opioid receptor. Two rabbits were immunized with the purified opioid receptor, and the immune response estimated using the solid phase ELISA technique. The data in Fig. 1 show the magnitude of the antibody response and the dose response characteristics of the antigen-antibody interaction in both rabbits. Both animals responded with relatively high titer. Pre-immune sera from the same animals did not show significant binding to the purified receptor (data not shown).

Immunoprecipitation of opioid binding component. The specificity of the polyclonal antibodies was determined by their ability to precipitate the opioid binding component from a solubilized preparation. In these experiments rat P_2 membranes were first solubilized with 1% TX-100 and the solubilized receptors partially purified by passage over a wheat germ lectin column. The wheat germ eluate was incubated with saturating concentrations of antiserum for 1 hour at room temperature, and the antigen-antibody complex precipitated using S. aureus. After immunoprecipitation, the supernatant was assayed for opioid binding.

Results are shown in Fig. 2. The activity remaining in the supernatant after immunoprecipitating with the pre-immune serum was considered as 100%

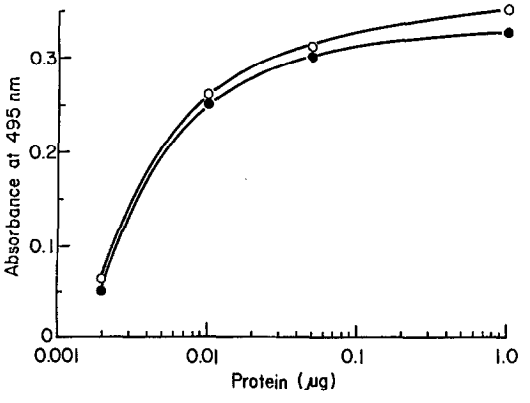

Figure 1. Antigen binding capacity of antiserum as determined by ELISA. The dose response curves for the sera obtained from rabbit$_1$(O-O) and rabbit$_2$ (●-●) immunized with purified receptor. All assays were performed with 0.025 ug of the purified receptor per well.

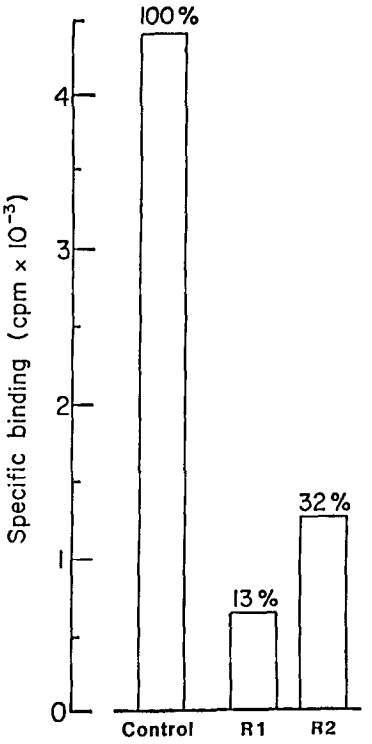

Figure 2. Specific binding of wheat germ eluate supernatant after immunoprecipitation. Rat P_2 membranes were solubilized with 1% Triton X-100 and partially purified by passage over a wheat germ lectin column. The partially purified material was incubated with saturating concentration of anti-receptor anti-serum. The antigen-antibody complex was precipitated by incubation with Stap-A and centrifugation in a microfuge. The supernatant was assayed for ^{3}H-Diprenorphine binding as described in the method section.

of the activity. When compared with the pre-immune serum, the antiserum produced by Rabbit 1 (R_1) was able to precipitate 87% of binding activity, with the remaining 13% of the activity recoverable in the supernatant. Likewise the antiserum from Rabbit 2 (R_2) precipitated 60% of the activity.

Effects of anti-opioid receptor antibodies on ^{3}H diprenorphine binding activity of the purified receptor. Immunization would result in antibodies directed against different determinants of the protein which may or may not include the active site. In order to investigate the possibility of the presence of antibodies directed against the opioid binding site on the receptor, the effects of the anti-opioid receptor antibody on ^{3}H-diprenorphine binding to the purified receptor was determined. As shown in Fig. 3, pre-incubation of the purified receptor with increasing

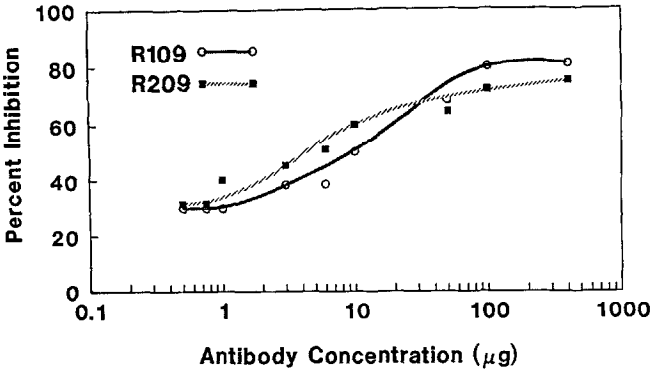

Figure 3. Effects of anti-opioid receptor antibodies on ^{3}H-diprenorphine binding activity of the purified receptor. (O-O) represents inhibition of binding obtained with Rabbit 1 and (■-■) represents inhibition obtained with Rabbit 2. Each data point represents the mean of 4 determinations.

concentrations of the immunoglobulin fraction (IgG) of the antiserum from R_1 and R_2 led to a concentration dependent inhibition of ^{3}H-diprenorphine binding activity of the purified opioid receptor. Maximum inhibition of ^{3}H-diprenorphine activity was achieved by 10 ug. of R_1 and 15 ug. of R_2. The same concentration of IgG from pre-immune serum did not inhibit ^{3}H diprenorphine binding to the opioid receptor protein.

Western blot analysis. To identify the protein component that the antibodies were directed against, rat brain membranes and NG108-15 neuroblastoma-glioma hybrid cell membranes were fractionated by SDS-PAGE, and the electrophorerograms analyzed by Western blot analysis. As shown in Fig. 4, the antiserum specifically reacted with a protein band of molecular weight of about 58K in rat brain membranes. This corresponds closely with the molecular size of the purified receptor protein used to prepare the antibodies (Cho, et. al., 1986).

In contrast, the antibodies reacted with a protein of approximately 45K in NG108-15 hybrid cell membranes (Fig. 5). Further evidence that this species is the opioid receptor in this system was provided by analyzing membranes from NG cells treated for 24 hours with 10 nM D-ala^{2}-D-leu^{5}-enkephalin (DADLE); under these conditions, there is a decrease, or down-regulation, of opioid receptors by about 80% (Law, et.

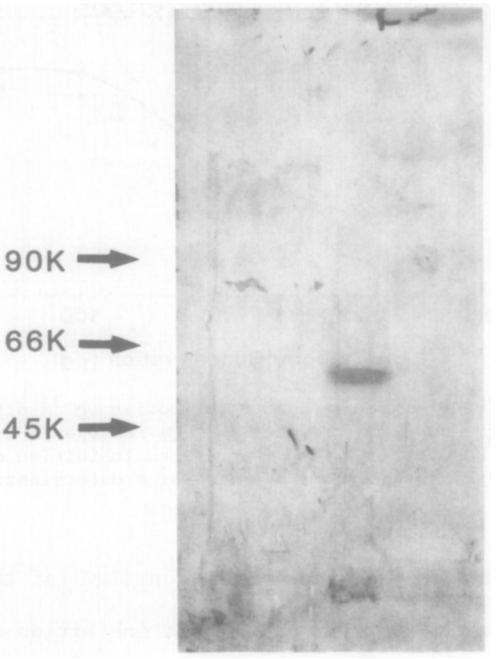

Figure 4. Western blot analysis of solubilized P_2 membranes using anti-receptor antiserum. See Methods section for details.

al., 1983). As seen in Fig. 5, the 45K band sensitive to the polyclonal antibodies was dramatically and specifically reduced under these conditions.

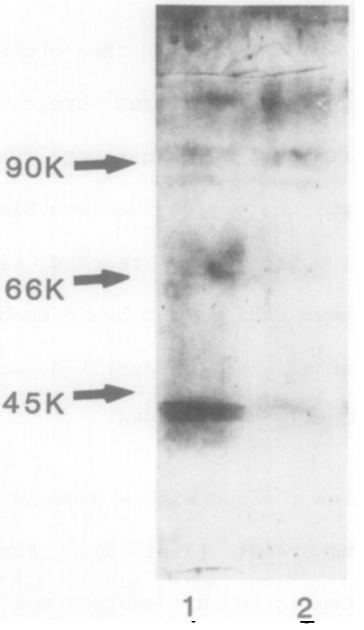

Figure 5. Western blot analysis of solubilized P2-P3 membranes of NG108-15 neuroblastoma-glioma cells. The solubilized membranes were processed exactly as given in the legend to Fig. 4. Lane 1, membranes from control, untreated cells; lane 2, membranes from cells pre-treated for 24 hours with 10 nM DADLE.

DISCUSSION

We describe here the preparation and assay of polyclonal antibodies against a purified opioid receptor. Two rabbits were immunized with the purified receptor and both rabbits responded with high antibody titers. The antigen binding capacity of the antiserum was dose-dependent. Pre-immune serum derived from the same animal did not show any significant immunoreactivity against the purified protein even at high concentration, indicating that the antibody response observed with the antiserum was of a specific nature.

The specificity of the antibodies was further established by their ability to 1) precipitate the opioid binding component from a solubilized preparation (Fig. 2); 2) inhibit ^{3}H-diprenorphine binding to the purified receptor in a dose-dependent manner (Fig. 3); and 3) react specifically with a 58 kD protein component of P_2 membranes (Fig. 4). This molecular weight is similar to that of the purified opioid receptor originally used to raise the antibodies.

Interestingly, however, the antibodies reacted with a different, 45K species in NG108-15 neuroblastoma-glioma hybrid cells (Fig. 5). Since these cells are known to contain a homogeneous population of the delta type (enkephalin-selective) opioid receptor, and since the 58K bovine brain species used to raise the antibodies has some mu selectivity (Cho, et. al., 1986), our results suggest that the distinct, yet antigenically similar, molecules present in these two systems correspond to mu and delta opioid receptors, respectively. The differences between the two remain to be elucidated, but since the 58K species is thought to be glycosylated (Cho, et. al., 1986), possibly the 45K species represents the same gene product with no or fewer carbohydrate residues.

With the demonstration of their specificity, these antibodies can now be used to characterize the purified opioid receptor further. In preliminary studies, we have shown that when injected into the brain, these antibodies

block morphine analgesia, indicating that the 58 K opioid receptor is pharmacologically relevant (unpublished data). Future work will include determining the molecular requirements for opioid binding and opioid receptor-mediated function and mapping the brain regional distribution of opioid receptors. These antibodies can also be used to purify the receptor in large quantity using a single purification step.

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