Inhibition of $\mu$ and $\delta$ but Not $\kappa$ Opioid Binding to Membranes by Fab Fragments from a Monoclonal Antibody Directed against the Opioid Receptor*

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Fab fragments from a monoclonal antibody, OR-689.2.4, directed against the opioid receptor, selectively inhibited opioid binding to rat and guinea pig neural membranes. In a titratable manner, the Fab fragments noncompetitively inhibited the binding of the $\mu$ selective peptide $[^3H]$enkephalin, and $\delta$ selective peptide $[^3H]$enkephalin. The $\mu$ binding site was not blocked by the Fab fragments. In addition to blocking the binding of $\mu$ and $\delta$ ligands, the Fab fragments displaced bound opioids from the membranes. When $\mu$ sites were blocked with $[^3H]$enkephalin, the Fab fragments suppressed the binding of $[^3H]$enkephalin to the same degree as when the $\mu$ binding site was not blocked. The Fab fragments also inhibited binding to the $\mu$ site regardless of whether or not the $\delta$ site was blocked with $[^3H]$enkephalin. This monoclonal antibody is directed against a 35,000-dalton protein. Since the antibody is able to inhibit $\mu$ and $\delta$ binding but not $\kappa$ opioid binding, it appears that this 35,000-dalton protein is an integral component of $\mu$ and $\delta$ opioid receptors but not $\kappa$ receptors.

The different types of opioid receptors have been difficult to characterize and classify. Only recently with the synthesis of selective peptides and alkaloids has the biochemical and pharmacological characterization of these opioid receptors been possible. The peptide DAGO1 and the alkaloid morphine are the most selective ligands for $\mu$ binding sites (1, 2). The peptides DPDPE and DSLET are the most specific ligands for the $\delta$ binding site (3, 4). The alkaloid U50,488H is the most selective ligand for $\kappa$ binding sites (5).

The endogenous opioid peptides have some opioid receptor selectivity. Met-enkephalin and Leu-enkephalin have the greatest affinity for $\delta$ binding sites but also some affinity for the $\mu$ site (1, 6, 7). $\beta$-Endorphin has almost equal affinity for the $\mu$ and $\delta$ binding sites (7, 8), though it may also bind to its own distinct opioid receptor, the $\kappa$ receptor (9, 10), and to a nonopioid receptor (11). The dynorphins have the highest affinity for the $\kappa$ opioid receptor (12, 13).

As a tool to probe the molecular basis of the multiple opioid receptors, a monoclonal antibody, OR-689.2.4, capable of partially inhibiting opioid binding to rat neural membranes, has been produced (14). This IgM was able to block and displace opioid ligands from rat neural membranes. The specificity of this immunoglobulin has been demonstrated by its inability to inhibit the binding of nonopioid ligands to neural membranes and the ineffectiveness of other mouse immunoglobulins to block the binding opioids to neural membranes (14). The antibody, directed against a 35,000-dalton protein, immunoprecipitated opioid binding sites from a solubilized preparation (14). Because it is an IgM with a molecular weight of 980,000, the ability of the antibody to penetrate tissue is hampered. As a consequence, a procedure was developed for obtaining Fab fragments, with a molecular weight of 48,000, from this IgM (15). This study describes the ability of OR-689.2.4 Fab fragments to selectively inhibit the different types of opioid binding sites in neural membranes.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Determining the Ability of OR-689.2.4 Fab Fragments to Inhibit Opioid Binding to Neural Membranes**—The Fab fragments from the IgM, OR-689.2.4, inhibited the binding of 1 nM $[^3H]$DAGO in a titratable manner as shown in Fig. 1. The longer the preincubation of membranes with the Fab fragments prior to the addition of $[^3H]$DAGO, the greater the inhibition obtained with a given concentration of Fab fragments. A 15-min preincubation of the rat neural membranes with the Fab fragments at 25°C was sufficient to cause significant blockade of 1 nM $[^3H]$DAGO. A 24-h preincubation at 4°C resulted in the greatest inhibition of the binding of 1 nM $[^3H]$DAGO. OR-689.2.4 Fab fragments at a final concentration of 250 nM inhibited the binding of 1 nM $[^3H]$DAGO to rat neural membranes by 28% with a 15-min preincubation at 25°C, by 35% with a 60-min preincubation at 25°C, and by 52% with a 24-h preincubation at 4°C. By increasing the number of photocopied sets published.

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concentration of Fab fragments and the time of preincubation, it was possible to achieve an 80% inhibition in the binding of \( ^{3}H \)DAGO to neural membranes.

The OR-689.2.4 Fab fragments also inhibited the binding of 3 nM \( ^{3}H \)DPDPE to rat neural membranes, as shown in Fig. 2. As with mu peptide, the longer the preincubation of the membranes with the Fab fragments the greater the inhibition obtained with a certain concentration of Fab fragment. A concentration of 400 nM Fab fragments resulted in a 50% inhibition of the binding of 3 nM \( ^{3}H \)DPDPE, when a 60-min preincubation at 25 °C was used. However with a 24-h preincubation at 4 °C, only 60 nM of Fab fragments was necessary to achieve a 50% inhibition in the binding of 3 nM \( ^{3}H \)DPDPE. Complete inhibition of the binding of \( ^{3}H \)DPDPE was obtained with 1 μM of OR-689.2.4 Fab fragments and a 24-h preincubation at 4 °C. Under identical conditions, the Fab fragments were able to inhibit the binding of the δ ligand slightly better than the μ ligand.

The ability of OR-689.2.4 Fab fragments to inhibit binding to rat neural membranes, which have less than 20% μ binding sites (20), and guinea pig cerebellum, which has been shown to contain 85% μ opioid receptors (21), was investigated. Membranes were preincubated with 500 nM OR-689.2.4 Fab fragments for 60 min at 25 °C. Under identical conditions, 500 nM OR-689.2.4 Fab fragments resulted in a 33% inhibition of the binding of 1 nM \( ^{3}H \)DAGO and a 50% inhibition of the binding of 3 nM \( ^{3}H \)DPDPE to rat neural membranes. After the preincubation, \( ^{3}H \)bremazocine at a final concentration of 0.1 nM was added to the samples. As depicted in Table I, in the absence of μ and δ blockers, the Fab fragments suppressed the binding of 0.1 nM \( ^{3}H \)bremazocine to rat neural membranes by 27%. However, in the presence of μ and δ blockers, the Fab fragments did not have a significant effect on the binding of \( ^{3}H \)bremazocine to rat neural membranes.

The binding of \( ^{3}H \)bremazocine to rat neural membranes is to μ and δ sites, due to the fact that μ sites account for less than 20% of the opioid binding sites. In the absence of μ and δ blockers, the Fab fragments inhibited the binding of 0.1 nM \( ^{3}H \)bremazocine to rat neural membranes by 27%, indicating that the Fab fragments could inhibit \( ^{3}H \)bremazocine binding to μ and δ sites. Table II shows the effect of the OR-689.2.4 Fab fragments on the binding of 0.1 nM \( ^{3}H \)bremazocine to guinea pig cerebellar membranes. The Fab fragments did not significantly suppress the binding of 0.1 nM \( ^{3}H \)bremazocine to guinea pig cerebellar membranes, regardless of whether μ and δ blockers were present. As previously reported (21) and confirmed in these studies, μ binding sites account for 84–88% of the opioid binding sites in the guinea pig cerebellum.

To determine that the lack of inhibition of \( ^{3}H \)bremazocine binding to guinea pig cerebellum by the Fab fragments was not the result of species differences in the molecular structure of the opioid receptor, we investigated the ability of the Fab fragments to inhibit the binding of 1 nM \( ^{3}H \)DAGO and 3 nM \( ^{3}H \)DPDPE to guinea pig neural membranes. As can be seen in Fig. 3, the OR-689.2.4 Fab fragments were able to suppress the binding of 1 nM \( ^{3}H \)DAGO and 3 nM \( ^{3}H \)DPDPE to guinea pig neural membranes, though the amount of inhibition attained with a given concentration of Fab fragments was slightly less than observed with rat neural membranes. The OR-689.2.4 Fab fragments interact with a component of the opioid receptor that is common between guinea pig and rat neural membranes. These studies suggest that OR-689.2.4 Fab fragments interact with a component of the opioid receptor that is common to μ and δ opioid receptors, but not μ opioid receptors.

Determining the Type of Inhibition of Opioid Binding by the OR-689.2.4 Fab Fragments—To determine whether the Fab fragments act as competitive or noncompetitive inhibitors of opioid binding, rat neural membranes were incubated with 500 nM OR-689.2.4 Fab fragments and varying concentrations of \( ^{3}H \)DAGO and \( ^{3}H \)DPDPE. Fig. 4 is a double reciprocal plot of the binding of \( ^{3}H \)DAGO at concentrations ranging from 0.1 to 6.4 nM. In the absence of Fab fragments, a Kᵦ value of 0.47 nM was obtained and the maximal number of binding sites was 50 fmo1/0.25 mg membrane protein. When membranes were preincubated with 500 nM OR-689.2.4 Fab fragments, an apparent Kᵦ value of 0.44 nM was observed, while the maximal number of binding sites was decreased to 28 fmo1/0.25 mg membrane protein. OR-689.2.4 Fab fragments act as noncompetitive inhibitors of μ binding sites.

When membranes were incubated with 500 nM Fab fragments followed by the addition of 0.3 to 19.6 nM \( ^{3}H \)DPDPE, a double reciprocal plot as depicted in Fig. 5 was obtained. A Kᵦ value of 6.1 nM and a Bₘₐₓ value of 40 fmo1/0.25 mg membrane protein were observed in control samples. In membrane samples incubated with Fab fragments, an apparent Kᵦ value of 9.1 nM was obtained, while the maximal number of binding sites decreased to 28 fmo1/0.25 mg protein. From these studies it can be concluded that OR-689.2.4 Fab fragments act as noncompetitive inhibitors of both μ and δ opioid binding to neural membranes.

Examining the Ability of OR-689.2.4 Fab Fragments to Displace Bound Ligand from Neural Membranes—In experiments designed to determine if the Fab fragments could displace bound \( ^{3}H \)DAGO, membranes were first incubated for 60 min with 1 nM \( ^{3}H \)DAGO. Varying concentrations of OR-689.2.4 Fab fragments were added for an additional 60-min incubation prior to filtration. Experiments directed at determining the ability of the antibody to block the binding of 0.5 nM \( ^{3}H \)DAGO were performed by first incubating membranes with Fab fragments. The Fab fragments could block and displace bound \( ^{3}H \)DAGO from membranes as shown in Fig. 6. While the Fab fragments were more effective at blocking the binding of \( ^{3}H \)DAGO, they were capable of displacing bound ligand.

Results of similar experiments using 5 nM \( ^{3}H \)DPDPE are shown in Fig. 7. As with the μ peptide, the Fab fragments could displace bound \( ^{3}H \)DPDPE. With a 60-min preincubation of membranes with the Fab fragments or \( ^{3}H \)DPDPE, 60 nM of Fab fragments resulted in the blockage of 30% of the binding sites, while a concentration of 500 nM was needed to displace 30% of the bound ligand.

Determining If OR-689.2.4 Fab Fragments Can Inhibit μ and δ Binding to Membranes When the Other Site Is Blocked with Ligand—To determine if the binding of a δ ligand to membranes resulted in a conformational change in the receptor so that the Fab fragments were no longer able to inhibit binding of μ ligands, the following experiment was performed. To block δ binding sites, membranes were incubated with 100 nM DPDPE for 30 min. OR-689.2.4 Fab fragments at a concentration of 250 nM were added. This concentration of Fab fragments displaced only 19% of bound \( ^{3}H \)DPDPE (see Fig. 7). After a 60-min incubation with the Fab fragments, 0.5 nM \( ^{3}H \)DAGO was added. As detailed in Table III, the Fab fragments were able to inhibit the binding of 0.5 nM \( ^{3}H \)DAGO to membranes to the same degree regardless of whether δ sites were or were not blocked by DPDPE.

To examine the reverse situation, DAGO at a concentration of 100 nM was used to block μ binding sites. The Fab fragments at a concentration of 250 nM displaced only 14% of bound \( ^{3}H \)DAGO (see Fig. 6). After the preincubation with DAGO and the addition of the Fab fragments, 5 nM \( ^{3}H \)
DPDPE was added to the membranes to determine if the Fab fragments could inhibit the binding of [3H]DPDPE when μ sites were blocked with ligand. As depicted in Table IV, the Fab fragments at a concentration of 250 nM inhibited the binding of 5 nM [3H]DPDPE by 38-40% regardless of whether the μ sites were blocked with DAGO. These results indicate that the binding of μ or δ opioids to membranes does not result in a conformational change of the receptor such that the Fab fragments become ineffective in inhibiting binding to the unblocked site.

**DISCUSSION**

Elucidating the molecular basis of the multiple opioid receptors has proven to be a difficult task. A monoclonal antibody to the opioid receptor is a tool that will help determine the biochemical basis of the opioid receptor. The monoclonal IgM OR-689.2.4 has been shown to be specific for the opioid receptor (14). Because of the large size of a mouse IgM, 980,000 daltons, the ability of this antibody to penetrate neural membranes is restricted. A procedure was developed for generating Fab fragments from an IgM, effectively reducing the active size of the antibody from a molecular weight of 980,000 to 48,000 (15). With the Fab fragments, inhibition of opioid binding to neural membranes of greater than 90% was obtained. With the whole IgM, maximal inhibition obtained was approximately 35%, probably a consequence of the IgM’s inability to sufficiently penetrate membranes (15).

The longer the preincubation of membranes with the Fab fragments, the greater the inhibition obtained with a certain concentration of Fab fragments. This is because most immunoglobulins have a much slower association and dissociation rate with the antigenic site than most receptor ligands have with their receptor. As a consequence, a 60-min incubation of membranes with the Fab fragments is not sufficient time for equilibrium to be reached. Thus, the longer the preincubation, the further toward equilibrium is the receptor-antibody complex. The Fab fragments inhibited opioid binding to μ and δ sites but not κ sites. The Fab fragments inhibited the binding of [3H]DPDPE slightly better than [3H]DAGO. Whether this finding would be true for all μ and δ ligands is not known.

Due to the fact that guinea pig cerebellum is a rich source of κ sites, this tissue was chosen in addition to rat neural membranes to investigate whether the Fab fragments could inhibit opioid binding to κ sites. The Fab fragments did not suppress the binding of [3H]bremazocine to κ sites in either rat or guinea pig membranes. Bremazocine has the highest affinity for κ opioid binding sites, but also binds to μ and δ sites (7). In rat neural membranes, where a significant proportion of the binding of 0.1 nM [3H]bremazocine was to μ and δ sites, the Fab fragments suppressed this binding by 27%. Under identical conditions, the Fab fragments inhibited 33% of the binding of 1 nM [3H]DAGO and 50% of the binding of 3 nM [3H]DPDPE to rat neural membranes. To determine that the Fab fragments’ inability to inhibit [3H]bremazocine binding to guinea pig cerebellum was not due to the antibody’s inability to recognize guinea pig opioid receptor, the ability of the OR-689.2.4 Fab fragments to inhibit μ and δ binding to guinea pig neural membranes was investigated. The Fab fragments inhibited the binding of [3H]DPDPE and [3H]DAGO to guinea pig neural membranes, indicating that the Fab fragments recognized a component of guinea pig μ and δ opioid receptor, but not the κ receptor (Fig. 3). The antigenic site on the 35,000-dalton protein that the antibody is directed against is present in guinea pig as well as rat neural membranes.

The enkephalins and δ-endorphin bind to μ and δ sites, but not significantly to κ sites (1, 6-8). Distinguishing between μ and δ opioid binding sites has always been most difficult. Many opioids can differentiate between κ sites and μ-δ sites, but not between μ and δ sites (7). A common high-affinity binding site, μ3, has been proposed for opioids and enkephalins (22). A physical separation by sucrose gradient centrifugation of solubilized μ and δ receptors from κ receptors has been obtained (23). The studies presented here suggest that there is a common component, a 55,000-dalton protein, shared by μ and δ opioid receptors but not the κ receptor.

The OR-689.2.4 Fab fragments are noncompetitive inhibitors of μ and δ binding to neural membranes. The antibody is acting at a site distinct from the ligand binding site. The Fab fragments appear to be exerting an effect similar to the inhibition of agonist binding seen with sodium (24). The antibody will, however, inhibit antagonist binding to neural membranes (14). The Fab fragments must be altering the receptor conformation in such a manner as to render it unable to bind opioids. A stimulation in the binding of nerve growth factor to the nerve growth factor receptor on PC12 cells has been observed when PC12 cells were incubated with a monoclonal antibody directed against this receptor (25). Monoclonal antibodies directed against different epitopes on a receptor can influence ligand binding to the receptor. The OR-689.2.4 antibody appears to be directed against a three-dimensional epitope of the 35,000-dalton protein, concluded from the fact that sodium dodecyl sulfate will destroy the antigenic site (14). When δ sites were blocked with DPDPE, the Fab fragments were able to inhibit the binding of [3H]DAGO to the same degree as when δ sites were not blocked with ligand. Under the conditions used, the binding of DPDPE to the δ site did not change the conformation of the receptor in such a manner as to render it unable to bind [3H]DAGO. The same results were obtained when the μ site was blocked, and binding to the δ site was measured. It has previously been suggested that μ and δ opioid receptors may be interconvertible (26). While the studies presented here do not rule out this possibility, the binding of DAGO to the μ site did not alter the receptor in a manner such that the Fab fragments could not block binding to the δ site, and vice versa.

In summary, Fab fragments from the OR-689.2.4 IgM recognize a component common to both μ and δ opioid receptors in rat and guinea pig neural membranes but not κ opioid receptors. This immunoglobulin should prove useful in the elucidation of the multiple opioid receptors.

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A Monoclonal Antibody Directed against the Opioid Receptor

E X P E R I M E N T A L  P R O C E D U R E S

Generating the O-S-489-3,4 Monoclonal Antibody and the Fragments. The IgM O-S-489-3,4 was produced by immunizing a BALB/c mouse with a partially purified peptide fragment of the rat opioid receptor labeled with \(^{125}\)I using polyethylene glycol (PEG). The monoclonal antibody was purified by affinity chromatography using the purified peptide fragment as the ligand. By its ability to block opioid binding to rat neural membranes, the IgM antibody was shown to be specific for the opioid receptor.

Marinating the Effect of O-S-489-3,4 on the Binding of Opioid Ligands to Rat and Guinea Pig Brain Membranes. Since antibodies, including neutralizing ones, were prepared from spleen cell and muscle washings, 25% for 30 min as previously described (12, 13). Membranes from rat and guinea pig brain and the membrane of the brain were prepared in a similar manner. Substrate released during incubation with neuraminidase was measured after the reaction was stopped with cold, using a specific neuraminidase assay. This assay used a specific neuraminidase protein, which was incubated with rat and guinea pig membrane protein in the presence of neuraminidase to determine the concentration of neuraminidase.

Determination of the Binding Capacity of the Fragments. To determine whether the O-S-489-3,4 Fm fragments were acting as competitive or noncompetitive inhibitors of opioid binding, we initiated the experiments with 1 nM \(^{125}\)I in various concentrations of the fragments for 30 min at 37°C. The binding capacity of the fragments was determined by the competition between the fragments and the ligand. The interaction of the fragments was determined by the competition between the fragments and the ligand.

The Ability of O-S-489-3,4 Fragments to Inhibit Binding to Membranes. The binding ability of the fragments was determined by the competition between the fragments and the ligand. The interaction of the fragments was determined by the competition between the fragments and the ligand. The interaction of the fragments was determined by the competition between the fragments and the ligand.

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M. A. Taupier, S. D. Reynolds, J. M. Bidlack, and E. M. Lord, manuscript in preparation.
A Monoclonal Antibody Directed against the Opioid Receptor

FIG. 1. Double reciprocal plot of the binding of [3H]DADLE to rat neural membranes in the presence and absence of OR-689.2.4 Fab fragments. After preincubation of 0.25 mg membrane protein with 0.2 ml OR-689.2.4 Fab fragments for 60 min at 25°C, [3H]DADLE at concentrations ranging from 0.1 to 1.6 nM were added for 60 min at 25°C. Closed circles represent binding to control samples. Closed circles represent binding to membranes preincubated with OR-689.2.4 Fab fragments. A K_d value of 0.47 nM and a B_max value of 56 fmol/mg protein were obtained. In control experiments, a B_max value of 58 fmol/mg protein was obtained. The experiment was repeated 4 times with similar results. The OR-689.2.4 Fab fragments acted as a noncompetitive inhibitor of [3H]DADLE binding to rat neural membranes.

FIG. 2. Effect of OR-689.2.4 Fab fragments on the binding of 0.1 nM [3H]DADLE to rat neural membranes.

| Condition     | Specific Binding | Inhibition |
|---------------|------------------|------------|
| Control       | 150 ± 15         | 1          |
| OR-689.2.4 Fab| 185 ± 23         | 27 ± 2     |

With α and β Blockers

| Condition     | Specific Binding | Inhibition |
|---------------|------------------|------------|
| Control       | 160 ± 17         | 3 ± 1      |
| OR-689.2.4 Fab| 162 ± 44         | 3 ± 1      |

Net neural membranes, 0.25 mg protein, were incubated with 150 nM OR-689.2.4 Fab fragments for 60 min. Specific binding at a final concentration of 0.1 nM was added and the incubation continued for an additional 30 min. They were then spun down and the supernatant was assayed for [3H]DADLE. The results were expressed as [3H]DADLE bound, and the data is presented as the mean ± SEM from 4 separate experiments.

TABLE 1

Effect of OR-689.2.4 Fab Fragments on the Binding of 0.1 nM [3H]DADLE to Rat Neural Membranes

| Condition     | Specific Binding | Inhibition |
|---------------|------------------|------------|
| Control       | 110 ± 13         | 1 ± 1      |
| OR-689.2.4 Fab| 112 ± 9          | 2 ± 1      |

With α and β Blockers

| Condition     | Specific Binding | Inhibition |
|---------------|------------------|------------|
| Control       | 890 ± 54         | 1 ± 1      |
| OR-689.2.4 Fab| 930 ± 58         | 1 ± 1      |

Guinea pig carotid membranes, 0.25 mg protein, were incubated with 150 nM OR-689.2.4 Fab fragments for 60 min. Specific binding at a final concentration of 0.1 nM was added and the incubation continued for an additional 30 min prior to filtration. Nonspecific binding was determined by the inclusion of 10 μM naloxone. DADO and DPDPE at final concentrations of 0.1 and 1 μM, respectively, served as α and β blockers. They were then assayed for [3H]DADLE bound with the Fab fragments. The data is presented as the mean ± SEM from 4 separate experiments.

TABLE 2

Effect of OR-689.2.4 Fab Fragments on the Binding of 0.1 nM [3H]DADLE to Guinea Pig Carotid Membranes
### Table III

| Condition                      | Specific Binding cpn | Inhibition |
|-------------------------------|----------------------|------------|
| Control                       | 1740 ± 49            |            |
| OR-689.2.4 Fab                | 1150 ± 74            | 34 ± 2     |
| DPDPE                         | 1620 ± 102           | 8 ± 1      |
| OR-689.2.4 Fab + DPDPE        | 1080 ± 62            | 37 ± 3     |

Rat neural membranes, 0.25 mg protein, were incubated with 100 nM DPDPE, in block 1 sites, for 30 min prior to the addition of 250 nM OR-689.2.4 Fab fragments. After a 60 min preincubation, 63 nM [3H]DPDPE was added. Samples were filtered 80 min later. Data is presented as the mean ± SEM from 3 separate experiments.

### Table IV

| Condition                      | Specific Binding cpn | Inhibition |
|-------------------------------|----------------------|------------|
| Control                       | 680 ± 15             |            |
| OR-689.2.4 Fab                | 440 ± 25             | 30 ± 1     |
| DAGO                          | 650 ± 34             | 5 ± 4      |
| OR-689.2.4 Fab + DAGO         | 130 ± 13             | 40 ± 6     |

Rat neural membranes, 0.25 mg protein, were incubated with 10 nM DAGO, in block 1 sites, for 30 min prior to the addition of 250 nM OR-689.2.4 Fab fragments for a 60 min preincubation. [3H]DPDPE was added at a final concentration of 5 nM and the incubation continued for 60 min. Data is presented as the mean ± SEM from 3 separate experiments.