Reconstitution of Solubilized Delta-Opiate Receptor Binding Sites in Lipid Vesicles*

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Delta-opiate receptors have been solubilized with the non-ionic bile salt detergent digitonin from NG108-15 cell membranes and reconstituted into lipid vesicles. Specific opiate binding was restored to soluble receptor preparations after supplementation with a brain lipid extract, and dilution below the effective detergent concentration. Saturable and specific opiate binding was measured for both membrane and vesicle preparations; dissociation constants (Kd) obtained from saturation isotherms of [3H]bremazocine binding were 1.3 and 4.2 nM, respectively. Relative affinity (I&) values of isotherms of [3H]bremazocine binding were 1.3 and 4.2 measured for both membrane and vesicle preparations; dissociation constants (Kd) obtained from saturation concentration. Saturable and specific opiate binding was also reconstituted after lectin affinity separation used in the reconstitution. Ligand binding was incompletely recovered after substituting pure, vesicle-forming phospholipid preparations. [3H]Bremazocine binding was also reconstituted after lectin affinity chromatography of solubilized receptor preparations, using conditions which likely effect the removal of endogenous lipid cofactors.

A photoaffinity cross-linking methodology was employed to verify recovery of the delta-opiate receptor after its solubilization from membranes and reconstitution. Two membrane-associated proteins (50 and 70 kDa) were covalently tagged with an azido analog of beta-endorphin(LCu) in cell membranes and subsequently identified by immunoblotting with antisera directed against this opioid. Labeling of the 50-kDa polypeptide was prevented by coincubating assay samples with a relative excess of (d-Pen(2,5)enkephalin. This opioid binding polypeptide was also present in solubilized/reconstituted receptor preparations.

The mu-, delta-, and kappa-opiate receptors mediate a variety of opiate responses including: the control and perception of pain, the regulation of water balance, appetite, and immune system function (properties reviewed in Refs. 1-3). Each receptor is characterized by a distinctive anatomical distribution, and differential selectivity toward the binding of opioid peptides, opiate alkaloids, and synthetic benzomorphan drugs. Little is known, however, about the membrane-associated structural elements that allow each receptor to recognize selectively pharmacologic ligands.

The clonal neuroblastoma cell line NG108-15, obtained by fusion of a mouse neuroblastoma with a rat glioma (4), has been well developed as a model system to study the biochemical basis of opiate receptor function. These cells express an unusually large, homogeneous population of delta-opiate receptors (5, 6). The opioid peptide agonist beta-endorphin (beta-lipotropin 61-91) binds to this site with high affinity: low concentrations (<1 nM) are needed to displace most of the specific binding of an opioid to neuroblastoma cells (7). The cyclic, penicillamine (d-Pen(2,5)enkephalin analog (DPPDE) is a selective ligand for the delta-opiate site and binds with low affinity to mu- and kappa-opiate sites (8). By contrast, the antagonist naloxone (a morphinan alkaloid) binds with poor affinity to the delta-opiate site, relative to its interaction at mu-opiate binding sites in brain (9). Non-hydrolyzable GTP analogs lower the affinity of the delta-opiate receptor for agonists by preventing coupling of the occupied receptor to GTP-binding proteins (10). These analogs do not effect the binding of drugs which act as antagonists at this site, such as naloxone and the benzomorphan bremazocine (11). Functional association of delta-opiate receptors with the pertussis toxin-sensitive G protein results in the inhibition of adenyl cyclase (12), whereas the ability of opioids to regulate calcium channel function in NG108-15 cells appears to be mediated via coupling to the GTP-binding protein Go (13).

Several laboratories have successfully employed the bile salt detergents CHAPS and digitonin to solubilize opiate receptors from a variety of sources, including NG108-15 cells (14-16). In each instance, however, CHAPS and digitonin do not replace the normal membrane lipid environment in proximity to the receptor and specific opiate binding cannot be measured in the presence of a significant detergent concentration. Only a small fraction of the total solubilized receptor

1 The abbreviations used are: DPPDE, (d-Pen(2,5)enkephalin; G-protein, guanine nucleotide binding protein; G., an inhibitory guanine nucleotide binding protein; Gs, a guanine nucleotide binding protein whose function is not clearly defined; GppNHP, 5'-guanylylimidodiphosphate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, WOA, wheat germ agglutinin, HSAB, N-hydroxy succinimimidyl-4-azidobenzoate.

2 A concentration of the detergent in question which is above its critical micellar concentration (cmc). An approximate cmc value for digitonin of 5 mM in water, and 1 mM in a solution of 1 M NaCl, was measured in the laboratory at 4 °C (unpublished data) as a function of the detergent solubilization of the dye azulene (method of Koda et al. (17)). Cmc values determined for CHAPS were closely comparable to those previously reported (18).
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FIG. 1. Specific opiate binding to NG108-15 cell membranes. A, specific [3H]bremazocine binding was determined as the difference between binding measured in the presence and absence of an excess of levorphanol (10 μM). Inset, Scatchard analysis of the data. B. The displacement of [3H]bremazocine from cell membranes by β-endorphin(Leu9) (Δ), DPDPE (●), levorphanol (○), and naloxone (▲) is presented as the fractional recovery of specific binding relative to that measured in the presence and absence of levorphanol. The membrane preparation and assay methodology is described under "Materials and Methods." The data are representative of duplicate experiments.

binding activity is typically recovered after further purification, suggesting that essential lipid and/or protein cofactors have been lost during chromatographic fractionation of the receptor. This view is consistent with work suggesting a specific lipid requirement for the interaction of opiates with their membrane binding sites (19, 20). These studies further suggest that a reconstitution methodology will be required in order to study lipid/protein cofactor association with the solubilized receptor, and to restore activity to soluble preparations following conventional (non-affinity) chromatographic purification steps.

As a first step to a detailed biochemical characterization of the delta-opiate receptor we now report conditions which permit the reconstitution in lipid vesicles of pharmacologically specific ligand binding to soluble and partially purified preparations of the receptor. Evidence is presented which establishes that the brain lipid extract used in the reconstitution successfully replaces lipid cofactor interactions lost during the solubilization, and adequately stabilizes the native receptor binding site polypeptide(s) during the process of detergent removal. In addition, an opioid binding protein has been identified by photoaffinity labeling and its presence demonstrated in vesicles that possess high levels of reconstituted ligand binding activity.

MATERIALS AND METHODS

RESULTS

Specific Opiate Binding to NG108-15 Cell Membranes—Fig. 1A shows a saturation isotherm for specific binding of the opiate ligand [3H]bremazocine to membranes prepared from NG108-15 cells. Scatchard analysis of the data (Fig. 1A, inset) confirms that the large number of binding sites that are expressed (Bmax = 1.2 pmol/mg of membrane protein) correspond to a population of high affinity sites with a single apparent affinity constant (Kd) of 1.3 nM. Similar results were obtained for [3H](β-Pen2,5)enkephalin: this enkephalin analog binds with high selectivity to the delta-opiate receptor (data not shown). The relative ability of different ligands to competitively displace [3H]bremazocine binding from these membranes (Fig. 1B) conformed to a rank order of pharmacologic affinity (β-endorphin(Leu9) > DPDPE > levorphanol > naloxone) that is consistent with previous reports of specific ligand interaction at a delta-opiate site (6–8).

Solubilization and Reconstitution of the Delta-opiate Receptor—Membranes from NG108-15 cells were solubilized with increasing concentrations of digitonin and the extracts reconstituted into lipid vesicles. Fig. 2 depicts the recovery of specific ligand binding activity to these vesicle preparations. Solubilization of the delta-opiate receptor from membranes necessitated using digitonin concentrations in excess of 10 mM (relative to a membrane protein concentration of 10 mg/ml); these conditions effected the solubilization of >30% of the 'Materials and Methods' are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

FIG. 2. Solubilization of receptors from NG108-15 cell membranes. Specific opiate binding was determined using a [3H]bremazocine concentration of 10 nM following the dilution of detergent extracts in Buffer B (○) or after reconstitution in brain lipid vesicles (●). The percent solubilization of protein (Δ) refers to the fraction of membrane protein recovered in supernatants following the ultracentrifugation of detergent extracts. Detergent extracts were obtained by incubating NG108-15 cell membranes with variable (0–40 mM) concentrations of digitonin. Membrane solubilization, reconstitution, and assay methodology are described under "Materials and Methods." The data are representative of duplicate experiments.
the membrane protein. The recovery of specific [3H]bremazocine binding to the vesicle preparations was strictly dependent upon the brain lipid concentration used during the reconstitution. Ratios of lipid to solubilized membrane protein and detergent exceeding 4:1 (w/w) and 0.81 (w/w), respectively, were optimal (Fig. 3). Negative staining/electron microscopy of the vesicle preparations showed that uniformly sized proteoliposomes 100–200 μm in diameter were formed when the solubilized receptor was reconstituted (data not shown). The presence of a lipid bilayer was, however, insufficient alone to restore receptor function. Pure, vesicle-forming lipids which vary in headgroup size and net charge did not successfully replace the total brain lipid extract. Brain phosphatidylcholine, and the synthetic, dioleoyl analog of phosphatidylglycerol, reconstituted only a fractional amount of the specific opiate binding activity solubilized from cell membranes (Table I). Brain phosphatidylserine readily forms vesicles but could not restore receptor binding activity. Specific [3H]bremazocine binding to the solubilized/reconstituted receptor was not a result of drug binding to lipid; there was no specific opiate binding to vesicles prepared from the total brain lipid extract. These data suggest that the readdition of one or more specific lipid cofactors present in the brain lipid extract is required to restore high levels of specific ligand binding activity to the solubilized receptor. In the absence of a reconstitution strategy only a small fraction (<5%) of the original ligand binding activity present in NG108-15 cell membranes was recovered (Fig. 2). This solubilized activity was measured in extracts obtained at digitonin/membrane protein ratios of 2–3:1 by directly diluting samples 15-fold in the assay; this lowers the concentration of digitonin below its critical micellar concentration and reduces the ionic strength of the assay samples. No opiate binding activity could be directly restored by simple dilution of the extracts obtained at higher detergent concentrations; this result is presumably due to the inhibitory effect of digitonin in the assay. Taken together, these results demonstrate that the solubilized receptor is inactive, but intrinsically stable, in the presence of detergent and can be reactivated after rehydration into an appropriate lipid environment.

A saturation isotherm for [3H]bremazocine binding to vesicle preparations is shown in Fig. 4A. Scatchard analysis of the data (Fig. 4A, inset) demonstrates the presence of a single, high affinity binding site. A linear best fit of the data yielded a dissociation constant value (Kd) of 4.2 nM for [3H]bremazocine, a 3.2-fold increase over the value of 1.3 nM measured in NG108-15 cell membranes. The maximal number of sites (B_{max}) measured for the reconstituted receptor was in excess of 7 fmol/mg of solubilized/reconstituted membrane protein. This value is consistent with a combined 6-fold enrichment of receptor activity during centrifugation steps which follow the detergent solubilization of cell membranes and reconstitution of the soluble receptor. That is, only 30% of the membrane protein need be solubilized from cell membranes to yield a maximal recovery of specific opiate binding activity, and only 33–40% of this soluble protein is typically recovered in lipid vesicles following the reconstitution of these membrane extracts. The results presented in both Fig. 4 and Table I demonstrate an overall recovery of receptor binding activity following its solubilization from NG108-15 cell membranes and reconstitution in brain lipid vesicles that was nearly quantitative.

Measurements of competitive drug binding to the solubilized/reconstituted receptor preparations demonstrated that the binding of opioid peptides (β-endorphin(Leu5), DPDPE) and morphinan alkaloids (levorphanol, naloxone) was preserved in lipid vesicles (Fig. 4B). Additionally, these ligands followed a rank order of relative binding affinity (β-endorphin(Leu5) > DPDPE, levorphanol > naloxone) which was comparable to that obtained for NG108-15 cell membranes. Shifts in the IC_{50} values for agonist binding which occurred following solubilization of the delta-opiate receptor from cell membranes were attributable to the loss of the receptor G_{i} interaction in solubilized preparations. This result was expected since large changes in agonist binding affinity were previously observed after solubilized mscarcin acetylcholine and mu-opiate receptors were reconstituted in the absence of added G_{i}/G_{o} (29, 30). The ability of the peptide agonist β-endorphin(Leu5) to compete for specific binding with the

### Table I

<table>
<thead>
<tr>
<th>Lipid</th>
<th>[3H]bremazocine binding</th>
<th>Recovery of activitya</th>
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</thead>
<tbody>
<tr>
<td>Brain extract</td>
<td>5875/4900</td>
<td>98/81</td>
</tr>
<tr>
<td>Phosphatidylcholine (brain)</td>
<td>1505/1640</td>
<td>24/19</td>
</tr>
<tr>
<td>Phosphatidylglycerol (dioleoyl)</td>
<td>1690/1850</td>
<td>22/24</td>
</tr>
<tr>
<td>Phosphatidylserine (brain)</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Brain extract (no receptors)</td>
<td>0/0</td>
<td>0/0</td>
</tr>
</tbody>
</table>

* Determined as that percentage of original, membrane-associated ligand binding activity recovered by reconstitution following the solubilization of membranes with digitonin.

* The difference between total and nonspecific binding was not statistically significant.

* Control binding to vesicles prepared in the absence of solubilized receptor.

Fig. 3. Lipid-dependent reconstitution of receptors. Varying amounts of brain lipid vesicles were added to fixed amounts of a detergent extract obtained by solubilizing cell membranes with 17.5 mM digitonin. Specific opiate binding to the vesicle preparations (♀) was determined using a [3H]bremazocine concentration of 11 nM. The percent recovery of protein (△) refers to the yield of protein in the sedimenting vesicles. The reconstitution and assay methodology employed is described under "Materials and Methods." The data are representative of duplicate experiments.
delta-opiate receptor antagonist \(^{3}H\)bremazocine in NG108-15 cell membranes was predictably impaired in the presence of the non-hydrolyzable guanosine 5'-triphosphate analog GppNHP. The concentration of \(\beta\)-endorphin(Leu\(^5\)) required to displace 50% of the specific \(^{3}H\)bremazocine binding in NG108-15 cell membrane preparations was increased more than 40-fold in the presence of 10 \(\mu\)M GppNHP, from 0.5 to 21 \(\mu\)M (Fig. 5A). Ligand binding to vesicle preparations containing the receptor was, however, unaffected by GppNHP addition (Fig. 5B). The IC\(_{50}\) value of 42 \(\mu\)M measured for the competitive binding of \(\beta\)-endorphin(Leu\(^5\)) in vesicle preparations differed only 2-fold from that determined for NG108-15 cell membranes in the presence of GppNHP. Differences in naloxone binding observed between membrane and vesicle preparations could not be explained by \(G\) protein dissociation and suggests the loss of some additional determinant of receptor function.

**Chromatography on WGA-Sepharose—**Reconstitution experiments performed with partially purified receptor preparations allowed us to clearly demonstrate that the conditions of the reconstitution are both necessary and sufficient to restore ligand binding to the solubilized delta-opiate receptor. To further purify the receptor detergent extracts of NG108-15 cell membranes were absorbed to a lectin affinity column and eluted from the column in a gradient of increasing acetoni trile concentration (data not shown). All chromatography steps were performed in the presence of 10 mM digitonin to ensure the effective sorting of hydrophobic membrane proteins. More than 50% of the receptor activity which originally bound to the WGA-Sepharose column was subsequently eluted with 0.5 M \(\beta\)-N-acetyl-D-glucosamine (Fig. 6). Activity was recovered in fractions 4–12 by removing detergent from the partially purified material in the presence of added brain lipid, as before. The maximal levels of specific \(^{3}H\)bremazocine binding recovered in fractions 5–8 represents a 19-fold purification of the delta-opiate receptor from NG108-15 cell membranes. Notably, >90% of the lectin-purified soluble protein was recovered by the centrifugation of vesicles following reconstitution. This finding suggests that the essential receptor binding site polypeptide(s) is an integral membrane glycoprotein which strongly associates with lipid during reconstitution. This view is also consistent with our previous observation that only a fractional amount of the membrane-associated protein present in detergent extracts of cell membranes, but almost all of the receptor activity, can be recovered in vesicle preparations.

**Identification of Receptor Binding Site Polypeptides—**A photoaffinity analog of \(\beta\)-endorphin(Leu\(^5\)) was used to characterize receptor binding site polypeptides present in NG108-15 cell membrane and solubilized/reconstituted vesicle preparations. These modified preparations of opioid peptide competitively displaced \(^{3}H\)bremazocine binding; this result demonstrates that the N-terminal azido-modification of \(\beta\)-endorphin(Leu\(^5\)) was mostly avoided. Several photocross reactive \(\beta\)-endorphin(Leu\(^5\)) species were generated by this method, four additional peaks eluted from a C-18 high performance liquid chromatography column in a gradient of increasing acetonitrile concentration (data not shown).

Two polypeptides that have apparent molecular masses of 70 and 50 kDa were identified by immunoblotting, using antibody directed against \(\beta\)-endorphin(Leu\(^5\)), after NG108-15 cell membranes were incubated with photoreactive \(\beta\)-endorphin(Leu\(^5\)) and exposed to ultraviolet light (Fig. 7A). Incubating cell membranes in the presence of a large relative excess (10 \(\mu\)M) of the enkephalin analog DPDPE blocked subsequent cross-linking to the 60-kDa protein; labeling of the 70-kDa protein was still apparent under these conditions. Levorphanol also prevented labeling of the 50-kDa protein (data not shown). This evidence established the identity of the 50-kDa polypeptide as a delta-opiate receptor binding site polypeptide. The 50-kDa polypeptide was also tagged by azido-\(\beta\)-endorphin(Leu\(^5\)) when vesicle preparations were used in the photoaffinity cross-linking studies. Membrane solubilization and vesicle reconstitution of the delta-opiate receptor yielded a 6-fold increase in specific ligand binding activity; an enhancement in the labeling of the 50-kDa protein relative to that of the 70-kDa protein was also observed (Fig. 7B).
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Fig. 5. Displacement of specific opiate binding by β-endorphin(Leu'). The displacement of [3H]bremazocine binding by β-endorphin(Leu') is presented as the fractional recovery of specific binding relative to that measured in the presence and absence of 10 μM levorphanol (△). Assays were performed using NG108-15 cell membranes (A) or using receptor preparations obtained by solubilizing membranes with 17.5 mM digitonin and reconstituting extracts in brain lipid vesicles (B). Assays were repeated (▲) after preincubating membrane and vesicle preparations at 25 °C in the presence of a 10 μM concentration of GppNHp. Measurements of control [3H]bremazocine binding (*10 pM levorphanol) were unaffected by GppNHp addition. The reconstitution and assay methodology employed is described under "Materials and Methods." The data are representative of duplicate experiments.

Fig. 6. Lectin affinity chromatography of the solubilized receptor. The recovery of specific opiate binding activity eluting from a 10-ml column of WGA-Sepharose 6MB (▲) was determined after reconstituting 2-ml fractions in brain lipid vesicles. The vesicle preparations were incubated with 10 nM [3H]bremazocine in the presence and absence of 10 μM levorphanol. The elution of protein from the column (△) in total accounted for 5% of the protein originally present in the detergent extract. The solubilization, chromatography, reconstitution, and assay methodology employed is described under "Materials and Methods." The data are representative of duplicate experiments.

The 70-kDa protein labeled in cell membranes appears to be a relatively abundant membrane-associated protein visible on sodium dodecyl sulfate-polyacrylamide gels by Coomassie Blue staining which nonspecifically binds β-endorphin. The identity of this protein is suggested by work showing that β-endorphin nonspecifically binds the 66-kDa serum albumin (31) and the plasma S-protein/vitronectin glycoprotein (32). Both of these proteins would only associate peripherally with cell membranes and are not likely to be incorporated into lipid vesicles under the reconstitution conditions employed in this study.

DISCUSSION

Characterization of the structural determinants contributing to opiate receptor function has been difficult owing to the typically small receptor number in cell membranes, and low yields of activity following detergent solubilization and partial purification. In this paper, evidence has been presented which shows that specific radioligand binding to delta-opiate receptors solubilized from NG108-15 cell membranes can be successfully restored by incorporating detergent extracts into lipid vesicles prepared from a total brain lipid extract. The recovery of activity was strictly dependent upon both the concentration of digitonin used during extraction, and the ratio of extracted protein to added lipid used in the subsequent reconstitution. A specific and saturable association of the radiolabeled antagonist [3H]bremazocine with vesicles was shown, and competitive dissociation of this binding interaction by opioid peptide and morphinan alkaloid agonists demonstrated. Relative differences in measurements of agonist binding affinities between vesicle preparations and the membrane-associated delta-opiate receptor were largely explained by dissociation of the solubilized receptor from GTP-binding proteins. It is unlikely that both delta-opiate receptors and Gi/Gi are incorporated into vesicles during reconstitution since the formation of a ternary complex following agonist binding would be expected if both were present.

Several criteria were used to establish that the brain lipid preparation employed in the reconstitution was both necessary and sufficient to restore specific ligand binding to the solubilized receptor. First, the recovery of activity was strictly
The novel photoaffinity cross-linking methodology used in these studies allowed independent confirmation of the reconstitution of a receptor binding site polypeptide in solubilized/reconstituted vesicle preparations of the delta-opiate receptor. Two membrane polypeptides (50 and 70 kDa) were covalently tagged with an azido-β-endorphin(Leu5) analog and identified with antibodies directed against β-endorphin(Leu5), but only the 50-kDa protein could be classified as a binding site polypeptide associated with the delta-opiate receptor. This was also the only polypeptide that could be labeled after solubilizing the receptor binding sites from cell membranes in digitonin, and reconstituting enhanced levels of specific opiate binding in brain lipid vesicles. At present, it is not known if this tagged 50-kDa polypeptide is a native receptor protein distinct from the 58-kDa protein previously affinity labeled in NG108-15 cell membranes with both an isothiocyanate analog of fentanyl (35), and an azido analog of enkephalin (36). The methodology used in this paper notably differs from these studies by avoiding chemical and isotopic modification of β-endorphin(Leu5) in its biologically active (enkephalin) portion.

Conventional chromatographic isolation of the delta-opiate receptor may now be possible since the efficient recovery of specific ligand binding can be accomplished by reconstitution after sorting receptors in the presence of detergent. This approach has previously been used by Fishman et al. (37) to purify the G-protein coupled vasopressin (IV) receptor 21,000-fold from rat liver membranes. By comparison, a theoretical purification of >10,000-fold will be required in order to purify the 50-kDa delta-opiate receptor binding site polypeptide to homogeneity from NG108-15 cell membranes. This is a requisite receptor purification strategy at present since opiate binding activity has not yet been reconstituted in the presence of a detergent, precluding the use of an effective affinity sorting strategy. The technique of affinity cross-linking and antibody detection described in this paper can be used as a highly sensitive tool to independently detect essential receptor polypeptides during chromatographic isolation of the solubilized receptor. By tagging the 50-kDa binding site polypeptide with azido-β-endorphin(Leu5) in NG108-15 cell membranes prior to detergent solubilization the delta-opiate receptor can then be located in eluting column fractions by standard immunoblotting techniques.

In conclusion, solubilized and partially purified preparations of the delta-opiate receptor have been used in studies aimed at characterizing determinants which are essential to its function. A lipid cofactor dependency has been established for the receptor, and a receptor binding site polypeptide identified. The feasibility of using reconstitution and cross-linking methodology as part of a purification scheme has also been demonstrated.

**Acknowledgment**—Skillful negative staining/electron microscopy studies of the solubilized/reconstituted delta-opiate receptor preparations were performed by Dr. D. H. Hall of the Department of Neuroscience. His expertise in performing this work is most appreciated.

**REFERENCES**

Reconstitution of the Delta-Opiate Receptor


Materials and Methods

Materials

[Detailed list of materials used in the experiments, including chemicals, reagents, and other necessary supplies.]