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MOLECULAR CHARACTERIZATION OF OPIOID RECEPTORS

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INTRODUCTION

Opioid receptors were identified biochemically in mammalian brain in the early 1970s (1–3), and since then their pharmacological properties have been extensively characterized (for reviews, see 4–7). However, little is yet known about the molecular properties of these receptors. Complete structural and functional characterization of a receptor requires its purification, reconstitution in a membrane environment, and, ultimately, the isolation and cloning of its gene. Progress in these areas has lagged considerably behind that for other cell surface receptors.

There are several reasons for this slow progress. First, opioid receptors have proved to be very difficult to solubilize in a form that retains ligand-binding activity, the conventional first step in most purification schemes. Opioid binding is eliminated or greatly reduced by low concentrations of most of the non-ionic detergents used to solubilize other cell surface receptors. At least part of this problem may be due to the more stringent criteria necessary to establish the existence of opioid receptors. The activity of most cell surface receptors is measured by specific, or displaceable, binding, which is defined as the difference between the amount of radioactive ligand bound in the presence and absence of a large amount of the same unlabelled ligand. A genuine opioid receptor, in contrast, must exhibit not only specific, but stereospecific binding—that which is selective for the l-forms of stereoisomeric agonists, such as Levorphanol and Levallorphan.

In fact, most laboratories where solubilization of opioid receptors has been reported have demonstrated the existence only of specific binding in their

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preparations. Stereospecific binding either is very low, or is not reported at all. While it is conceivable that opioid receptors lose stereospecificity upon solubilization, obviously the inability to demonstrate it casts doubt on the pharmacological relevance of the preparation.

A second obstacle to purification of opioid receptors is that no simple biochemical process mediating opioid-receptor function has yet been identified. Opioid receptors have been classically defined as those promoting antinociception in mammals or, in some cases, by their effects in certain in vitro tissue systems. Recent work has indicated an association between opioid receptors and several other functional molecules in cell membranes, including adenylate cyclase (8, 9) and ion channels (10–13). However, none of these molecules has been demonstrated to play a role in the process of antinociception. Thus, there is still no definitive biochemical test to confirm the functional relevancc of a putative isolated opioid receptor.

Finally, opioid receptors are heterogeneous. At least three different classes are present in brain, mu (μ), delta (δ) and kappa (κ), which differ in their ligand selectivity and in their pharmacological effects (14, 15). Other receptor types may exist, in brain or in peripheral tissues (16, 17), as well as subtypes for μ (18, 19) and κ (20) receptors. Because most opioid ligands are not completely selective for a single receptor type, it is difficult to isolate a single type, which complicates pharmacological characterization.

Despite these problems, however, substantial progress has been made in recent years toward the purification and molecular characterization of opioid receptors. The introduction of new nonionic detergents, especially 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate (CHAPS), has often led to higher yields of solubilized opioid-binding activity. Purification of specific opioid-receptor types has been facilitated by the use of tissue sources pure or enriched in one type, such as NG108-15 neuroblastoma-glioma hybrid cells (δ opioid receptors) and human placenta (κ receptors), as well as by the development of synthetic ligands with relatively high specificity for a particular opioid receptor type. Several new covalent opioid ligands have been synthesized that are useful in identifying and purifying opioid-binding proteins in the presence of denaturing solvents; thus, the problem of maintaining ligand-binding activity during purification is circumvented. Idiotypic and anti-idiotypic antibodies against opioid ligands have been developed and used to purify opioid receptors retaining binding activity.

As purified or partially purified preparations of opioid receptors have become available, investigators have also begun to characterize them, deploying the full armory of tools made available by modern molecular biology. Several groups have now prepared antibodies to opioid receptors, which can be used to map the distribution of receptors in the brain, to determine the regions in the receptor molecule involved in ligand binding and other functions, to compare different opioid receptor types, and in further purification of

the receptor. At least one group has also cloned a putative opioid receptor, making possible for the first time elucidation of its amino acid sequence, and, to some extent, its secondary structure.

In this article, we briefly review these developments and discuss some of their implications for the structure and function of opioid receptors. We also briefly consider work aimed at determining the second messengers associated with opioid receptors, which may mediate their pharmacological effects.

SOLUBILIZATION, PURIFICATION AND MOLECULAR WEIGHT ANALYSIS OF OPIOID RECEPTORS

There are two basic approaches used to purify cell surface receptors; the first solubilizes the receptor in a nondenaturing detergent that preserves ligand-binding activity, and then isolates it from other membrane components by various fractionation steps, such as affinity chromatography, with ligand-binding activity monitored at each step. The alternative approach is to label the receptor with a radioactive, covalently bound ligand, solubilize it with a denaturing detergent, and follow its purification by means of the radioactive label.

The first approach is generally preferred, as binding activity must be retained if the purified receptor is to be reconstituted and tested for function. An additional advantage, as applied to opioid receptors, is that it may be possible to purify more than one receptor type from a single solubilized preparation, by using several affinity columns that differ in their selectivity for these receptor types. When opioid receptors are covalently labelled, the selectivity of purification is largely dependent upon the selectivity of the covalent ligand, though some additional selectivity may be obtained by protecting particular receptor types with reversibly bound ligand during the labelling process. In any case, once the covalent-labelling process has occurred, no further selectivity is possible.

On the other hand, if a suitable covalent label can be found, the second approach to purification is usually faster and easier, and the molecular weight of the putative receptor can be determined by SDS gel electrophoresis, even without purification. And though covalently labelled opioid receptors lack ligand-binding activity, in principle they can be used to purify active receptors in an indirect way, by means of cDNA cloning techniques. This is discussed in further detail below.

Purification of Opioid Receptors Retaining Ligand Binding

For several years after their discovery, a number of laboratories tried unsuccessfully to solubilize opioid receptors in a variety of relatively mild detergents. In one of the first successful studies, Bidlack et al (21) reported solubilization of rat brain membranes with Triton X-100; they then purified

the opioid-binding material by means of affinity chromatography, using 14-β-bromoacetamido-morphine. The resulting preparation bound dihydromorphine and several other opioids with nM affinity, but the specific binding activity (cpm/mg. protein) was at least an order of magnitude lower than theoretically expected for a pure receptor. SDS gel analysis revealed three polypeptides of molecular weights 25–50,000.

Simon and colleagues solubilized opioid receptors from toad and later rat and other mammalian brain preparations with digitonin (22). Using opioid ligand- and lectin-affinity chromatography, they purified an opioid-binding protein from bovine striatum to theoretical homogeneity (23). However, protein estimation was difficult because of the small amounts isolated and the presence of detergent. The opioid-binding material had a mol wt of 300–350 kd under nondenaturing conditions, and 65 kd on SDS gels. The μ nature of this opioid-binding material was not demonstrated directly, but was suggested by its ability to bind the μ-selective ligand D-ala^2-N-met^4-enkephalin-gly-ol (DAMGO) and β-endorphin, and its isolation from a source low in δ receptors.

Maneckjee et al (24) reported partial purification (500-fold) of a μ-specific opioid receptor from rat brain, using CHAPS as solubilizer; SDS gel electrophoresis indicated the presence of 3 peptides, of molecular weights 94kd, 42kd, and 35kd, respectively. Subsequently, they prepared a more highly purified receptor preparation from bovine striatum (25), using a combination of affinity chromatography and hydroxyapatite chromatography. Studies with polyclonal antibodies prepared to this protein (discussed further below) indicated that the 94 kd component was involved in opioid binding.

Using a combination of affinity chromatography, lectin chromatography, and gel filtration, Cho et al (26) reported purification from bovine brain of a 58 kd protein selective for opioid alkaloid ligands. A novel feature of this protein was that it required acidic lipids possessing unsaturated fatty acids in order to manifest binding activity; neither the protein nor the lipids alone possessed significant opioid binding (27). The binding affinities of ligands to this reconstituted material were lower than the corresponding values for binding to brain membranes, but the rank order of binding affinities to the two preparations were highly correlated (26, 27).

Ueda et al (28, 29) used a similar method to obtain a protein consisting primarily of a 58 kd band. When this preparation was reconstituted with the purified G-proteins G_i or G_o, a large increase in displacement of ^{3}H-naloxone binding by the μ agonist DAMGO was observed, and this increase was sensitive to GTP. Mu agonists, but not δ or κ agonists, also stimulated GTP binding and GTPase activity in this preparation. Thus this work suggested that μ opioid receptors may exert their in vivo effects through a G-protein.

As the above discussion shows, many of the solubilized opioid receptor preparations initially reported did not bind κ ligands. However, Chow &

Zukin (30) reported that CHAPS-solubilized rat brain membranes contained κ- as well as μ-binding species, as assayed by ^{3}H-bremazocine binding. The material eluted as two peaks of 50 and about 250 kd, respectively. Itzhak and colleagues (31–33) solubilized guinea pig brain membranes using digitonin in the presence of high NaCl concentrations, and found that δ binding was associated with material of 750–875 kd, while κ-binding was associated with material of about 400 kd.

DeMoliou-Mason & Barnard (34) used digitonin extraction in the presence of 10 mM $MgCl_2$ to solubilize material that bound dynorphin-(1-9), a putative κ ligand, as well as μ- and δ-selective ligands. Subsequently, they characterized this binding material by gel filtration (35). Delta ligands were associated with material over 500 kd, while both μ alkaloids and dynorphin-(1-9) were associated with both this high molecular weight material and with lower molecular weight material.

Simon et al (36) purified κ opioid receptors from digitonin-solubilized frog brain membranes. An affinity column consisting of DADLE coupled to Sepharose-6B was used to isolate μ, δ, and κ receptors from the solubilized preparation, and κ receptors were separated from the other two by gel filtration. The extent of purification was over 4,000, based on pmol of ^{3}H-EKC bound/mg. protein. SDS gel analysis revealed two bands, of 65 and 58 kd.

Ahmed and coworkers (37, 38) reported purification of κ opioid receptors from human placental tissue, which, is thought to be highly enriched in this specific type. The protein had a mol wt of 63 kd, and exhibited high affinity (nM range) binding for κ opioid ligands such as bremazocine, ethylketocyclazocine, U-50,488H and dynorphin 1-8. The iodinated receptor migrated as a single band on SDS gels, but since the purified receptor's binding capacity (fmole/mg. protein) was only about half of the theoretical value, the authors were not certain that this preparation was homogeneous.

Purification and Analysis of Covalently Labelled Opioid Receptors

Because it has proved so difficult to solubilize opioid receptors in a form retaining ligand-binding properties some investigators have used an alternative approach in which the receptor is labelled with a radioactive, covalently bound ligand. The receptor can then be solubilized with a denaturing detergent, and purification followed by means of the radioactive label. The fact that many opioid ligands are peptides has made this approach especially attractive, for in addition to synthesizing and testing compounds capable of reacting covalently with opioid receptors, some researchers have covalently attached opioid peptides to the receptor by various bifunctional cross-linking reagents.

Some of the earliest attempts to purify opioid receptors used the covalent-

labelling approach. However, in most cases, it could not be demonstrated that the label specifically bound to opioid receptors (39). More recently, a number of covalent opioid ligands have been synthesized that have proven useful for selectively eliminating opioid receptor types, including β-funaltrexamine (FNA), chlornaltrexamine (CNA), 2-(4-ethoxybenzyl)-1-diethylaminoethyl-5-isothiocyanatobenzimidoazole (BIT), N-phenyl-N-[1-(2-(4-isothiocyanato) phenylethyl)-4-piperidinyl]pro-panamide (FIT), 6-desoxy-oxymorphone (FOXY), and 6-desoxy-6β-fluoronaltrexone (cycloFOXY) (40–43).

Simonds et al (44) covalently labelled δ receptors on NG108-15 neuroblastoma-glioma cells with triated FIT, which served as a marker to follow the receptors in subsequent purification steps. Then antibodies to protein-conjugated FIT were prepared and used to construct an affinity column. When detergent-solubilized hybrid cell membranes were applied to this column, the FIT-receptor complex was preferentially bound, thus effecting a major purification step. When this step was combined with an additional lectin-affinity column elution, the receptor-FIT complex was purified to essentially homogeneity. SDS gel analysis indicated that it had a molecular weight of 58 kd.

Newman & Barnard (45) synthesized DALECK, an enkephalin derivative possessing a chloromethyl ketone group at its C-terminus. The ligand was capable of binding both reversibly and irreversibly to rat brain opioid receptors, depending on the pH. Reversible binding occurred to both μ and δ receptors, while the irreversible binding was highly specific for μ receptors. Analysis of the irreversibly bound material on SDS gels indicated a single species, of molecular weight 58 kd. However, another group, using the same ligand at higher specific activity, reported labelling of additional bands of higher and lower molecular weight (46).

Liu-Chen & Phillips (47) labelled bovine striatal membranes with ^{3}H-β-funaltrexamine, and showed that the irreversible portion of this binding was specific for μ opioid receptors. SDS gel analysis indicated that the label was associated with material in the molecular weight range 68–97 kd. The diffuse migration pattern was presumed to be due to the attachment of variable amounts of carbohydrate, for the labelled material was adsorbed by wheat germ lectin-Sepharose.

Bochet et al (48) labelled rat brain and NG108-15 cell membranes with the photoaffinity δ ligand [D-thr^2,leu^5][tyrosyl 3,5-^{3}H]Enkephalyl threonine (azido-DTLET). In rat brain, the ligand labelled two bands of 44 and 34 kd, and a 33 kd band in NG cells. Yeung (49) prepared a photoreactive enkephalin derivative, which labelled a 46 kd protein in rat brain and spinal cord membranes. Photolabelling was inhibited by μ, δ, and κ ligands, suggesting that the 46 kd band was a peptide shared by all three receptor types.

Zukin & Kream (50) first applied the cross-linking technique using D-ala^2-D-leu^5-enkephalinamide (DADLE) as ligand. The material so labelled had a

molecular weight of 380 kd under nondenaturing conditions, and 35 kd on SDS gels. However, as the only available group for cross-linking on DADLE is the terminal amino group, which is presumably directly involved in binding, it could be argued that during cross-linking, DADLE was moved from its binding site.

We cross-linked ^{3}H-β-endorphin to brain membranes that had previously been solubilized with the nonionic detergent Brij 36-T. This opioid peptide contains several internal amino groups that could serve as the site of covalent attachment without interfering with the binding process. SDS gel analysis of the solubilized, cross-linked material revealed a broad spectrum of species, from 2–200 kd (51).

Howard et al (52, 53) covalently labelled opioid receptors in brain membranes by cross-linking ^{125}I-β-endorphin with the bifunctional reagent bis[2-succinimido-oxycarbonyl-oxy-O-ethyl] sulfone (BSOCOES). Membranes were incubated with the radioactive ligand, then centrifuged to remove free ligand, and incubated with the cross-linking reagent. By carrying out the original incubation with β-endorphin in the presence of unlabelled ligands selective for μ, δ, or κ receptors, and analyzing the cross-linked products on SDS gels, they concluded that β-endorphin labelled both μ and δ receptors, and that the latter could be distinguished by molecular weight. The μ receptors, identified as bands cross-linked in the presence of δ and κ agonists, were associated with a major 65 kd band and a minor 38 kd band, while the δ receptors consisted of a major 53 kd band and a minor 25 kd band. The major 53 kd band, but not the 65 kd band, was also observed in cross-linked membranes from NG108-15 hybrid cells, which contain only δ opioid receptors.

Bero et al (54), using a similar technique, found that ^{125}I-β-endorphin labelled three peptides of molecular weight 108, 73, and 49 kd. However, the relationship of these three species to μ, δ, and κ receptors was not clear. Preincubation with unlabelled β-endorphin was capable of blocking much or most of the cross-linking to each of these species, but other ligands were much less effective. Thus, etorphine, which binds to all three opioid receptor types, blocked 60% of the cross-linking to the 73 kd band, but less than 40% to either of the other two bands. The μ-selective ligand DAMGO and the κ-selective U-50,488H blocked 30–50% of the binding to the 10 and 73 kd bands, while the δ-selective ligand DPDPE was ineffective in blocking cross-linking to any of the species.

Target Analysis of Opioid Receptors

Many laboratories have now reported full or partial purification of opioid receptors, suggesting that rapid advances in our understanding of these receptors at the molecular level are now possible. However, one major outstanding

problem is considerable variety in the molecular size of these preparations, as reported by different investigators. When the molecular weight was determined under nondenaturing conditions, such discrepancies are to be expected, as they may be due to association of the binding proteins with other membrane proteins, as well as with detergent micelles. Differences in molecular weights as determined on SDS gels, on the other hand, are more difficult to reconcile, as they must reflect distinctly different species.

It is possible that different opioid-receptor types have different molecular weights, and some support for this is provided by cross-linking experiments in which μ- or δ-selective ligands are used to protect sites from cross-linking (53, 54). However, discrepancies in reported molecular weights exist even for putative opioid receptors of the same type.

On the other hand, a molecular weight of approximately 60 kD has been found by some groups for all three major opioid receptor types (23, 26, 28, 36, 37, 44, 45). This suggests that these three opioid-receptor types could all be closely related molecules. For example, they could be identical gene products that are modified differentially after translation, or even identical species that assume different binding specificities depending on their association with other molecules in the cell membrane.

An alternative approach to determining the molecular weight of a membrane receptor, which does not require its extraction from its membrane environment, is target analysis. In this technique, the cell membrane is subjected to electron beam irradiation of various intensities, and the degree of inactivation of the receptor, as measured by ligand binding or some functional assay, is determined as a function of degree of irradiation. If one assumes that bombardment by a single electron is sufficient to inactivate the receptor, then the molecular size of the latter can be calculated from the irradiation vs inactivation curve.

Because target analysis is conducted on the receptor in situ, it can be used to estimate the molecular weight of different opioid-receptor types in the same experiment, simply by conducting the binding assays using ligands selective for these different types. Target analysis can also distinguish between the actual ligand-binding component, and any larger receptor complexes, consisting of the binding component in association with other molecules. This is done by conducting the assays for the receptor in the presence or absence of conditions favoring association of the binding component with the other components.

In one of the earliest applications of this technique to opioid receptors, McLawhon et al (56) studied the δ opioid receptor in NG108-15 cells, using either ^{3}H-DADLE or ^{3}H-naloxone as ligand, and obtained a value of 200 kd. In contrast, Lai et al (57) estimated a value of 110 kd for both μ and δ receptors in rat brain. Tao et al (58) also analyzed rat brain opioid receptors by

target analysis, assaying ligand binding with ^{3}H-diprenorphine, which binds to μ, δ, and κ opioid receptors. In the presence of D-ala^2-D-leu^5-enkephalin (DADLE), NaCl and Gpp(NH)p, the estimated molecular size of the receptor was 313 kd, but in the presence of only NaCl, or NaCl and Gpp(NH)p, the estimated size was 165 kd. In the presence of Gpp(NH)p, the size was 217 kd, but 286 kd in the presence of Mg^{++}.

Tao et al (58) suggested that the 165 kd value obtained in the presence of NaCl or NaCl and Gpp(NH)p was the size of the uncoupled receptor. In the presence of DADLE, NaCl and Gpp(NH)p, the receptor was coupled to a GTP-binding component of 96 kd (including not only the α subunit, but the β and γ subunits as well), and a Na^+-binding component of 52 kd. In the presence of Gpp(NH)p, it was coupled only to the Na^+-binding component, whereas in the presence of Mg^{++}, it was coupled only to the GTP-binding component.

Ott et al (59) applied target analysis to opioid receptors in rat, guinea pig, and frog brain, as well as in NG108-15 neuroblastoma-glioma hybrid cells. By determining binding to different ligands, they were able to estimate the molecular weights of μ, δ, and κ opioid receptors. Furthermore, they also conducted assays in the presence or absence of Na^+, Mg^{++} and GTP, which are required for coupling of receptors to G-proteins. They found that all three opioid-receptor types had a molecular weight of 98 kd in the presence of ions and GTP, and a molecular weight of 56 kd in their absence. The latter value, though much smaller than the size reported by other laboratories using target analysis, is in close agreement with the 58–65 kd values for purified opioid receptors reported by several other groups (see above). Moreover, the 44 kd increase observed in the presence of ions and GTP is consistent with association with the α subunit of the inhibitory G-proteins G_i (α_i), which is approximately 40 kd in molecular weight.

However, subsequent studies by this group led them to conclude that the 100 kd species does not include a G-protein (60). Moreover, in agreement with Tao et al (58), they found evidence for a Na^+-binding component in association with the opioid receptor. In the presence of 100 mM Nat^+, the inactivation curve became biphasic, leading Ott et al (60) to conclude that a Na^+-binding component that regulates opioid binding is destroyed at low doses of radiation. The component was estimated at 168 kd.

Target analysis thus leads to molecular size estimates of opioid receptors that are considerably greater than most investigators have obtained from SDS gel analyses of purified or covalently labelled receptors (see above). This suggests that, in situ, the receptors may be complexed with other components of unknown function. However, other studies (57–59) are all consistent with notion that under a given set of conditions different opioid-receptor types are approximately the same molecular weight. Ott et al (59) found identical

molecular weights for μ, δ, and κ receptors, and Lai et al (57) reported identical molecular weights for μ and δ receptors. Although Tao et al (58) assayed opioid binding with ^{3}H-diprenorphine, which should bind to all three receptor types, the plot of log inactivation against radiation dose was linear under all conditions; this result suggests that the receptors being measured were of a single size.

MOLECULAR CHARACTERIZATION OF OPIOID RECEPTORS

Now that purified opioid receptors are available, their detailed molecular characterization may finally proceed, following methods well established for other cell-surface receptors. Purification of a cell-surface receptor is just the first step in its molecular characterization. Antibodies to the receptor can be prepared and used in a variety of studies, including the mapping of functional domains within the receptor, as well as determining its cellular and brain-regional localization. The receptor can be reconstituted into a membrane, with the demonstration of some functional activity. The receptor DNA can be cloned, transfected into a cell line, and its pharmacological properties determined and compared to that of in situ receptors. This section describes the work done in this area.

Preparation and Characterization of Antibodies to Opioid Receptors

As well as helping in characterizing purified receptors, antibodies can also be used as a rapid and efficient means of purification. Although antibodies are simplest to raise against purified receptor preparations, they can also be prepared against preparations that are only partially pure. Moreover, the receptor need not bind ligand. Antibodies can be made to denatured receptors, such as those identified by covalent labelling with a tritiated ligand, and partially purified by SDS gel electrophoresis. Antibodies to receptors can even be prepared in the absence of receptors, by preparing antibodies to ligand, then using these antibodies as antigen to prepare anti-idiotypic antibodies. Once such antibodies are available, they can be efficiently screened by their ability to inhibit opioid binding in vitro.

Several recent reports illustrate the power and flexibility of this approach in purifying opioid receptors. Simonds et al (44) covalently labelled δ receptors on NG108-15 neuroblastoma-glioma cells with tritiated FIT, which served as a marker to follow the receptors in subsequent purification steps. Then antibodies to protein-conjugated FIT were prepared and used to construct an affinity column. When detergent-solubilized hybrid cell membranes were applied to this column, the FIT-receptor complex was preferentially bound,

thus effecting a major purification step. When this step was combined with an additional lectin-affinity column elution, the receptor-FIT complex was purified to essentially homogeneity. It had a molecular weight of 58 kd.

An alternative immunological approach, which has the considerable advantage of requiring no receptor preparation to begin with, is to prepare anti-idiotypic antibodies. Antibodies are first raised to an opioid ligand, which in principle should contain a site similar to the ligand-binding site of the receptor. These antibodies are then used as an antigen to raise anti-antibodies, which should be directed against this binding site.

Using β-endorphin as the original opiod ligand, Gramsch et al (61) prepared a monoclonal antibody that exhibited binding characteristics similar to opioid receptors. They then used this as an antigen to prepare monoclonal anti-idiotypic antibodies; the resulting hybridoma clones were screened using Fab fragments of the anti-β-endorphin antibody. Two of the positive clones secreted monoclonal antibodies that were able to displace μ and δ, but not κ, opioid ligands from rat brain membranes. This profile matches the selectivity of β-endorphin. both antibodies also inhibited opioid inhibition of adenylate cyclase in NG108-15 neuroblastoma-glioma cells.

Another group reported the presence of anti-idiotypic antibodies to β-endorphin in serum of patients suffering from major depressive disorder (55). Binding of the antibodies to brain membranes was inhibited by several opioid ligands. In addition, the anti-idiotype bound to a 60 kd peptide in Western immunoblots.

Several groups have now prepared antibodies to opioid-receptor preparations that were previously purified in their laboratories. As discussed earlier, Bidlack et al (21) reported purification of an opioid receptor from Triton-solubilized rat brain membranes, using affinity chromatography. Subsequently, this group prepared a monoclonal antibody to this material, which proved to be directed against the 35 kd band observed on SDS gels. This mab was capable of partially inhibiting opioid binding to the solubilized preparation (62), though long incubation periods were required. Moreover, Fab fragments prepared from the antibody rapidly and completely inhibited binding of μ and δ opioid ligands to brain membranes. (63).

Maneckjee et al (25) prepared a polyclonal antibody to the highly purified opioid binding protein that they had previously isolated from bovine striatum. This antibody inhibited selectively the binding of μ opioids to rat brain membranes, and also selectively precipitated a 94 kd band from detergent-solubilized striatal membranes.

We recently purified to homogeneity an opioid-binding protein (OBCAM) from bovine brain (26), and isolated the cDNA coding for this protein (64; see below). Both monoclonal and polyclonal antibodies have been prepared to this protein, as well as polyclonal antibodies to peptides corresponding to

portions of the predicted amino acid sequence of the cDNA (64–66). The monoclonal antibodies, or Fab fragments derived from them, inhibit opioid binding to both the purified protein and to brain membranes, with binding of μ, δ, and κ ligands all affected (66). The polyclonal antibodies to the purified protein also inhibit opioid binding to brain membranes (65).

Although OBCAM shows some selectivity toward μ alkaloid ligands, both the monoclonal Fab fragments and the polyclonal antibodies inhibited opioid binding to NG108-15 cell membranes, which contain exclusively δ opioid receptors, as well as to guinea pig cerebellum membranes, which are enriched in κ opioid receptors. This suggests that the three major opioid-receptor types in mammalian brain contain common antigenic epitopes. However, Western blot analysis revealed that the polyclonal antibodies interacted with a 63 kd band in guinea pig cerebellum, and with a 39 and a 58 kd band in NG108-15 cells. In addition, a polyclonal antibody raised to a peptide corresponding to a portion of OBCAM's predicted amino acid sequence specifically adsorbed proteins of 39 and 58 kd from detergent-solubilized NG108-15 cell membranes (S. Roy, unpublished data).

To determine whether either of the two bands recognized by polyclonal antibodies to OBCAM in NG108-15 cells is involved in opioid-receptor function, the cells were treated with 10 μM DADLE for 12–48 hours. This treatment is known to result in down-regulation, or decrease, of opioid receptors in these cells (67). Under these conditions, the 39 kd, but not the 58 kd, band was decreased, in a time-dependent, naloxone-reversible fashion.

This result was somewhat surprising, in view of the above evidence that a 58 kd protein binds opioids in NG108-15 cells. However, OBCAM is thought to contain carbohydrate and, in fact, its predicted amino acid sequence has a molecular weight of just 38 kd (64). Thus it is conceivable that the 39 kd protein isolated from NG108-15 cells is closely related to OBCAM, but has little or no carbohydrate.

Cloning of Opioid Binding Proteins

As discussed earlier, Cho et al (26) purified an opioid-binding protein from bovine brain that was selective for μ alkaloids. The cDNA coding for this opioid-binding protein was then cloned by standard procedures (64). The amino acid sequence of a portion of the opioid-binding protein was determined, oligonucleotide probes corresponding to these sequences were synthesized, and these probes used to screen a cDNA library from bovine brain. A single DNA sequence was inferred that contained all the sequences of the probes. This sequence was determined, and translated to give the amino acid sequence of the opioid-binding protein (Figure 1).

The protein consists of 345 amino acids, with a calculated molecular weight of 37.9 kd. The discrepancy between this value and the molecular

Figure 1 Predicted amino acid sequence of opioid-binding protein (OBCAM), in single letter code. Potential N-linked glycosylation sites are underlined, and cysteines flanking the three immunoglobulin domains are boxed.

weight of the originally purified opioid-binding protein (58 kd) suggests that the protein might be glycosylated. This conclusion is supported by two other observations: (*a*) the opioid binding protein binds to lectin affinity columns (26); and (*b*) there are six potential glycosylation sites in the protein's sites in the protein's amino acid sequence, consisting of an asparagine residue in close proximity to a serine or threonine (see Figure 1, underlined sequences).

Although it has not yet been possible to show that transfection of the cDNA into cells lacking opioid receptors confers opioid-binding activity, other indirect evidence indicates the pharmacological relevance of this opioid-binding protein. An antibody to a portion of the predicted amino acid sequence was used to construct an affinity column. This column was capable of specifically adsorbing from detergent-solubilized brain membranes a protein that bound opioids in in vitro assays (64). Moreover, a monoclonal antibody raised to the purified protein (66) inhibited opioid binding to this affinity-purified material. These results indicate that the cloned sequence indeed codes for the purified opioid-binding protein.

A search of the NBRF-PIR database revealed that this opioid-binding protein had significant homologies to several members of the immunoglobulin superfamily (64; Table 1). This is a group of proteins characterized by repeating domains flanked by cysteine residues (68, 69). The highest degree of homology was to molecules involved in cell adhesion or cell recognition, including the vertebrate proteins neural cell adhesion molecule (N-CAM) and myelin-associated glycoprotein (MAG), and the invertebrate proteins fasciclin II and amalgam. The sequence of each of these proteins is 20–25% homologous with that of the opioid-binding protein. Somewhat lower significance values, also suggestive of an evolutionary relationship, were found for several peptide receptors, including those for interleukin-6 (IL-6) and platelet-derived growth factor (PDGF).

Sequence analysis is therefore consistent with the notion that this protein functions as an neuropeptide receptor, while suggesting that it could also play

Table 1 Homologies of opioid-binding protein (OBCAM) with members of the immunoglobulin superfamily

Protein	Search score[a]		z-value[b]	
	Initial	Aligned	Initial	Aligned
Amalgam	84	327	9.39	29.89
N-CAM	136	147	24.51	11.62
Fasciclin II	75	93	8.80	11.59
CEA	71	151	5.87	11.18
MAG	99	206	10.60	11.12
L1	75	117	6.67	8.83
T-Cell R (α)	83	83	7.54	6.90
Ig κ V	74	82	9.15	6.76
Il-6 R	43	76	2.28	6.47
Poly Ig R	44	94	2.07	6.31
PDGFR	53	102	3.26	4.81
Ig λ V	55	58	5.94	4.57
T-Cell R (γ)	52	70	4.16	4.36
CD2	44	56	2.19	3.08

[a] The amino acid sequence of OBCAM (Figure 1) was compared to sequences in the NBRF-PIR database using the FASTP program (111). This algorithm does not compare the full sequences of the test protein and the proteins in the database, but only a subsequence of highest similarity. The initial score is determined by totalling identical matches as well as conserved replacements, according to a standard amino acid weighting matrix (112). The aligned score is determined after allowing for insertions and deletions in the two sequences being compared. Initial scores of greater than 50, and aligned scores of greater than 100, are usually considered suggestive of an evolutionary relationship (111).

[b] The proteins in Table 1 were subsequently compared with OBCAM using the RDF test for sequence similarity (111). In this test, the alignment score of each pair is compared with the mean of a similarly derived set of scores obtained by comparing 20 randomized versions of each sequence. Significance scores (z-values) are defined as the number of standard deviations by which the aligned score of the original (non randomized) pair exceeds the mean of the randomized aligned scores. Z-values of > 3, > 6 and > 10 are considered, respectively, possibly, probably and definitely indicative of an evolutionary relationship between two proteins of undetermined functional similarity (111). However, on the basis of OBCAM's highly significant homologies with some members of the Ig superfamily, z values of > 3 obtained with other known members of this family can definitely be considered significant (112).

The proteins are listed in descending order of aligned z-scores. All of the protein sequences were obtained from the NBRF-PIR database, except for amalgam (113), fasciclin II (114), and L1 (115). In addition to those proteins listed, several others had aligned z-scores of 4–6 when compared to OBCAM, but their homologies are not considered significant, as they are not members of the Ig superfamily. Abbreviations: N-CAM, neural cell adhesion molecule; CEA, carcinoembryonic antigen precursor; MAG, myelin associated glycoprotein; L1, neural adhesion molecule; IL-6 R, interleukin-6 receptor; PDGF R, platelet-derived growth factor receptor.

a role in cell adhesion. Accordingly, it has been named OBCAM, or opioid-binding cell-adhesion molecule. Interestingly, a recent study has shown that opioids can modulate cell-cell interactions of monocytes (70). Moreover, the homologies of OBCAM with immunoglobulins and immune cell receptors such as the T-cell receptor and the IL-6 receptor are consistent with a growing body of evidence linking opioids and the immune system (71). In view of this relationship, it is of great interest that antibodies to OBCAM block interleukin (IL-1) binding to T-cells (S. Roy, unpublished). The IL-1 receptor is also a member of the immunoglobulin superfamily (72).

Nevertheless, the homology of this opioid-binding protein with members of the Ig superfamily is somewhat surprising, because some opioid receptors, namely the δ type in NG108-15 neuroblastoma x glioma hybrid cells and in mammalian striatum, are coupled via a G-protein to adenylate cyclase (73, 74; see below). This has suggested that these receptors are structurally and functionally related to a group of cell-surface receptors that are associated with G-proteins, which include the β_1-, β_2-, and α_2-adrenergic, m_1 and m_2 muscarinic, serotinin 1a, and 1c, and substance K receptors (75–79). All these receptors have seven hydrophobic regions in their interior, which are thought to span the membrane.

Although the second messenger(s) mediating the actions of OBCAM has not yet been determined, the sequence of this protein suggests that either it does not interact with G-proteins, or if it does, the interaction is quite distinct from that of those receptors with multiple membrane-spanning regions. In fact, because of its sequence homologies, it seems more likely to function as a type III tyrosine kinase growth factor receptor, such as the PDGF receptor. Activation of these receptors results in phosphorylation of several intracellular substrates and of the receptor itself, and, in some cases, may also induce a number of other cellular responses. However, while OBCAM contains the extracellular Ig domains characteristic of these receptors, it does not possess the structural motifs in the C-terminal portion that are characteristic of the catalytic domain in tyrosine kinase growth factor receptors. In fact, OBCAM's predicted amino acid sequence appears to lack both the transmembrane and intracellular regions found in all types of these receptors (80).

An attempt has also been made to clone the δ opioid receptor of NG108-15 hybrid cells (P. Y. Law, personal communication), without beginning with purified receptor. Total mRNA was isolated from NG108-15 cells and used to synthesize a "library" of cDNA, i.e. DNA containing all the mRNA sequences found in NG108-15 cells. This cDNA library was fractionated by size, then inserted by a plasmid vector into a glioma cell line with no detectable opioid binding.

A key to the success of these studies was the construction of the subtraction

probes, which were used to enrich or "prescreen" the initial colonies for opioid-receptor cDNA. To make these probes, advantage was taken of the fact that several treatments can selectively alter the number of opioid receptors in clonal cells. Prolonged treatment of NG108-15 hybrid cells with opioid agonist, such as 100 nM DADLE for 24 hours, results in a naloxone-reversible decrease, or down-regulation, of as much as 80% in receptor number (67). In addition, treatment of the PC cell subtype PC12h, which ordinarily contains undetectable levels of opioid receptors, with nerve growth factor for 14 days results in the appearance of high levels of δ opioid receptors.

Accordingly, mRNA was prepared from these cells under both high receptor (naloxone-reversed, down-regulated NG108-15 cells or NGF-treated PC12h cells) and low receptor (down-regulated NG108-15 hybrid cells or normal PC12h cells). The mRNA from the high receptor cells was used to make cDNAs, which were then hybridized with mRNA from low receptor cells. This hybridization in effect "subtracts" all the common mRNa from the high and low receptor conditions and, in theory, leaves cDNA highly enriched in opioid-receptor DNA.

After such screening, the colonies that tested positively for both subtraction probes were screened for opioid binding. Several candidate cDNAs that bestowed both opioid binding and opioid-mediated adenylate cyclase inhibition on these cells are currently under investigation.

POSSIBLE SECOND MESSENGER SYSTEMS IN OPIOID ACTION

Like other cell surface receptors, opioid receptors are presumed to act by modulating the activity of other functional molecules in the cell. Recent work has indicated that opioid receptors may be associated with several such second messenger systems, though none has been shown to play a role in opioid-induced analgesia. This discussion is limited to the two second messenger systems for which the best evidence exists of an association with opioid receptors, adenylate cyclase, and ion channels. It should be emphasized that these two systems are not necessarily exclusive. Some studies suggest that opioid actions on ion channels may be mediated through adenylate cyclase.

Adenylate Cyclase

The best-characterized second messenger is cyclic AMP, which is synthesized by the enzyme adenylate cyclase. The activity of this enzyme is modulated by a large number of cell surface receptors, including the α_2 and β_1 and β_2 adrenergic, muscarinic, serotonin, dopamine, and substance K. Some of these receptors stimulate adenylate cyclase activity, while others inhibit it. In all

cases, however, the effect on cyclase is mediated by a GTP-binding protein (G-protein). Stimulatory receptors are coupled to a G-protein that activates cyclase (G_s), while inhibitory receptors activate an inhibitory G-protein (G_i) (82).

Because of the large number of hormones and neurotransmitters that modulate adenylate cyclase activity, this enzyme had long been a prime candidate for the second messenger of opioid effects. Early efforts to demonstrate this in brain could not be reproduced, but Sharma et al (74) found that in cultured NG108-15 neuroblastoma-glioma cells opioid agonists inhibited both basal and prostaglandin E_1 (PGE_1)-stimulated adenylate cyclase. This inhibition was dose-dependent and naloxone-antagonizable, and the inhibitory potencies of a series of opioid agonists correlated well with their binding affinities to these cells, as well as with their in vivo pharmacological potencies. However, comparison of the binding affinities and inhibitory potencies for a series of opioid agonists revealed that the receptor was selective for δ agonists, such as leucine and methionine enkephalin and β-endorphin. Morphine and other opioid agonists that preferentially interact with μ receptors had significantly lower binding affinities and IC_{50} values for adenylate cyclase inhibition. In fact, the latter opioids act as partial agonists in these cells, unlike the δ agonists, which act as full agonists (83).

As had previously been shown with other cell surface receptors coupled to adenylate cyclase (82), the inhibitory effect of opioids was mediated by a GTP-binding protein, G_i. Thus, opioid inhibition of adenylate cyclase in NG108-15 cells could be blocked by substituting for GTP its non-hydrolyzable analog Gpp(NH)p, and by pretreatment of the cells with pertussis toxin, which ATP-ribosylates G_i (73, 84).

The NG108-15 system attracted opioid researchers' special interest because of the demonstration that after continuously exposing the cells for several hours to an opioid agonist, there was a gradual loss of opioid inhibition of adenylate cyclase, whereas cyclic AMP levels returned to normal (85). Furthermore, withdrawal of agonist increases adenylate cyclase activity to levels above that of control cells. These effects are at least superficially analogous to opioid tolerance and dependence, respectively, suggesting that these cells would make a useful model system for studying these phenomena.

Law et al (9) and Cooper et al (86) subsequently demonstrated opioid inhibition of adenylate cycalse in mammalian neostriatum. As with NG108-15 cells, δ type receptors were involved. Childers (87) reported that opioid inhibition of adenylate cyclase could be detected in whole brain membranes if the latter were pretreated with pH 4.5 buffer.

More recently, there have been reports that μ and/or κ opioid receptors are also coupled to adenylate cyclase. Schoffelmeer and colleagues (88, 89) reported that D_1-dopamine-stimulated adenylate cyclase in rat neostriatum

was inhibited by both μ and δ agonists. Chneiweiss et al (90) found that μ and δ, but not κ, opioid agonists inhibited adenylate cyclase in cultures of embryonic striatum. Tallman and associates (91, 92) reported that morphine as well as DADLE inhibited adenylate cyclase in frontal cortex, dorsal raphe and locus coeruleus as well as neostriatum. These researchers also found that chronic morphine treatment increased adenylate cyclase activity, but only in LC. A large increase in pertussis toxin-mediated ADP-ribosylation of G-proteins also occurred under these conditions.

Attali et al (93) reported that κ opioid agonists inhibited adenylate cyclase in membranes of both rat spinal cord and spinal cord-dorsal root ganglion cultures. There was a loss of this inhibition after chronic treatment of cells with etorphine or U-50,488H. Interestingly, this effect was heterologous, as α_2 and muscarinic agonists also had reduced effects under these conditions; thus the chronic κ opioid treatment presumably affected not opioid receptors, but some post receptor process.

Crain and his associates (94, 95) also reported that κ opioid agonists inhibited adenylate cyclase in cultured spinal cord-ganglion explants from fetal mice; δ, but not μ, agonists were also effective. The effect was blocked by pertussis toxin, implicating a G_i-mediated process. As with NG108-15 cells, chronic morphine treatment of the explants resulted in a tolerance-like reduction of opioid inhibition.

Frey & Kebabian (96) reported that opioids inhibit release of prolactin from a pituitary tumor-cell line, and that this inhibition was associated with a stimulation of adenylate cyclase activity. Kazmi & Mishra (97) found that both μ and δ opioid agonists inhibited adenylate cyclase in SH-SY5Y neuroblastoma cells.

In conclusion, it appears that all three major opioid receptor types are coupled to adenylate cyclase, in at least some tissues. However, there still is no evidence that adenylate cyclase mediates opioid analgesia, or for that matter, any other known pharmacological effects of opioids in vivo. In light of the evidence that μ and κ receptors, and perhaps also δ receptors, are themselves heterogeneous (18–20), it is conceivable that adenylate cyclase is coupled to opioid receptor subtypes that are not involved in analgesia.

Ion Channels

Early studies revealed that a number of opioid agonists were able to inhibit the firing of neurons in several brain regions, suggesting that opioid receptors might be coupled to ion channels (98). In detailed electrophysiological studies of the rat locus coeruleus and guinea pig submucous plexus, North and colleagues (10, 11, 99) found that both μ and δ opioid agonists activated K^+ channels in these tissues, resulting in membrane hyperpolarization, conductance increase, and inhibition of firing, while κ opioid receptors inhibited

K^+-stimulated Ca^{++} channels. Consequently, they postulated that both μ and δ opioid receptors belong to a family of receptors that include M_2 cholinergic, α_2 adrenergic, D_2 dopamine, 5-HT_1, adenosine, GABA and somatostatin.

Werz & MacDonald obtained similar results with the mouse dorsal root ganglion (DRG) preparation (12, 100). In this system, μ and δ agonists decreased the calcium-dependent action potential duration with an increase in action potential after hyperpolarization, and the effect was blocked by intracellular injection of cesium. In contrast, dynorphin A, a κ agonist, also reduced the calcium-dependent action potential duration, but the effect was blocked by cadmium, but not by cesium. Similar results were obtained by Attali et al (101) in rat spinal cord-DRG explants. Several lines of evidence indicated that the coupling was mediated by a G-protein, including inhibition by pertussis toxin, and the observation that κ agonists also inhibited forskolin-stimulated adenylate cyclase in this system. Crain et al (102) reported that tolerance to the effect of opioids on action potential duration developed upon chronic treatment.

There is also considerable pharmacological evidence linking opioids to altered calcium channels. Contreras et al (103) found that morphine analgesia in mice was enhanced by the Ca^{++} channel blockers diltiazem, flunarizine, nicardipine, and verapamil. Flunarizine, and verapamil, along with nifedipine, also reduced the intensity of tolerance that developed from a single dose of morphine in a slow-release preparation. Likewise, Shah et al (104) found that verapamil potentiated morphine-induced inhibition of bladder emptying, and, in a clinical study, Boldt et al (105) reported that cardiac patients receiving nimodipine, but not nifedipine, required less fentanyl for pain relief. Kavaliers (106, 107) reported that i.p. administration of the Ca^{++} channel blockers nifedipine and verapamil reduced inhibition of morphine analgesia by FMRFamide, but not by naloxone. I.p. administration of Ca^{++} channel blockers enhanced analgesia induced by DAGO and DPDPE, but Ca^{++} channel agonists reduced the opioid analgesia.

Gandhi & Ross (108) reported that the κ-selective ligand U-50,488H decreased binding of ^{3}H-nimodipine, a calcium channel antagonist, to rat brain membranes; kinetic analysis indicated the effect was on B_{max}. Morphine had no effect in this system. Adamson et al (109) found that dynorphin reduced internal [Ca^{++}] in synaptosomes, as determined by using the fluorescent dye quin 2. They concluded that this effect was independent of action on the voltage-sensitive calcium channel.

At least one report links opioids to Na^+ channels. Iwama et al (110) found that U-50,488H decreased the synaptic response in CA3 guinea pig hippocampal neurons in vitro, but did not affect membrane potential or the spike generation. U-50,488H also inhibited the depolarization induced by glutamate

or by veratrine. The authors concluded that U-50,488H acts on a slow-closing Na^+ channel in this system.

SUMMARY AND CONCLUSIONS

Though opioid receptors are more difficult to purify and characterize than other cell surface receptors, significant progress has been made in the past several years. At least a dozen groups have now reported purification of opioid-binding proteins, either in a form that retains ligand-binding properties, or in a covalently bound form. Although there are some discrepancies in the molecular weights of these proteins, it is significant that many investigators have reported a molecular weight of about 60 kd for the receptor, regardless of whether it is of the μ, δ, or κ type. This finding, together with immunological evidence, suggests that different opioid receptor types may be highly similar, and could conceivably even share a common ligand-binding subunit.

Several groups have prepared monoclonal or polyclonal antibodies to purified opioid-binding proteins, which should be useful in mapping the brain regional distribution of the opioid receptors, determining the regions in the peptide involved in ligand binding and association with second messengers, and in determining the relationships among different opioid receptor types. One group has in fact already established an antigenic similarity between a μ-selective opioid-binding protein in mammalian brain, and the δ opioid receptor in NG108-15 neuroblastoma-glioma hybrid cells.

One group has reported cloning of the cDNA for a purified opioid-binding protein. Somewhat surprisingly, its predicted amino acid sequence places it in the immunoglobulin superfamily, with strongest homologies to cell-adhesion molecules such as N-CAM. MAG, amalgam and fasciclin II, as well as receptors for peptides such as PDGF and interleukin-6. However, this is consistent with evidence that opioids can modulate cell-cell interactions of monocytes, and provides further support for links between opioids and the immune system.

The second messengers mediating opioid actions are still unknown. Opioid agonists affect the activity of adenylate cyclase and ion channels in some tissues, but neither has been shown to mediate opioid analgesia. The sequence homologies of the purified opioid-binding protein OBCAM with tyrosine kinase growth factor receptors suggest additional possibilities for second messengers.

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