The Next Frontier in the Molecular Biology of the Opioid System

The Opioid Receptors

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In memory of ***Edward Herbert***

Contents

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Abstract

The analgesic and euphoric properties of some plant alkaloids such as morphine have been known and exploited for centuries. In contrast, only during the last twenty years have we begun to unravel the molecular basis by which opiates exert their effects, mechanisms important to our general understanding of the nervous system. The analgesic response to opiates is the result of a cascade of biochemical events that are triggered by the interaction of the opiate with specific macromolecular components found on the membranes of nervous system tissues, the opioid receptors. The endogenous ligands of these receptors are small peptides, the opioid peptides. Although much has been learned about the structures and the mode of synthesis of the opioid peptides, little is understood about the structure of their receptors. The application of molecular genetic techniques was of great importance to the studies of the opioid peptides. It is now expected that this same technology will unravel the physical mysteries of the opioid receptors.

Index Entries: Opioid receptors; molecular biology; physical characteristics of the different types of opioid receptors.

Introduction

The opioid receptors are macromolecular components of plasma membranes that bind opioid ligands specifically, as determined by three criteria: (1) a high affinity for opioids, (2) a saturable opioid binding pattern, and (3) a binding pattern which is stereospecific and naloxone reversible (Dole et al., 1975). These criteria have historically constituted the definition of opioid receptors.

The opioid receptors are implicated not only in pain perception, addiction, and withdrawal but also in hormone secretion, response to injury, and gastrointestinal motility. The molecular basis of their participation in all these phenomena is unknown. These receptors predominantly have been studied biochemically by measuring ligand binding affinities or pharmacologically by analyzing the effects of the ligand-receptor interaction on the release of neurotransmitters. Although these studies have led to the discovery that there are different classes of opioid receptors, they were not directly able to address the problem of the molecular structure of the receptors. We do not know, for example, whether the ligand recognition process necessitates interaction of the opioid ligand with one or more macromolecules that could be part of the receptor complex, or if the apparent opioid receptor heterogeneity reflects the existence of different receptor molecules. Also, since the experiments that have attempted to purify opioid receptors have been plagued by the low abundance and lability of the receptors, our best hope to understand the structure and to analyze, at a molecular level, the activation of the opioid receptors lies with the use of a technology that can overcome these obstacles, recombinant DNA technology.

This review is intended to give a molecular biologist's view of the opioid receptors. The opioid receptors are analyzed with the intent to understand their physical structure. In this respect, the separation of the opioid receptors to several types is of major importance and serves as the common thread in our discussion. We use the term opiate for the products of the opium poppy and their congeners. Upon the discovery of the opioid peptides, the previously named opiate receptors naturally become coined opioid receptors and, consequently, opioid refers to any compound that interacts specifically with the opioid receptor.

History

The theory that opiates produce their effects by interacting with specific receptors developed from observation of the rigid stereochemical and structural requirements for their analgesic action. In the mid-1960s, Portoghese (1965) suggested the existence of multiple opiate receptors based on the relationship between the molecular structure of opiate drugs and their analgesic effect. In 1973, the characterization of stereospecific opiate binding sites (Simon et al., 1973; Pert and Snyder, 1973; Terenius, 1973) and the evidence of their nonuniform distribution in the central nervous system (Hiller et al., 1973; Kuhar et al., 1973) set the foundation for a molecular analysis of the mode of opiate action. A few years later, Martin and coworkers (Martin et al., 1976; Gilbert and Martin, 1976) found that the pharmacological profiles of several opiates were sufficiently different to postulate the existence of multiple opiate receptors.

The discovery of opiate receptors reinforced the possibility of the existence of endogenous ligands. The search for these ligands was based on two of their pharmacological properties: their ability to inhibit acetylcholine release in the guinea pig ileum assay and the reversible antagonism of this inhibition by naloxone (Kosterlitz and Waterfield, 1975). This search culminated in 1975 when Hughes and Kosterlitz (Hughes et al., 1975) purified and sequenced the first two opioid peptides: the pentapeptides methionine- and leucine-enkephalin. Since then nearly two dozen different peptides, all sharing the common enkephalin sequence at their amino terminus, have been characterized as opioid peptides. Later, the advent of recombinant DNA technology has allowed several laboratories to demonstrate that these peptides are produced by proteolytic cleavages of only three different polypeptide precursors; proopiomelanocortin, POMC (Nakanishi et al., 1979; Roberts et al., 1979), proenkephalin (Noda et al., 1982; Gubler et al., 1982; Comb et al., 1982), and prodynorphin (Kakidani et al., 1982; Civelli et al., 1985).

The most striking result from this work was the demonstration that the POMC polypeptide is the precursor for the hormone ACTH, as well as the neuropeptide, beta-endorphin (Herbert et al., 1981). This constituted unequivocable proof of a direct relationship between the endocrine and the nervous systems. This discovery established the opioid system as a model for studying neuroendocrine systems and underlined the physiological importance of the opioid system.

Since these discoveries, many studies related to the opioid peptides or their receptors have been completed, and their results have been the subject of several reviews (Hughes and Kosterlitz, 1983; Paterson et al., 1984). One of the major conclusions of these studies is the extent of the complexity of the opioid system. Opioid peptides and their receptors are present in several tissues in addition to the nervous and the endocrine tissues; in particular, in cells of the immune system (Teschemacher and Schweigerer, 1985). The opioid system might, therefore, regulate physiological responses that are of clinical interest. Indeed, at least forty different physiological responses have been proposed to be affected by the opioid peptides or by their receptors (Holaday, 1985); although for most of the cases analyzed, the implication of the opioid system in the physiological responses is anecdotal or stems from extrapolation from animal studies. In some cases, however, the involvement of the opioid system is clear. These instances include: the response to pain described as analgesia or antinociception, some feeding behavior resulting in appetite suppression, the inhibition of hormone release in the case of prolactin and growth hormone, effects on respiration, well documented antiperistaltic effects on the gastrointestinal tract, and the phenomena of addiction and withdrawal (Fig. 1). The cascade of

THREE MAJOR TYPES OF OPIOID RECEPTORS

RECEPTOR TYPE	μ	δ	κ
LIGAND BINDING SPECIFICITY			
ENDOGENOUS AGONIST	methorphamide	met-enkephalin leu-enkephalin	dynorphin neo-endorphin
PROTOTYPIC AGONIST	morphiceptin DAGO	DADLE DPDPE	ketocyclazocine U 50,488
TISSUE DISTRIBUTION			
Caudate putamen	++++	++++	++++
Nucleus accumbens	++++	++++	+++
Temporal cortex	+++	+++	0
Amygdala	+++	++	++
Substantia nigra	+++	+	+
Thalamus	+++	0	+
Raphe nuclei	+++	0	++
Habenula	++	0	+
Hippocampus	++	+	0
Olfactory tubercle	+	+++	+++
Hypothalamus	+	0	++
Guinea pig ileum	+	-	+
Mouse vas deferens	+	+	+
Rabbit vas deferens	-	-	+
Hamster vas deferens	-	+	-
NG108-15	-	+	-
N4TG1	-	+	-
PHYSIOLOGICAL PROFILES			
Analgesia	+	+	(-)
Addiction and Tolerance	+	+	+
Appetite Suppression	-	-	+
Hormone release inhibition:			
-Prolactin	+	-	+
-Growth hormone	-	+	-
Antiperistalsia	+	(-)	-
Respiration	+	+	(-)
CELLULAR RESPONSES			
Association to K^+ Channels	+	+	-
Association to Ca^{++}Channels	-	-	+
Adenylate cyclase inhibition	ND	+	ND

Fig. 1. The three major types of opioid receptors differentiated on the basis of their ligand binding specificities, tissue distributions, physiological profiles, and cellular responses. The tissue distribution data relative to the central nervous system were obtained from Mansour et al. (1987), the physiological profiles from Holaday (1985). ++++ to 0 indicate decreasing relative densities of the opioid receptors from very dense to undetectable, and is valid only for the central nervous system data. (–) possible involvement, ND: not established.

events that result in these responses can be traced to a single reaction, the recognition of an opioid by its specific receptor. The opioid receptor is, therefore, key to our understanding of the resulting physiological and behavioral responses.

The Different Types of Opioid Receptors and the Cellular Responses to Their Activation

The complex nature of the opioid system stems from the heterogeneity of its two primary components, the numerous opioid peptides and their different receptors. The complexity of the opioid peptides is evident by their different structures, whereas the heterogeneity of the opioid receptors is based on their different pharmacological properties, a concept introduced by Martin (Martin et al., 1976).

In a detailed analysis using the chronic spinal dog model, the pharmacological profiles of several types of opioids in neurophysiological and behavioral tests were found to be different. Moreover, morphine and the analogs used were unable to substitute for each other in the prevention of withdrawal symptoms in dogs made dependent on one of the drugs. Based on these results, Martin postulated the existence of three types of opioid receptors, which he named for the prototypic drugs used in the study: mu for morphine, kappa for ketocyclazocine, and sigma for SKF 10,047 (*N*-allylnormetazocine). The discovery of the enkephalins led to the question of whether these peptides act via the same receptors as those through which the opiate drugs had been shown to act. Kosterlitz and his group (Lord et al., 1977) found that the opiate alkaloids were more effective than enkephalins in inhibiting the electrically induced contractions of the myenteric plexus of the guinea pig. But the enkephalins were more potent than the opiates in the mouse vas deferens bioassay. The inhibition of contraction of the mouse vas deferens by enkephalins was comparatively less sensitive to reversal by naloxone. These results indicated the existence of a new receptor type, the delta receptor, named after its site of origin, the vas deferens.

Since then, the sigma receptor has come to be viewed as pharmacologically distinct from the other opioid receptors because some of its binding characteristics are not naloxone reversible. On the basis of pharmacological and ligand binding studies, some investigators have also further delineated two of the opioid receptors as mu1 and mu2 (Pasternak et al., 1983) and kappa1 and kappa2 (Attali et al., 1982). In addition, a different receptor has been attributed specifically to beta-endorphin, the epsilon receptor (Schulz et al., 1981) and one to naloxone, the lambda receptor (Grevel et al., 1985). In general, however, the opioid receptors are divided in only three major types mu, delta, and kappa, because these three types are distinguishable on the basis of four criteria: their differences in ligand selectivities, their diverse anatomical distribution patterns, their differing physiological and behavioral profiles, and the differences in the cellular responses to their activation (Fig. 1).

Ligand Selectivities

Three major approaches have been used to characterize binding of opioids to the receptors. In the first, the kinetic parameters of the interaction of a labeled opioid with a binding site, that is, the equilibrium dissociation constant (K_d) and the maximum number of binding sites, have been determined directly by analysis of saturation curves (Hill, 1910; Scatchard, 1949). In the second approach, the affinity of unlabeled opioids is estimated indirectly, as the inhibition constant (K_i), from their ability to displace the binding of labeled ligands of known characteristics (Cheng and Prusoff, 1973). In the third approach, the selectivity of ligands at the opioid binding sites are tested in experiments where the binding site is protected against inactivation by the technique of site-directed alkylation (Simon and Groth,

1975; Robson and Kosterlitz, 1979; Smith and Simon, 1980).

When analyzing binding data, it is important to realize that it is not possible to reproduce in vitro the exact environmental conditions in which the opioid receptor is found in vivo. The components and media used in the in vitro assay profoundly affect the binding characteristics of opioids. For example, it is well known that some cations are inhibitory to opioid binding and that the opioid receptors are extremely labile and sensitive to proteolysis (Simon and Groth, 1975; Blume, 1978). As a result, binding reactions are in general carried out in the artificial milieu of 50 m*M* Tris-HCl solution at pH 7.4. These technical constraints have to be kept in mind while trying to deduce something about the opioid receptors from the binding data.

The separation of the opioid receptors into three types is based on the use of synthetic analogs that are specific to one particular type and can therefore serve as a reference for determining the selectivity of other opioids. The prototypic analogs for the mu receptor are Tyr-Pro-Phe-Pro-NH2 (morphiceptin) and Tyr-DAla-Gly-MePhe-Glyol (DAGO); for the delta receptor, Tyr-DAla-Gly-Phe-DLeu-OH (DADLE); Tyr-DPen-Gly-Gly-Phe-DPen-OH (DPDPE); and for the kappa receptor U50, 488. Some endogenous opioid peptides have also been found to be class-selective. Metorphamide has the highest affinity to the mu site (Weber et al., 1983), and the two enkephalins are strong delta binders (Lord et al., 1977), whereas the dynorphins (Goldstein et al., 1979) and the neo-endorphins are essentially kappa ligands. The other endogenous peptides are not as selective and, in the majority of the cases, are able to bind efficiently to two or more types of receptors. Beta endorphin, for example, has about the same affinity to mu and delta sites as do the hepta- and the octapeptides derived from proenkephalin (McKnight et al., 1983). Morphine, the first alkaloid purified, is a strong mu ligand. With regard to the binding spectra of antagonists, there is as yet no compound that presents a high degree of selectivity.

Tissue Distribution

Opioid receptors have been localized in both central and peripheral nervous systems. Two technical approaches have been used. The first consists of measuring receptor levels and affinities after isolation of tissues; the second detects receptors by autoradiography following in vitro binding of labeled ligands to tissue slices. This second method allows for a more detailed anatomical mapping of opioid receptors.

Early binding experiments of opioid ligands to brain membranes revealed that there were marked variations in the number of receptors in different regions of the rat brain (Chang et al., 1979, 1981; Ninkovic et al., 1981; Simon et al., 1980). In general, higher concentrations of opioid receptors were found in the phylogenetically newer structures of the brain, such as the diencephalon and telencephalon; and among these areas, the limbic stuctures showed highest concentration. It is noteworthy that the enkephalins have a distribution similar to that of opioid receptors (Atweh and Kuhar, 1983).

A variety of studies indicate that, in the rat brain, mu and delta receptors have somewhat different distributions, although they are present together in the majority of brain tissues. The thalamus, the amygdala, the substantia nigra, the raphe nuclei, the habenula, the hippocampus, and the hypothalamus appear to be enriched in mu receptors as compared to delta receptors, whereas the olfactory tubercle is relatively enriched in delta sites. The distribution of kappa receptors in these tissues is different from those of mu and delta (Fig. 1). Similar studies done on human brain have shown that, although there are species differ-

ences, the overall distribution of the different opioid receptors is similar to what is found in the rat (Pfeiffer et al., 1982).

Histochemical localization of opioid receptors in rodent and primate brains has revealed more detail about the specific distribution of each receptor type. Opioid receptors have been found associated with the sensory system, with limbic structures and with neuroendocrine cells (Atweh and Kuhar, 1983). A quantitative anatomical distribution of the three receptor types in the rat brain has also been achieved (Mansour et al., 1987).

Opioid receptors have also been localized in peripheral organs. They are present in afferent, sympathetic, and parasympathetic neurons, in the enteric nervous system, in the adrenal gland, and in the reproductive organs (Paterson et al., 1984). The best studied tissues are those that are used for defining the pharmacological profiles of the opioids. Consequently, we know that the guinea pig ileum contains mu and kappa receptors, that the vas deferens prepared from hamster has only delta receptors, from rabbit only kappa receptors, and the mouse vas deferens contains all three types (Kosterlitz, 1985). Finally, opioid receptors have been found in some neuroblastoma cell lines, in particular the cell lines N4TG1 and NG108-15, which both express delta receptors (Klee and Nirenberg, 1974; Amano et al., 1972).

Physiological Profiles

Opioid receptors have been implicated in the modulation of important physiological activities. In general, it has been observed that injections of opioid peptides into the CNS or peripheral sites produce the same spectrum of effects as injections of alkaloid opioids like morphine. An important consideration to remember when interpreting these data is the high metabolism of endogenous peptide in the animal. Because of the variability in exogenous peptide proteolysis, it is difficult to differentiate the effects of each receptor type (Malfroy et al., 1978; Sullivan et al., 1980).

Beta-endorphin has a greater antinociceptive potency (Loh et al., 1976) than enkephalins, implying that mu receptors are important in antinociception and analgesia. The delta receptors, however, are also implicated in these responses as indicated by the fact that stable delta agonists are potent antinociceptive agents (Frederickson et al., 1981). On the other hand, kappa agonists have been implicated in visceral analgesia. Opioid receptors, especially kappa, are thought to be involved in appetite suppression (Morley et al., 1983). The mu receptor has been shown to be responsible for prolactin release inhibition, whereas the delta receptor inhibits growth hormone release. These two receptors are also involved in depressing respiration, the primary cause of death from opioid overdose. The antidiarrheic action of opioids is mediated by the mu and to a certain extent the delta receptors. Finally, the phenomena of addiction and tolerance are mediated by all three receptor types, as evidenced by the fact that all the endogenous and synthetic opioids are addictive, the major problem in opioid pharmacology (Wei and Loh, 1976; Holaday, 1985) (Fig. 1).

Cellular Responses to the Activation of the Opioid Receptors

To a certain extent, the three opioid receptor types can also be differentiated by the biological actions they mediate. In general, opioid receptors can mediate two types of effects in the cell, an electrophysiological and a biochemical effect (North, 1979; West and Miller, 1983).

Acutely, opioid binding to receptors inhibits neuronal firing and neurotransmitter release. Three model systems have been used in these studies: the myenteric plexus, the locus coeruleus, and the sensory neurones of the dorsal

root ganglion. The transmitters utilized by these neuronal populations are acetylcholine, norepinephrine, and substance P, respectively. The inhibition of acetylcholine release from the myenteric plexus has served to pharmacologically define opioid receptor activity (*see above*). In the locus ceruleus, opioid binding to mu receptors hyperpolarizes the cells and increases the potassium conductance (Williams et al., 1982). A secondary effect is the depression of the rate of rise and peak amplitude of calcium action potentials (North and Williams, 1983). In the dorsal root ganglion, several lines of evidence suggest that the mu and delta receptors are also coupled to potassium channels, whereas kappa receptors are coupled to calcium channels. Both of these effects result in a presynaptic reduction of voltage-dependent calcium influx (Werz and MacDonald, 1982, 1983; Gross and MacDonald, 1987; North, 1986).

The long-term biochemical effects of opioid receptor activity have been best studied in the neuroblastoma cells, NG108-15, and implicate the involvement of the second messenger cAMP (Klee et al., 1975). The delta receptors in these cells are coupled to a pertussis toxin-sensitive GTPase activity and binding of a ligand results in an inhibition of adenylate cyclase (Koski and Klee, 1981). There is, therefore, evidence that the delta receptor, upon binding of an agonist, interacts directly with a Gi protein complex that hydrolyses GTP and reacts with adenylate cyclase to inhibit cAMP production. It has also been found that Gi regulatory proteins can regulate the opening of potassium channels (Sasaki and Sato, 1987; Yatani et al., 1987) and that Gi proteins are present in the myenteric plexus and the locus ceruleus (North et al., 1987). Therefore, it has been suggested that, in nervous tissues or cells in culture, the first interaction of the receptors upon opioid binding is with a Gi protein complex. This complex then acts on two systems, either a channel and/or the adenylate cyclase. The factors determining the choice of one system over the other are unknown.

The concept that the opioid receptors interact with a G protein is important because it relates the opioid receptors to a growing family of neuroreceptors. This family includes the adrenergic, the muscarinic, the histaminergic, the dopaminergic, and several neuropeptide receptors (North et al., 1987).

Physical Characteristics and the Purification of the Opioid Receptors

Analysis by radiation inactivation or by molecular sieving indicate that the bioactive opioid receptor is a protein complex with an apparent MW varying between 300,000 and 400,000 daltons (Paterson et al., 1984). These complexes are made of several proteins, one (or few) of which is the ligand binding protein, the opioid receptor *sensu strictu*. The other subunits may be implicated in the intracellular response to opioid binding described in the preceding section or might result from random polypeptide associations occurring during the receptor preparation. The first polypeptide that has to be characterized is the ligand binding protein, commonly referred to as the opioid receptor.

As discussed above, the separation of the opioid recetors into three types is based on their binding affinities and very little is known concerning the physical differences between these types.

Three models can account for the heterogeneity of the opioid receptors: first, the three opioid receptors are structurally different and are coded by different genes; second, the opioid receptors or at least two of them are coded by different but sequence-related genes; and third, the opioid receptors have the same polypeptide backbone but differ in post-translational modifications or in their interactions

with other proteins such as the second messenger carriers (Bowen et al., 1981). At the present time, there is no definite data to favor one model over the others.

Using Schwyzer's concept (Schwyzer, 1980) to describe the information content of peptides, one can envision the active site of the receptor as being divided into two domains: the message domain that recognizes the common amino terminal sequence of the enkephalins, Tyr-Gly-Gly-Phe, and the address domain that recognizes the rest of the opioid peptide sequence. This model suggests that one part of the opioid receptor is common to the different classes and, therefore, that the opioid receptors must be, at least partially, homologous. Also, in view of analysis performed on other neuroreceptors (Kubo et al., 1986; Stevens, 1987; Hall, 1987), the possibility that the opioid receptors and in particular mu and delta, are coded by homologous if not identical genes is very attractive.

The bulk of our knowledge about the physical nature of the opioid receptor stems from the experiments that have attempted their purification. Two purification approaches have been followed: the labeled ligand binding approach and the affinity chromatography approach.

The Labeled-Ligand Binding Approach

In this approach, a labeled opioid ligand is bound and covalently crosslinked to the receptors in the membrane serving as a marker in subsequent purification steps. Since binding of the ligand is carried out on native receptor-membrane complexes, artifacts caused by aberrent protein–ligand interactions are minimized. But because the ligand binding site is irreversibly occupied, subsequent binding assays during purification or reconstitution are impossible.

This approach has been used by Klee and coworkers (Simonds et al., 1985). [^{3}H]-fentanylisothiocyanate was crosslinked to opioid receptors found in the membranes of the neuroblastoma cell line NG108-15. Several proteins were nonspecifically labeled, but one of M_r 58,000 was shown to bind opioid ligands stereospecifically. This protein was further purified to apparent homogeneity by antifentanylisothiocyanate affinity chromotography, lectin chromatography, and SDS-polyacrylamide gel electrophoresis. These results also showed that the 58,000 dalton protein is glycosylated. Using this strategy, enough protein was purified to allow an analysis of its amino acid composition. A similar strategy has been employed in which the enkephalin peptide analog [^{3}H] DALECK was crosslinked to rat synaptic brain membranes (Newman and Barnard, 1984). Here, too, it was found that DALECK irreversibly labels several polypeptides, but in particular a M_r 58,000 protein that may correspond to the mu class of opioid receptors. Finally, [^{125}I] human beta-endorphin has been crosslinked to opioid receptors present in NG108-15 cells and in rat or bovine brain membranes. It was shown that different labeling patterns were obtained when mu or delta binding sites were crosslinked (Howard et al., 1985). The delta receptor had a M_r 53,000, whereas the mu had a M_r 65,000. The differences in M_r in these studies might be accounted for by differences in sample preparation and in the gel systems used.

Taken together, these experiments indicate that the opioid receptors are proteins in the range of 58,000 daltons and that they are glycosylated.

The Affinity Chromatography Approach

The purification of solubilized opioid receptors by affinity chromatography relies on their ability to recognize immobilized opioid ligands. The receptor population isolated by

this technique can be tested for binding potency and can be obtained in sufficient quantities as to allow sequencing. The major drawback of this approach is the difficulty in preserving biological activity during the various purification steps.

Prior to purification of ligand binding proteins, membrane–receptor complexes must be dissociated and receptor proteins freed from membrane lipids and extraneous membrane associated proteins. This implies solubilization of the membranes. Since the opioid receptors are particularly sensitive to detergents (Zukin and Maneckjee, 1986), this step can result in loss of biological activity. The solubilized membrane proteins are then chromatographed on an opioid affinity column. An enriched population of opioid receptors is specifically eluted and its binding characteristics determined. The ligand binding polypeptide contained in this enriched protein mixture is then further purified by means of different biochemical techniques.

One of the first purifications of opioid receptors used rat brain membranes as receptor source, Triton X100 as detergent and a derivative of morphine, 14-beta-bromoacetamidomorphine, conjugated to Sepharose as affinity matrix (Bidlack et al., 1981). A 2000-fold purification was achieved. This enriched mixture was composed of several proteins in particular three major one of M_r 23,000, 36,000, and 43,000. By solubilizing rat brain membranes in the detergent CHAPS, and retaining opioid receptors on another morphinan column (Hybromet), a 500-fold purification was achieved. Electrophoresis of this material revealed the presence of three proteins M_r 35,000, 44,000, and 94,000 (Maneckjee et al., 1985). More recently, two studies have succeeded in purifying opioid receptors to apparent homogeneity. Using bovine striatal membrane proteins solubilized in digitonin, Gioannini et al. (1985) have purified opioid receptors on a beta-naltrexylethylenediamine Sepharose column followed by a wheat germ agglutinin chromatography step. The opioid receptor was purified to homogeneity as judged by the level of its binding activity and the appearance of a single band with a MW of 65,000 daltons. Finally, the mu opioid receptor of rat brain has been isolated after solubilization in Triton X100, by chromatography on a succinylmorphine column, gel filtration step, wheat germ agglutinin chromatography, and isoelectrofocusing polyacrylamide gel electrophoresis. The opioid receptor was purified to homogeneity and has a M_r 58,000 (Cho et al., 1986).

From these affinity chromatography approaches, two physical characteristics of the ligand binding subunit of the opioid receptor can be derived: its molecular weight is approximately 58,000 daltons and it is glycosylated. These conclusions are in agreement with the results obtained using the ligand binding approach.

Example of a Partial Purification of the Rat Mu Receptor

We have attempted to purify rat mu opioid receptor using the affinity chromatography approach. The difficulties in purifying the opioid receptors to homogeneity are considerable. Although it has been reported that this task has been achieved (Simonds et al., 1985; Gioannini et al., 1985; Cho et al., 1986), we will use some of our own experiments to illustrate the kind of problems that have to be overcome. The techniques used in obtaining an enriched opioid receptor preparation have already been described (Bidlack et al., 1981) (*see above*).

Rat neural membranes were prepared from brain without the cerebellum and were solubilized in 1% Triton X100 in the presence of protease inhibitors. Excess detergent was removed by incubation in the presence of Biobeads SM2 and the proteins were concentrated

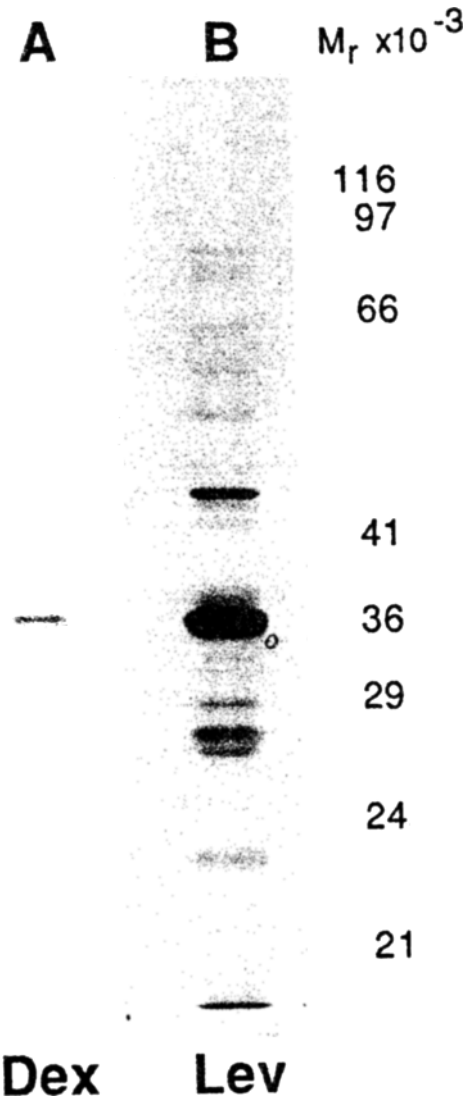

Fig. 2. SDS polyacrylamide gel of stereospecifically eluted protein from the BAM affinity column. Solubilized rat brain membrane proteins were chromatographed on a BAM column as described in the text. Proteins retained on the matrix were eluted first with dextrorphan (100 μM) lane A and then levorphanol (100 μM) lane B. These proteins were concentrated, treated in the presence of SDS and 2-mercaptoethanol and electrophoresed on a 10% Laemmli/SDS slab gel, which was stained with silver. Molecular weight standards are indicated.

by ultrafiltration in 50 m*M* Tris pH 7.4 buffer.

A morphine derivative, 14-beta-bromoacetamidomorphine (BAM), conjugated to omega-aminohexyl-Sepharose 4B, served as affinity matrix. This morphine derivative showed some of the properties of a mu type ligand, suggesting that a receptor isolated using this affinity matrix would be of the mu class. The solubilized membrane proteins were applied to the BAM column in 50 m*M* Tris pH 7.4 at 4°C. The column was washed extensively in the same buffer and the bound opioid receptor was eluted with an opioid agonist in Tris buffer. The displacing agent was used at a concentration of 10–100 μ*M* because the affinity column has between 6 and 10 μmol of binding sites/mL of gel. (In preliminary studies where the concentration of the opioid agonist was lowered, the opioid active fraction was eluted over a larger volume.) A small quantity of proteins were eluted by opioid agonist and consist of several different polypeptides. This complex was reproducibly eluted, and, moreover, as described below, represents an important enrichment in bioactive opioid receptors.

Elution of the proteins bound to the BAM column was achieved using different opioid related ligands, and the eluted proteins were first analyzed by SDS polyacrylamide gel electrophoresis (Fig. 2). If the BAM column is incubated in presence of dextrorphan, an inactive opiate stereoisomer, essentially no polypeptides are eluted (the 35 kD polypeptide seen on the autoradiogram is also present in the wash preceding the elution). On the other hand, if the active isomer levorphanol is used to elute the BAM associated proteins, a complex misture of polypeptides is detected consisting of three major proteins of 23, 36, and 43 kD (kD = 1000 daltons) as well as several minor ones in the 28–30 kD and in the 55–80 kD ranges. The detection of these proteins by silver staining confirms the previously described analysis using Coomassie blue staining (Bidlack et al., 1981). Some proteins are differentially detec-

ted by the two staining procedures; in particular the 23 kD polypeptide is poorly stained by silver. The fact that this protein mixture is eluted by levorphanol and not by dextrorphan indicates that the elution is stereospecific. Indeed, we have extended these results by showing that other opioid agonists or even opioid peptides elute the same proteins as levorphanol (Fig. 3, and unpublished results).

The protein mixture eluted from the BAM column retained binding activity. Before the column, the solubilized proteins bound [^{125}I] beta-endorphin with a K_d value of 4.2 n*M* and a Bmax of 98 fmol/mg protein. Approximately 0.015% of those proteins are eluted with levorphanol and this mixture has a K_d of 2.2 n*M* for beta-endorphin and a Bmax value of 125 pmol /mg of protein. The affinity chromatography step therefore resulted in a 1200-fold purification of the opioid ligand binding protein.

It can be calculated, on the basis of a 60 kD mol wt, that a homogenous opioid receptor will represent about 12,000 pmol/mg of protein. We therefore need to increase our purification of the opioid receptor by another factor of 100 to reach homogeneity. Such an enrichment can succeed in one step if one applies reverse-phase high-performance liquid chromatography.

We have analyzed our BAM-eluted receptor preparation by chromatographing the proteins in a 25–95% acetonitrile gradient on a C4 column matrix (VYDAC). A typical protein profile is shown in Fig. 3. Five different peaks are clearly resolved, which, upon analysis by SDS-polyacrylamide gel electrophoresis have been demonstrated to be respectively and sequentially the 23, 60, 36, 43, and another 36 kD. These proteins correspond to the proteins detected by SDS-gel electrophoresis before the HPLC step. This purification has resulted in the separation of the major 36 kD proteins into two distinct peaks. The detection of polypeptides by absorbance at 214 n*M*, instead of by silver staining, gives a better evaluation of the amount of 23 kD protein in this preparation. Although five peaks have been clearly resolved, several other proteins are also present in minor quantities.

The next step was to determine which of the eluted proteins was the genuine opioid-ligand binding protein. Since the HPLC step denatures proteins, the opioid receptor cannot be assayed for its binding activity. Therefore, we were obliged to apply a different criterion for its identification. One of the physical constants known for this receptor is its molecular weight, around 58,000 daltons. We have detected a protein of 60 kD, which represents less than 5% of the total protein. This polypeptide is therefore a possible opioid receptor candidate based on its size and concentration. Another physical characteristic attributed to the opioid receptor is its glycosylated nature. Preliminary experiments indicate that our 60 kD protein might be retained on a lectin column, and therefore could fit the two criteria for an opioid receptor.

Interestingly, all the proteins eluted from the BAM affinity column show a stereospecific interaction with the matrix material. When analyzed by HPLC, proteins eluted with the stereoactive agonists levorphanol or morphine (Fig. 3) showed very similar patterns of absorption, whereas the inactive isomer dextrorphan eluted practically no protein. In addition, if the highly specific mu opioid peptide analog DAGO (*see* Fig. 1) was used, the eluted proteins displayed a pattern similar to the ones obtained with the other active agonists (data not shown). This analysis, therefore, demonstrates the stereospecificity of the opioid receptor preparation and also points to the 60 kD as the most likely candidate for being the ligand-binding protein. Work is in progress to further characterize this protein.

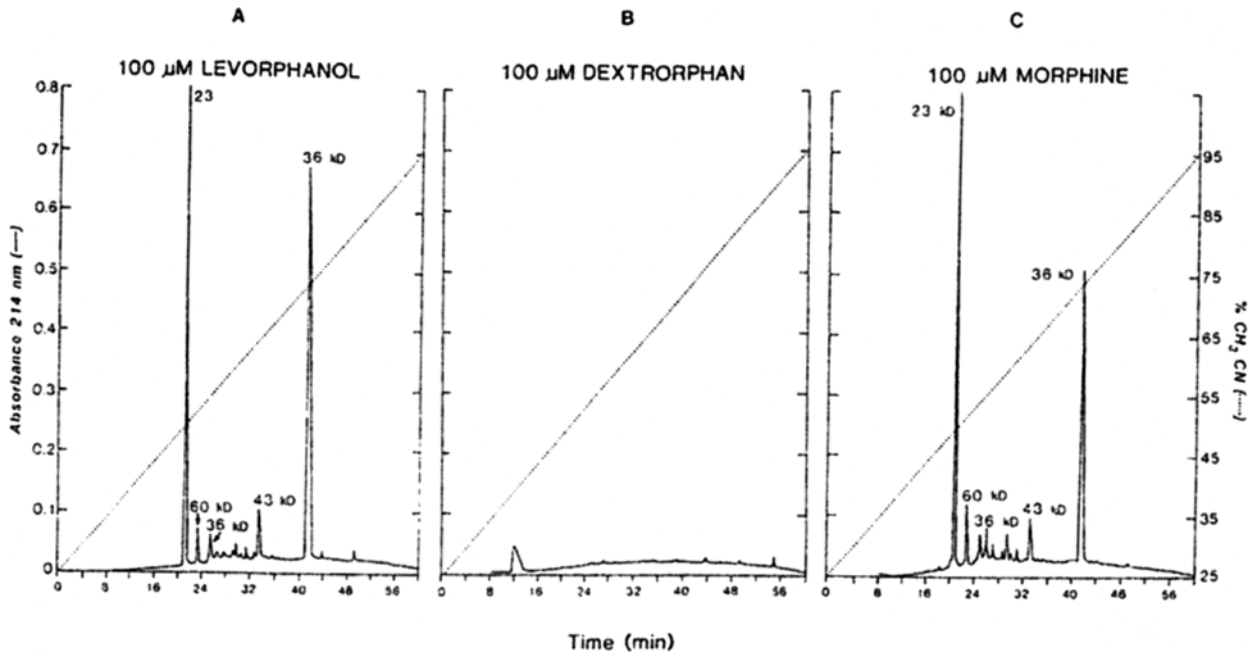

Fig. 3. HPLC absorbance profiles of the proteins eluted from the BAM affinity column. Proteins eluted as described in Fig. 2 were loaded onto an HPLC C4 reverse phase column (VYDAC) and eluted with an acetonitrile gradient of 25–95%. Absorbance was monitored at 214 nm. The Figure presents the profiles of the proteins eluted by levorphanol and dextrorphan prepared as in Fig. 2 or eluted by morphine from a different preparation. The different fractions were analyzed by SDS gel electrophoresis to determine the molecular weight of the protein present in the different peaks (kD = 1000 daltons).

Strategies for Cloning of the Opioid Receptors

As pointed out in this review, the physical analysis of the opioid receptors has been hampered by their low abundance and by their lability. In order to study structure–activity relationships or the molecular basis of opioid receptor involvement in physiological responses, it is necessary to clone these receptors. Several approaches can be adopted toward this goal.

The first, more traditional approach, consists of purifying the receptor to homogeneity using one of the biochemical strategies described above. The pure receptor is subjected to partial proteolysis, individual peptide fragments are recovered by HPLC and their sequences are determined. These sequences are used to predict and synthesize oligonucleotide probes specific to the receptor sequence. In parallel, the total mRNA population of a tissue or a cell line known to express opioid receptors is prepared. These mRNA molecules are enzymatically copied into their complementary DNA form. The cDNA molecules are inserted into procaryotic vectors, which are then used to transform bacteria. These transformed bacteria, each having incorporated a single, different recombinant vector, are plated separately. This population of transformed bacteria corresponds, therefore, to a representation of the original mRNA population. (Alternatively, genomic DNA libraries can also be constructed.) The

synthetic oligomers then serve as hybridization probes to isolate a cDNA (or a gene) coding for the opioid receptor. This isolated clone is used as a template to determine the entire sequence of the opioid receptor and, moreover, as a probe to study opioid receptor gene expression.

To verify the authenticity of the opioid receptor clone, this isolated clone must be capable of inducing the synthesis of bioactive opioid receptors. Techniques have been established for the transfer of cDNAs (or their corresponding genes) into eucaryotic cells. Provided that the isolated cDNA is inserted in a plasmid containing a transcriptional promoter (Okayama and Berg, 1982), this cDNA should be able to direct the expression of bioactive opioid receptors. The presence of newly synthesized receptors on the membranes of transfected cells can be measured in binding assays. This experiment not only ascertains the nature of the isolated clone, but also represents the first step in the study of opioid receptor gene expression.

The second approach does not require prior isolation of the receptor protein. Instead, the receptor is cloned on the basis of its biological activity, i.e., a molecule able to bind an opioid, this binding being reversible by an antagonist such as naloxone. For this approach to succeed, a large population of different cDNAs (or large fragments of genomic DNA) from a tissue known to express high level of opioid receptors are transferred into eucaryotic cells that do not express the receptors. Transfectants that have incorporated exogenous DNA are selected according to traditional techniques (Southern and Berg, 1982), and are tested for their ability to express opioid receptor by binding analyses. The DNA of those positive transfectants is then analyzed for integration of exogenous fragments. If pools of cDNAs are transfected, the opioid receptor specific cDNA can be isolated by gradually decreasing the size of the analyzed pool to unity. When genomic DNA fragments are randomly transfected, more than one contiguous exogenous fragment can be integrated. Therefore, the transfected DNA fragments are further purified by secondary transfection.

This step consists of repeating the transfection procedure using the DNA of the primary transfectant as the source of exogenous genes. Several different variations of this procedure can be envisioned, in particular, cDNA libraries enriched in opioid receptor sequences can be constructed through mRNA subtraction (Hedrick, 1984). In addition, injection of mRNA into frog oocytes can be used as a method to test for the synthesis of opioid receptors. It has been shown that mRNAs microinjected into *Xenopus laevis* oocytes can direct the synthesis of a variety of receptors that can be detected by electrophysiological measurements (Grundersen et al., 1984). By combining this detection procedure and the mRNA subtraction approach described above, it may be possible to isolate a cDNA specific to an opioid receptor. This strategy has been successfully employed in the cloning of a serotonin receptor (Lubbert et al., 1987). There is also the possibility of expressing an opioid receptor cDNA in procaryotic cells; however, in view of the requirements necessary to maintain the biological activity of this receptor, it seems very unlikely that such an expression experiment would be successful.

The expression approach presents the advantage of directing the cloning procedure toward a biologically active receptor. In the case of the more traditional approach, demonstration of the nature of the isolated clone will require its expression and analysis of its binding characteristics. These requirements make the cloning of the opioid receptor a challenging project. This problem, however, is currently being overcome for numerous similar receptors (Bonner, 1987) and, indeed, these experiments will assist in the cloning of the opioid receptor technically, as well as conceptually.

Prospects: Molecular Biology of the Opioid Receptor

An emerging concept in the field of neuroreceptors is that these receptors can be divided into two classes: the ligand-gated ion channel receptors and the G protein-coupled receptors (Noda 1982, 1982; Grenningloh, 1987; Schofield, 1987; Kubo, 1986; Nathans and Hogness, 1983; Young, 1986; Dixon, 1986; Peralta, 1987; Bromer, 1987; Hall, 1987; Stevens, 1987). The receptors constituting these two different classes display similarities in structure and in sequence. It is possible that they are part of two distinct gene families. The opioid receptors, at least the mu and delta types, belong to the G protein-coupled class, as indicated by their biological activities. They might, therefore, share some sequence homology with the receptors of this family, notably the beta-adrenergic, the opsin, the mas oncogene, or the muscarinic receptor, the only four receptors thus far cloned (Dixon, 1986; Kubo, 1986a, 1986b, Peralta, 1987; Nathans and Hogness, 1983; Young, 1986). This hypothesis can serve as a basis for a third cloning approach. The salient common feature between the G protein-associated receptors lies in their conformation; they all have seven transmembrane domains. In this approach, this feature becomes a criterion when analyzing a possible opioid receptor clone.

An opioid receptor cDNA or gene can be isolated by screening cDNA or genomic libraries under conditions that allow the detection of not only one but several G protein-related receptors.

This approach has already been successfully used in isolating related genes (Bonner, 1987). Since not only the opioid receptor, but also other G protein-related receptors, will be detected by this approach, expression of the isolated clone will be necessary to determine its specificity.

The possible homology between the G protein-coupled receptors make the cloning of the opioid receptor an even more attractive task. At the time of writing this review, no neuropeptide-specific receptors have been cloned. Gaining insight into the structure of such a receptor will help not only our understanding of the mechanism of neuropeptide recognition by its receptor but also might shed some light on the evolutionary process that led to the appearance of those receptors. Also, a clone specific to one of the opioid receptors will probably be the best tool for resolving the problem of the molecular basis of opioid receptor heterogeneity.

Acknowledgments

We thank Alan North and Eckard Weber for their critical review of this manuscript, and Diane Arrington for her expert manuscript preparation. The research presented in this review was supported by research grants from the National Institute of Diabetes, Digestive and Kidney Diseases (DK-37231) and from Cambridge NeuroScience Research, Inc.

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