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Review: Josef Rudinger Memorial Lecture 2002‡

The Chemistry of the Opioid Receptor Binding Sites

KÁLMÁN MEDZIHRADSZKY*

Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Budapest, Hungary

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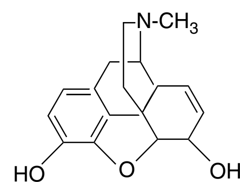
Abstract: Since the discovery of the opioid receptors and their endogenous ligands an immense research work has been devoted to the exploration of their specificity, the mechanism of ligand binding and ligand–receptor interactions. One of the main goals has been the location and characterization of the binding sites. The present review compiles the results achieved in this field in the last quarter of a century, and puts some questions concerning the success of these efforts. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: opioid receptor; affinity labelling; binding sites; receptor specificity; opioid peptides

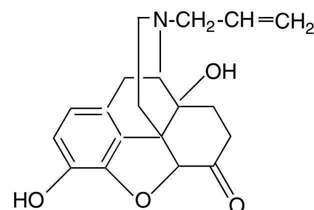
INTRODUCTION

The biological effects, such as euphoria and analgesia, of opium, the dried latex obtained from poppy-head were known to the ancient people in the Mediterranean and the Middle East well before our era. Its main active ingredient, morphine, named after Morpheus, the Greek god of dreams, was the first alkaloid isolated in crystalline form by the German chemist Sertürner in 1805 [1]. Through the centuries, morphine proved to be the most effective pain-relieving drug, possessing, however, significant undesirable side effects such as tolerance and physical dependence.

The effective pharmacological dose of morphine is very small and its action is highly stereoselective. This can be explained only by the existence of very specific receptors in the living organism. As it seemed unlikely that a plant alkaloid would have its own stereospecific receptors on neuronal membranes, an extensive search for a putative endogenous opioid ligand in mammalians was initiated. As is known, this research work culminated in the discovery of the two enkephalins, the first opioid peptides of known structures, by Hughes, Kosterlitz



Morphine



Naloxone

* Correspondence to: Prof. Kálmán Medzihradszky, Research Group of Peptide Chemistry, H-1518 Budapest, PO Box 32, Budapest, Hungary; e-mail: medzy@szerves.chem.elte.hu
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BIOGRAPHY

Dr Kálmán Medzihradszky is at present Emeritus Professor at the Eötvös University, Budapest. He finished his studies at the same University in 1950, and was Prorector (1980–1983) and Dean of the Faculty of Sciences (1983–1989). He has been involved in peptide chemistry from the beginning of his career (bacterial polyglutamic acid, 1950–1956, synthesis of human ACTH, 1959–1967, structure-activity studies on α-melanotropin (1968–1975) and opioid peptides (from 1976 on). He has been member of the Hungarian Academy of Sciences since 1982.

and coworkers in 1975 [2]. As the opiate receptor possesses therefore not only ligands with alkaloid like and peptide structure as well, it is better named an opioid receptor. The existence of a common receptor is proved also by the fact that the opioid effects exerted by either of the two ligand families can be antagonized by the same compound, naloxone. On this basis, a series of further opioid peptide agonists has been discovered.

From investigations with different ligands and antagonists the presence of opioid receptors could be established not only in the central nervous system but in other organs such as guinea-pig ileum or mouse vas deferens as well, two systems which later became important experimental *in vitro* test objects. From these experiments and binding studies it appeared that more than one receptor type existed. Studying the effects of morphine alkaloids Martin and co-workers observed [3,4] that the symptoms caused by the withdrawal of different opiates could not be suppressed by the administration of another opioid compound, concluding that it is likely that these act on different opioid receptors. The authors distinguished μ-, κ- and σ-receptors, this palette was later enriched by the δ-type, which was supposed to be responsible for the binding of peptide ligands [5]. At present we know that the opioid receptor family is much more abundant, there exist subclasses or subtypes as well (for short reviews see [6,7]).

Although at the beginning characterization of the individual receptor types was performed by using selective synthetic compounds of alkaloid structure, it became interesting to know whether these receptors possess their own specific endogenous ligands as well. Of the discovered natural peptides no fully selective compound was found, but their selectivity could be greatly improved by systematic structure modifications. Table 1 comprises the presently known and most used receptor selective opioid peptide derivatives.

According to the general concept developed through structure–activity studies the 'message' centrum responsible for the biological effect of these peptides lies in the *N*-terminal part bearing the protonated tyrosine moiety, while the *C*-terminal part is the 'address' sequence responsible for the receptor specificity. Determination of the exact selectivity is difficult because of the inhomogeneity of the receptor populations in the test systems applied. Recently, Mansour *et al.* [15] used pure cloned μ- δ- and κ-receptors, and compared the selectivities of known opioid peptides in these systems . They found that none of these peptide ligands could be ordered to a single pure receptor, so the former thesis about the putative message and address segments of enkephalins could not be corroborated. According to their opinion the whole sequence of enkephalins is necessary for the binding to μ- and

Table 1 Specific Peptide Ligands of the Opioid Receptors

Receptor	Peptide ligand	Short name	Reference
μ	Tyr-D-Ala-Gly-MePhe-Gly-ol	DAGO or DAMGO	[8]
	Tyr-Pro-Phe-Pro-NH_2	Morphiceptin	[9]
δ	Tyr-D-Ala-Gly-Phe-D-Leu	DADLE	[10]
	Tyr-D-Pen-Gly-Phe-D-Pen (Pen–Pen bridged)	DPDPE	[11]
	Tyr-D-Ser-Gly-Phe-Leu-Thr	DSLET	[12]
	Tyr-D-Thr-Gly-Phe-Leu-Thr	Deltakephalin or DTLET	[13]
κ	D-Pro^{10}-dynorphin-(1–11)	DPDYN	[14]

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δ-receptors, and an additional basic core renders the molecule capable of binding to the κ-receptor.

A number of valuable reviews can be found in the literature on the different opioid receptors and their endogenous ligands [16–19].

THE CHEMICAL STRUCTURE OF THE OPIOID RECEPTORS

With the aid of more or less selective ligands one can discriminate and characterize the individual receptor types. From these investigations it became very likely that these are independent, structurally different protein molecules. A next possible step, at least from the chemical point of view, could be the determination of the mechanism of interaction between the receptor and ligand, and of the chemical structure of the ligand binding site. With knowledge of these structural elements it may become possible to establish the receptor conformation and eventual conformation changes as a result of receptor–ligand binding, and finally the details of signal transduction mechanism.

From the first cDNA cloning experiments published during 1992–1993 (NG108-15 neuroblastoma-glioma hybrid cells, δ-receptor: Evans *et al.* [20]; Kieffer *et al.* [21]; rat brain κ-receptor: Chen *et al.* [22]; rat brain δ- and μ-receptor: Fukuda *et al.* [23]; rat brain μ-receptor: Thompson *et al.* [24]) we know that all of these receptors belong to the guanine-nucleotide binding protein (G-protein) family, containing seven transmembrane domains. The *N*-terminal part of their protein chain is on the extracellular side of the membrane, the *C*-terminal part can be found inside the cell. They are glycoproteids, the sugar components bound to asparagine residues as *N*-glycosides. The *C*-terminal segment contains a palmitoyl chain, bound through a cysteine residue and submerged in the lipid layer. A cystine residue binds the first and second extracellular loop.

Comparison of the structures of the three receptor types shows that the sequences of the transmembrane domains and of the intracellular loops are very similar, in some places even identical. There are significant divergencies, however, in the structures of the *N*- and *C*-terminal segments, and differences in the chain length can also be found in these regions. The longest is the μ-receptor (398 amino acids), shorter is the κ-receptor (380 amino acids) and the shortest is the δ-receptor (372 amino acids). If we begin the numbering of the individual amino acids at the *N*-terminus of each receptor, different values

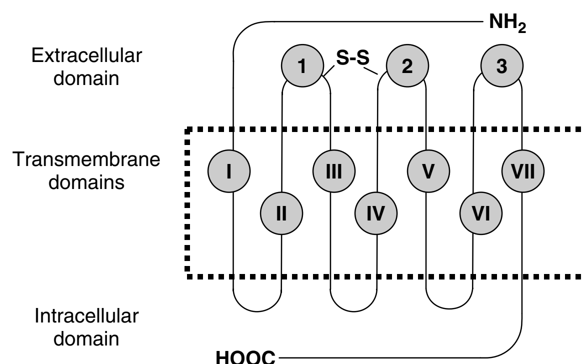

Figure 1 Position of the opioid receptor in the cell membrane.

for the corresponding residues will be obtained, as the terminal amino acids occupy different points of an imaginary whole chain.

The knowledge of the primary structure of the receptors still does not answer the question, as to what the mechanism of the receptor-ligand binding is, could the functional groups of the receptor protein be determined, or in other words, is it possible to map or topochemically characterize the receptor binding site? A probable answer is that first the functional groups responsible for the ligand binding have to be characterized and, as a second step, their position along the polypeptide chain has to be determined. The functional groups can be recognized by reactions with specific reagents, and their location in or near the binding site determined by affinity labelling. In the latter case a receptor selective ligand should bear a chemically reactive substituent which in itself does not influence the binding, but following the reversible binding the ligand forms an irreversible covalent bond with the receptor. If, at the same time, the ligand is radiolabelled, after partial enzymatic degradation and separation of the radioactive fragment, the position of the binding site in the receptor, or even its primary structure could be determined.

This review intends to give an overview of experiments aimed at achieving this goal.

Sulfhydryl Groups on the Opioid Receptor

Pasternak *et al.* [25] observed in 1975 that the opioid receptor could be inactivated with sulfhydryl reagents: iodoacetamide in a concentration of 0.1–5.0 mM, and *N*-ethylmaleimide at 10 μM inhibited the binding of opiate alkaloids, more strongly that of the agonist (dihydromorphine), and to a lesser degree that of the antagonist (nalorphin).

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Previous addition of the ligand to the receptor prevented the inhibition, indicating that the sulfhydryl group to be alkylated lies in the neighbourhood of the ligand-binding site.

After 5 years Smith and Simon examined the same phenomenon with peptide ligands: *N*-ethylmaleimide irreversibly inhibited the binding of tritiated DADLE, and addition of the peptide ligand protected the binding site from alkylation [26]. Perhaps even more important was the observation that the protecting effect of the peptide ligands (D-Ala^2,Leu^5-enkephalin or D-Ala^2,Met^5-enkephalin) and that of alkaloid-ligands (morphine or naltrexon) was significantly different, indicating the presence of opioid receptors of different specificity. From the pseudo first order kinetics of the inactivation observed, the authors came to the conclusion that in this reaction a single sulfhydryl group is involved. On the other hand, Mullikin-Kilpatrick *et al.* [27], observed that on inactivation by *N*-ethylmaleimide of the NG108-15 hybrid cell line opioid receptors the previous addition of ligands or sodium ions differently influenced the binding of agonists and antagonists showing rather that two sulfhydryl groups participate in ligand binding. Much later Ofri and Simon [28] in the μ-opioid receptor from bovine striatal membrane also demonstrated the presence of two sulfhydryl groups, as μ-agonist binding was found to be much more sensitive to *N*-ethylmaleimide than antagonist binding, especially in the presence of sodium ions, which effects the binding of the two types of ligands in opposite directions. From the two sulfhydryl groups one is assumed to be essential only for agonist binding, the other for antagonist and agonist binding. As we will see later, site directed mutagenesis studies are indicative of the involvement of even more sulfhydryl groups in ligand binding.

Two further findings rendered the presence of sulfhydryl groups on the receptor probable. Hazum *et al.* [29] observed that opioid receptors in neuroblastoma cells form clusters on addition of fluorescent labelled enkephalin and this phenomenon does not occur when the receptor is preincubated with *N*-ethylmaleimide. Similarly, receptor conformation change by the formation of a Cu-S-complex may be responsible for the analgesic effect caused by the ICV administration of Cu^{2+} ions into mouse brain [30].

Affinity Labelling through Sulfhydryl-disulfide Interchange

The chemical sensitivity and the pH dependent nucleophilic character of the sulfhydryl group offers an excellent possibility for affinity labelling. In 1987 Bowen *et al.* [31] synthesized an enkephalin derivative elongated at the *C*-terminus by a cysteine residue (D-Ala^2,Leu^5,Cys^6-enkephalin). This δ-specific ligand irreversibly inhibited by 50% the binding of tritiated DPDPE. Its disulfide-containing dimer was an even more effective affinity label. The latter reaction points clearly to a thiol-disulfide interchange. On the other hand, the irreversible binding of the cysteine containing ligand could be explained only by interaction with a receptor disulfide group, supposing that during the incubation no oxidation of the thiol-ligand took place.

A Japanese research team [32,33] performed a series of experiments to achieve irreversible binding with similar enkephalin analogues (D-Ala^2, cysteamine^5-enkephalin, D-Ala^2,Leu^5,cysteamine^6-enkephalin). Of these, the dimer of the first mentioned compound was able to bind covalently to μ- and δ-receptors [34], suggesting the receptor contained an essential thiol group which may interact with the ligand's *C*-terminal functional group. The same team later reported that an enkephalin derivative containing the so-called leucine-thiol moiety (D-Ala^2,Leu(CH_2SH)^5-enkephalin) became covalently bound to the receptor sulfhydryl group via disulfide formation after prolonged incubation [35]. Again this would mean the interaction between a ligand-thiol and a receptor-disulfide group or the involvement of an oxidation process.

Unambiguous results could be achieved by the application of disulfide derivatives of the leucine-thiol-enkephalin. This compound, activated by disulfide formation with a thiomethyl-, or 3-nitro-2-pyridine-sulfenyl (Npys) group could be irreversibly bound to μ-receptors from rat brain or guinea pig ileum [36,37].

Comparing this with the observation that enkephalin derivatives containing Npys residues in different positions do not bind irreversibly to the

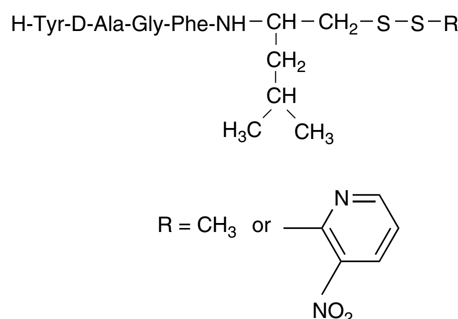

Figure 2 Activated DALE-analogues.

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receptors [38], the authors concluded that the μ-receptor contained a reactive sulfhydryl function accessible only for the disulfide group at the enkephalin *C*-terminus. Irreversible binding takes place, however, on the incubation of rat brain δ-receptors with D-Ala^2,Leu^5,Cys(Npys)^6-enkephalin and this reaction can be reversed by treatment with dithiothreitol. Regeneration of δ-receptors in this way is more rapid than that of the μ-type, indicating the easier accessibility of δ-receptor thiol groups [39]. The more recent results on the affinity labelling with nitrophenylsulfenyl derivatives have been summarized by Shirasu and Shimohigashi [40].

Shimohigashi and his group, already in the knowledge of the primary structure of cloned receptors, tried to localize the reactive sulfhydryl groups in the neighbourhood of the binding site by measuring the binding of dynorphin analogues [41]. From these peptides, containing Cys(Npys) residues in different positions of the dynorphin sequence, D-Ala^2,Cys(Npys)^{12}-dynorphin-A-(1–13)-amide blocked only the μ- and δ-receptor, while D-Ala^2, Cys(Npys)^8-dynorphin-A-(1–9)-amide bound irreversibly to all receptor populations. This means that there is no sulfhydryl group near the dynorphin 12 position in the κ-receptor.

An alkaloid-based affinity label, S-activated 6-sulfhydryl dihydromorphine has also been prepared for labelling the μ-opioid receptor [42].

Maleimido Derivatives

The successful application of *N*-ethylmaleimide for the inhibition of thiol-proteinases and in the characterization of reactive cysteine residues on the ligand binding sites of different receptors gave the idea for the synthesis of maleimide containing affinity labels. It should be kept in mind, however, that the pH optimum of the Michael addition between maleimide and mercapto compounds lies around 7, and the reaction is not fully selective, the side chains of lysine and histidine in lysozyme and ribonuclease are also capable of being alkylated if the reaction time is sufficiently long [43,44]. It is also noteworthy that hydrolysis of maleimides to nonreactive maleamic acids occurs above pH 8 [45].

The maleimido group can be introduced into the peptide amino terminus or at the side chain of a lysine residue. If it affects the biological properties of the compound adversely, as in the case of the opioid peptides, the reactive group can be formed at the carboxyl terminus as well, e.g. through a hydrazide function. Here we can distinguish between direct coupling of the maleoyl group to the hydrazide moiety or rather acylation of the hydrazide by maleoyl amino acid. Examples for both are described by Keller and Rudinger [46]. In the same year in independent experiments Rich *et al.* synthesized maleoyl-glycyl-oxytocin [47] and Krojidlo *et al.* [48] another maleoyl-glycyl-oxytocin derivative. These compounds have been prepared for the affinity labelling of the oxytocin receptor. Although they reacted readily with compounds bearing sulfhydryl groups, they were unable to inactivate the receptor, which may indicate that the maleimido group could not reach the supposed cysteine residue in the ligand binding site.

For the introduction of maleimido groups an alternative procedure was elaborated by Wünsch and co-workers [49]: acylation of peptide amino-terminus by the *N*-hydroxy-succinimide derivative of maleoyl-β-alanine. An intramolecular side reaction between the maleimido group and a histidine residue in favourable position could be observed [50].

For the affinity labelling of the opioid receptors through the well established sulfhydryl groups on the binding site Medzihradszky and co-workers synthesized maleimido derivatives of opioid peptides [51,45]. First the Pro-Gly-Phe-Leu-Thr pentapeptide has been acylated on the *N*-terminus by maleoyl tyrosine, in order to obtain a δ-specific antagonist (cf.[52]). Affinity labels of agonist properties could be prepared by introducing the reactive group into the carboxyl terminus by acylating the peptide hydrazide with maleoyl glycine. Maleimido peptide derivatives prepared this way are listed in Table 2.

Most of the synthesized ligands bound irreversibly to the opioid receptors of a membrane homogenate prepared from rat brains. It was remarkable that binding increased with the length of the peptide chain, the heptapeptide containing D-alanine showed an 86% irreversibility at a concentration of 10 μM. The last peptide in Table 2, derived from

Table 2 Maleoyl-glycyl Derivatives of Opioid Peptide Hydrazides

Tyr-Gly-Gly-Phe-Leu-NH-NH-Gly-**Mal**
Tyr-D-Ala-Gly-Phe-Leu-NH-NH-Gly-**Mal**
Tyr-Gly-Gly-Phe-Met-Arg-Phe-NH-NH-Gly-**Mal**
Tyr-D-Ala-Gly-Phe-Met-Arg-Phe-NH-NH-Gly-**Mal**
Tyr-D-Ser-Gly-Phe-Leu-Thr-NH-NH-Gly-**Mal**

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DSLET [12] proved to be a highly selective irreversible δ-agonist exerting a nearly quantitative inhibition of the receptor at a concentration of 10 μM [53]. This compound seems the best of the maleimido derivatives described so far for mapping the receptor binding site.

It should be mentioned that there was no observable irreversible binding in the isolated organs (GPI or MVD), although the peptide specificity was the same as measured on brain receptors. This is a recurrent phenomenon that can be explained by discrepancies in the receptor populations of the test systems or by circumstances influencing the accessibility of reactive functional groups [45,54,55].

Diazomethyl Ketones

Peptide diazomethyl ketones are selective inhibitors of thiol-proteinases if their sequence corresponds to the specificity of the enzyme, as their reactivity against structurally unrelated mercapto compounds, such as mercapto-ethanol or glutathione is negligible [56–58]. At the same time diazomethyl ketones are capable of reacting in a non-selective way when irradiated by ultraviolet light.

There is only one example of attempting to affinity label the opioid receptors: Belcheva *et al.* [59] prepared the D-Ala^2,Met^5-enkephalin diazomethyl ketone. The compound lost most of its δ-specificity compared with that of the parent peptide and showed only 30% irreversible inhibition of the receptor in a concentration of 100 μM on UV irradiation. There are of course, no data about which structural element of the receptor reacted with the intermediary carbene.

Histidine on the Opioid Receptor

The presence and participation in ligand binding of histidine in the opioid receptor derived from rat brain synaptosome fraction was demonstrated by Roy and Ng [60]. The histidine imidazol group could be ethoxyformylated in a rapid reaction by ethoxyformic anhydride (diethyl-pyrocarbonate, C_2H_5O-CO-O-CO-OC_2H_5), and the receptor treated in this way almost lost its ligand binding (tritiated etorphine) property. If the receptor is saturated with opioid ligand (Met-enkephalin) beforehand, the inactivation fails to come about, proving that the histidine residue occupies a position in the binding site.

The importance of the histidine residues in the ligand binding became evident through site directed mutagenesis studies as well (see later). It is not clear, however, whether it proves a direct participation of histidine in the binding process or if these are just conformational changes caused by the elimination of the imidazole function. An unambiguous answer to this question could perhaps be obtained by affinity labelling of the histidine residues involved in ligand binding.

Chloromethyl Ketones

In order to inhibit serine-proteinases, where an Asp-His-Ser amino acid triade is responsible for the catalytic activity, Schoellmann and Shaw [61] were the first to prepare chloromethyl ketones: tosyl-phenylalanine chloromethyl ketone was the covalently bound inhibitor of chymotrypsin and tosyl-lysine chloromethyl ketone that for trypsin. The chloromethyl group alkylated the histidine imidazole ring in the active centre, mostly because of its proximity to the ligand binding site rather than for its extraordinary reactivity. Thereafter the synthesis of a series of peptide chloromethyl ketones for the inhibition of various serine proteinases has been reported (for a review see [62]). In 1980 Pelton and co-workers published the preparation of chloromethyl ketones of leucine-enkephalin and its D-Ala^2 analogue [63]. Owing to the enhancement of their *C*-terminal apolarity compared with the parent compounds possessing free carboxylic groups, these peptides showed increased affinity to the GPI receptors, but they could be washed out and did not show any irreversibility.

Somewhat later Venn and Barnard also synthesized the D-Ala^2,Leu^5-enkephalin chloromethyl ketone (DALECK) [64]. Their compound proved to be an alkylating affinity reagent irreversibly inhibiting the electrically stimulated contractions in a mouse vas deferens tissue preparation, and partially inactivating a defined population of enkephalin receptors in rat brain preparations. The authors did not characterize the nucleophile bound covalently to the alkylating reactant; only several years later did Kondo *et al.* [36] suggest the possible involvement of a receptor sulfhydryl group.

At about the same time as the beginning of an extensive search for affinity labels of the opioid receptor, the author of the present review and his co-workers published the preparation of the two enkephalin chloromethyl ketones [65], the experimental details of this synthesis appeared in Hungarian [66]. Investigating the biological effects of DALECK Szücs *et al.* [67] demonstrated

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its irreversible binding to a membrane fraction prepared from rat brain, the label was stable under denaturing conditions, the binding was pH dependent and maximal at pH 9.0. It could be concluded that a nucleophile, deprotonated at this pH value (imidazole or sulfhydryl group) could be the reactive counterpart of the chloromethyl ketone. Intracisternally given DALECK showed a long-lasting, dose-dependent antinociceptive effect in the rat tail-withdrawal test. This effect could be reversed by naloxone, indicating that in this test system no irreversible binding took place. A possible explanation is that DALECK is able to interact with more than one receptor subtype, its effect being reversible on the μ- (morphine) receptor and irreversible on the δ- (enkephalin) receptor. This explanation may also be valid for earlier observations, namely that DALECK reacts reversibly in the guinea-pig ileum [63] but irreversibly in the mouse vas deferens test [64].

Newman and Barnard [68] prepared the tritiated DALECK as well, making its application possible in the low concentrations necessary for specific labelling. In this way not only the bound ligand quantity could be determined, but also the molecular mass (58 KDa) of the binding μ-receptor protein. Again, no reference to the possible reactive nucleophile was mentioned. With the aid of an even more active radiolabelled DALECK at a nanomolar concentration [69] the ligand's preference for the μ-binding sites could be confirmed [70,71]. Depending on the origin (rat or frog) of the receptor fraction, the binding of 110–130 fmoles ligand to 1 mg receptor protein could be established [72,73].

The μ-directing effect of the chloromethyl ketone group is so strong that by this substitution even the δ-specificity of DADLE could be altered in the μ-direction (DADLECK) [74]. Of course there is no change if the parent peptide itself is μ-specific (conversion of DAGO to Tyr-D-Ala-Gly-MePhe-CH_2Cl) [75]. Indeed, DAGO-chloromethyl ketone (DAMCK) tritiated in the tyrosine residue up to a specific radioactivity of 56.8 Ci/mmole proved to be a highly μ-specific affinity label. In addition, treatment of rat brain membranes with *N*-ethylmaleimide abolished more than 85% of the chloromethyl ketone binding, indicating the involvement of sulfhydryl groups in the binding to the μ- opioid receptor [76]. In the light of other's observations [77,78] it still remains questionable whether or not a histidine residue is the coupling partner of the chloromethyl ketone derivatives.

As the chloromethyl ketone functional group endows the ligands with a powerful μ-specificity, it was difficult to find opioid peptides which keep their δ- or κ-character after conversion to the chloromethyl ketone derivative. Starting from the fact that δ-specificity depends mainly on a free carboxyl terminus, building in a *C*-terminal aspartic acid allows the introduction of a chloromethyl ketone group while keeping the second carboxyl group intact. In fact the synthesized peptides (AECK and DAACK, Table 3) preserved their δ-specificity but their irreversible, covalent binding proved to be limited [79]. On the other hand, the reason for their moderated alkylating property may be an indication of structural deviations between the binding sites of the different receptor subtypes.

Synthesis of a κ-specific chloromethyl ketone derivative was also successful: dynorphin-(1-10)-Gly^{11}-chloromethyl ketone (DYNCK) bound to a membrane fraction rich in κ-receptors from frog brain with an affinity similar to that of the dynorphin-(1-10)-decapeptide and it blocked the receptors irreversibly in a concentration dependent manner (90% inhibition at 50 μM ligand concentration) [80]. Most of the chloromethyl ketones synthesized by this research team are compiled in Table 3.

As an illustration Table 4 shows the degree of irreversible binding to the naloxone sensitive sites of the chloromethyl ketones at different concentrations.

As mentioned before, most of these chloromethyl ketones did not show an irreversible reaction in the *in vitro* pharmacological tests (guinea-pig ileum, mouse vas deferens assays). Although these observations are sometimes not consistent with other's results, the different experimental conditions applied in the *in vitro* tests compared with those used in the binding experiments may be responsible for the discrepancies.

Table 3 Enkephalin Chloromethyl Ketones

Abbreviation	Structure
LECK	Tyr-Gly-Gly-Phe-Leu-CH_2Cl
DALECK	Tyr-D-Ala-Gly-Phe-Leu-CH_2Cl
DADLECK	Tyr-D-Ala-Gly-Phe-D-Leu-CH_2Cl
DAMCK	Tyr-D-Ala-Gly-MePhe-CH_2Cl
AECK	Tyr-Gly-Gly-Phe-Asp(CH_2Cl)
DAACK	Tyr-D-Ala-Gly-Phe-Asp(CH_2Cl)
DYNCK	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg--Pro-Gly-CH_2Cl

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Table 4 Percent Irreversible Binding of Peptide Chloromethyl Ketones

Peptide	1 μM	5 μM	10 μM	50 μM	100 μM
DALECK	36	65	75		
DAMCK	50	70	75		
DYNCK	25	40	70	80	
DADLECK		30	40	50	70

Nitrogen Mustard Derivatives

Compounds containing the bis-(2-chloroethyl)-amino (nitrogen mustard) group have already been used in cancer chemotherapy. These are unspecific biological alkylating agents, e.g. chlorambucil is able to esterify carboxylate groups in proteins [81], melphalan reacts with a number of nucleophiles, among others with the imidazole ring of purine bases.

It seemed obvious to use these mustard derivatives as affinity labels. Chlorambucil can be introduced by acylation of peptide *N*-termini, and melphalan, being an amino acid, can easily be built into any position of the peptide chain. The only difficulties encountered are practical ones, reactivity of the nitrogen mustard group renders these compounds chemically labile during experimental work, such as synthesis, and the application of great caution is advisable [82].

Mustard derivatives were first used for affinity labelling of the angiotensin II receptor. For this purpose Paiva *et al.* [83] acylated the hormone with chlorambucil, Park *et al.* [84] modified the peptide by melphalan. A few years later Hsieh and Marshall [82] prepared Ac-Asn[1],Mel[8]-angiotensin II, which proved to be an effective competitive antagonist of the hormone without demonstratable irreversible action. Melphalan analogues of other natural polypeptides

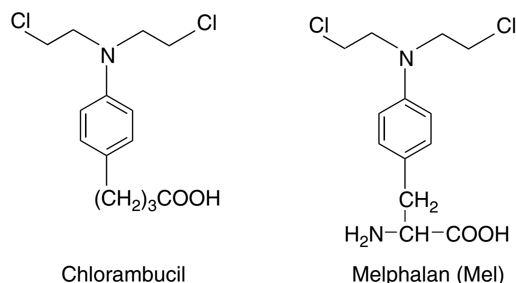
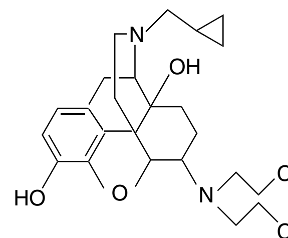


Figure 3 Nitrogen-mustard derivatives.

(α-melanotropin: [85], bombesin: [86]) for receptor targeting have also been synthesized.

The first nitrogen mustard derivative of opioid ligands, a morphine-based compound, named chlornaltrexamine was synthesized by Portoghese *et al.* [87]. It was an antagonist with long lasting effect, covalently bound to the opioid receptor. According to their observation the binding site was identical to that of naloxone, because preincubation of the receptor with naloxone prevented the alkylating effect of chlornaltrexamine [88].



Chlornaltrexamine

A series of melphalan-substituted DALE analogues have been synthesized in the author's laboratory [89–91]. These are shown in Table 5.

D-Ala[2],Leu[5],Mel[6]-enkephalin methyl ester, an enkephalin analogue elongated by melphalan on the *C*-terminus had the same receptor specificity as the parent peptide in a membrane preparation from rat brain homogenate. For a 30–80% irreversible binding, the application of a 10–100 μM ligand concentration was necessary. Previous treatment with non-reactive ligands partially protected the receptor from alkylation. Addition of melphalan alone did not influence the naloxone binding property of the receptor, even when used at a concentration of 50 μM, indicating that the melphalan-substituted peptides do act as affinity labels.

Of these analogues, an especially interesting one is Mel[1],D-Ala[2],Leu[5]-enkephalin methyl ester,

Table 5 Melphalan Analogues of D-Ala[2],Leu[5]-enkephalin Methyl Ester

Tyr-D-Ala-Gly-Phe-Leu-**Mel**-OMe
Mel-D-Ala-Gly-Phe-Leu-OMe
Tyr-D-Ala-Gly-**Mel**-Leu-OMe
Mel-D-Ala-Gly-**Mel**-Leu-OMe

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in which melphalan occupies the *N*-terminal position, substituting the tyrosine residue, thought to be indispensable in exerting the opioid activity. This analogue proved to be a good agonist, with properties similar to those of the parent D-Ala^2,Leu^5-enkephalin, except that its binding to the DALE binding site was somewhat weaker, which, of course can be explained by the presence of the methyl ester instead of the carboxyl group thought to be important for δ-specificity. It inhibited by 50% the naloxone binding sites at a 1 μM concentration, and by 90% the DALE binding sites at a concentration of 10 μM [91].

That a terminal tyrosine in an opioid peptide can be substituted by melphalan without losing the biological activity is worth consideration from the point of view of the ligand-receptor binding, but its discussion would, however, exceed the frames of this review. It is very likely that it does not have anything in common with the alkylation process, because the peptide containing the corresponding diol, 4-bis(2-hydroxyethyl)-amino-phenylalanine, which is of course not an alkylating agent, binds to the receptor with the same affinity as does the melphalan derivative [92].

Synthesis of irreversible antagonists has been attempted by Lovett and Portoghese by substituting melphalan into the Phe^4 position of *N*,*N*-dialkylene-enkephalins [93]. These Mel^4-analogues showed reduced activity in the *in vitro* (GPI and MVD) tests and they were only marginally effective as irreversible antagonists. Despite the structural relationship the Phe^4 position does not seem to be a favourable site for substitution with melphalan (c.f. [94]).

In order to find affinity labels for the κ-opioid receptor, Sartania *et al.* [94] synthesized a number of melphalan-substituted Met^5-enkephalin-Arg^6, Phe^7 derivatives (Table 6).

Although the starting peptide possessed a definite κ-specificity [95], only the heptapeptide containing melphalan at the *C*-terminus preserved this property. This compound irreversibly inhibits by 75% the naloxone binding in guinea-pig brain membranes at a concentration of 10 μM and is a good candidate for mapping the receptor binding site.

As the β-chloroethyl-amino group is not a selective functional moiety, it is uncertain whether the alkylated amino acid side chains belonged to a cysteine or histidine residue.

Table 6 Melphalan Analogues of Met^5-enkephalin-Arg^6, Phe^7

Mel-Gly-Gly-Phe-Met-Arg-Phe
Tyr-Gly-Gly-**Mel**-Met-Arg-Phe
Tyr-Gly-Gly-Phe-Met-Arg-**Mel**

Peptide Epoxides

With knowledge of the presence of sulfhydryl groups in the opioid receptor and their role in ligand binding it is conceivable to design affinity labels from opioid peptides by building in epoxy-containing functional groups. Biologically active natural epoxy-peptides, like the cytotoxic Chlamydocin, a cyclopeptide containing an epoxy-substituted fatty acid [96] or E64, an epoxy-succinyl dipeptide [97,98] are known from the literature. The latter irreversibly inhibits thiol-proteinases by alkylating the cysteine residues in the enzyme active centre. Based on the structure of these natural products, for a possible utilization of their biological activity in the therapy a number of synthetic works has been published. Bernardi *et al.* synthesized alkylating cyclopeptides by substitutions with glycidic acid and epoxy-succinic acid [99], and for the inactivation of cysteine-proteinases epoxy-succinyl dipeptides have been prepared by Gour-Salin [100] and Korn and co-workers [101]. The mechanism of enzyme inhibition by the epoxy-succinyl groups has been discussed by Bihovsky *et al.* [102].

While the epoxy-succinyl group converts the peptide *N*-terminus into an alkylating structural unit, Albeck and co-workers [103,104] used peptides containing an epoxide group on the carboxyl terminus for the inhibition of cysteine-proteinases. The corresponding halogene-methyl ketones served as starting materials for building up the epoxy-substituent, and these could be converted by reduction to halohydrines which in turn were closed to epoxides by treatment with alkali. The authors established that these epoxides are weak electrophiles, and stable under basic or neutral conditions. For the inhibition of thiol-proteinases

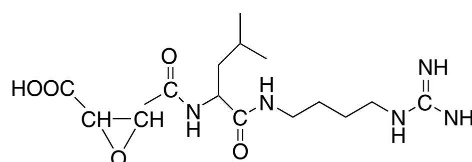

Figure 4 Structure of E64.

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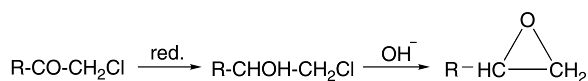


Scheme 1 Synthesis of epoxides from chloromethyl ketones.

pH 7.0–8.0 is favourable and the inhibitory effect depends on the configuration of the epoxide group.

Only attempts are known for the synthesis of epoxy-derivatives of opioid peptides, in order to develop a new class of affinity labels for the opioid receptor. Treatment of D-Ala^2,Leu^5-enkephalin by epichlorohydrine gave a mixture of mono- and disubstituted epoxy-propyl-enkephalins, but separation of the compounds, or working out a method to produce solely the monoalkylated derivative encountered difficulties (Magyar *et al.*, unpublished results). More successful was an experiment to form an epoxide function on the peptide carboxyl terminus, esterification with glycidyl alcohol by the aid of an earlier described method [105] led to peptide-glycidesters with good alkylating properties [106]. An *N*-epoxy-succinyl derivative of enkephalin has also been described [106], this compound is expected to be an irreversible antagonist of the opioid receptor (c.f. [107]).

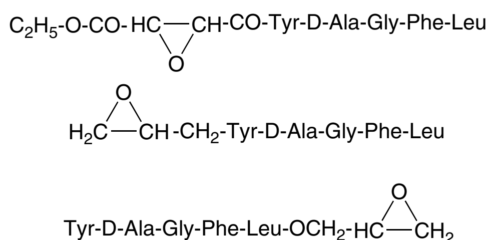


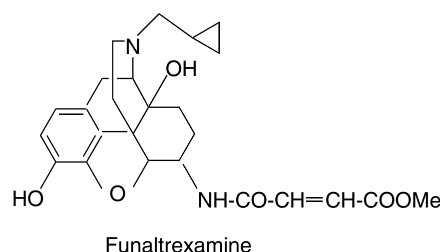
Figure 5 Epoxy-substituted enkephalin derivatives.

Miscellaneous Affinity Labels

Bromoacetyl and diazoacetyl derivatives of α-melanotropin for the preparation of affinity labels have been synthesized by Eberle and co-workers [108]. As the *N*-terminus of melanotropin contains an acetyl group, substitution by the alkylating, in case of the diazoacetyl moiety even photoreactive group is expected to retain the binding and biological activity of the parent hormone rendering it suitable for affinity labelling of the receptor protein.

Derivatives of the δ-opioid selective peptide Tyr-Tic-Phe-Phe containing the alkylating p-bromoacetamide group on the phenyl ring of Phe^3 or Phe^4 have been synthesized and used for affinity labelling of cloned δ-receptors. The Phe^4 derivative exhibited high affinity to the binding site resulting in an 85% wash resistant inhibition of the receptor [109]. Enkephalin-based antagonists containing bromoacetamido phenylalanine have also been synthesized by Maeda *et al.* [110].

We have seen previously that the nitrogen mustard chlornaltrexamine developed by Portoghese *et al.* [87] proved to be a good irreversible affinity label. As this compound was highly reactive, the same authors synthesized funaltrexamine, a fumaramate derivative, expected to possess greater selectivity in covalent bond formation with opioid receptors [111,112].



Funaltrexamine

Tritiated β-funaltrexamine covalently labelled μ-opioid receptors in bovine striatal membranes, as well as μ-receptors in the brain membranes of the guinea-pig, rat and mouse. This specific binding was greatly reduced by naloxone [113]. Receptors labelled in this way proved to be glycoproteids, removal of the *N*-linked carbohydrates resulted in a specifically labelled protein band of molecular mass 43 KDa.

β-Funaltrexamine was irreversibly bound to the cloned μ-opioid receptor as well [114], this specific binding was potently inhibited by μ-, but not by δ- or κ-ligands. Receptors labelled with tritiated β-funaltrexamine migrated as one band with a mass of 67 KDa by electrophoresis. Upon removal of carbohydrates the receptor showed a mass of 40 KDa. Similar investigations with μ/κ chimeras showed that the region between the middle of the third intracellular loop and the *C*-terminus of the μ-receptor is necessary for this irreversible binding. By site-specific mutagenesis, the exact location of this alkylation site was determined [115].

A novel affinity label, fentanyl isothiocyanate, has been used for the characterization of the δ-opioid receptor from NG108-15 hybrid cells by Klee *et al.*

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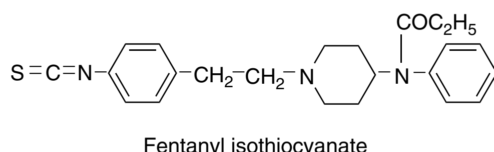

Fentanyl isothiocyanate

[116]. The labelled receptor had a molecular-mass of 58 KDa.

δ-Specific affinity labels from peptide antagonists have been prepared by Maeda *et al.* [110], substituting the Phe^4 residue of *N*,*N*-dialkyl-enkephalins by isothiocyanate or bromoacetamido groups. Although the affinity of these derivatives to the receptor was greatly reduced, *N*,*N*-dibenzyl-Phe(NCS)^4,Leu^5-enkephalin bound irreversibly to the δ-receptor. The same research team synthesized the Tyr-Tic-Phe-Phe peptide antagonist substituted on each of the Phe residues by the isothiocyanate group, both peptides were able to inhibit radioligand binding to δ-receptors in a wash-resistant manner at a concentration of 10 μM [117].

Photoaffinity Labelling

Much less selective than the labelling methods dealt with so far is photoaffinity labelling [118]. Corresponding to the general principles of affinity labelling, here too a ligand recognized by the receptor bears a specific substituent, which in this case is itself unreactive but upon irradiation becomes activated and irreversibly bound to the receptor binding site. The photoligand must contain a readily detectable, in most cases radioactive tag, which allows easy identification of the receptor protein. After partial enzymatic or chemical degradation the fragments of the macromolecule that have been covalently modified can be separated and analysed by mass spectrometry or sequence determination. As labelling occurs at nonspecific functional groups, employment of a great excess of affinity label may result in reactions with positions far from the binding site. To avoid this non-specific labelling a highly selective ligand with great affinity is needed enabling it to effectively occupy the binding site in nanomolar concentrations. Moreover, as UV irradiation itself can destroy the receptors, a trustworthy, ligand-dependent inactivation can be achieved only under carefully controlled experimental conditions. Perhaps these uncertainties are responsible for the fact that by photoaffinity labelling up to now only the specificity and mass of receptor subunits could be determined and no data are available on the location and chemical structure of the ligand binding sites.

In the past few decades numerous methods of photoaffinity labelling were developed, they are critically summarized in a number of recent reviews [119–122]. In most cases the precursor groups become activated upon irradiation through a carbene [123] or nitrene [124] intermediate. These reactive species form covalent bonds not only with the well-known functional groups of the receptor but with aliphatic side chains thought to be unreactive as well. Photoaffinity labelling of polypeptide hormones by ligands containing the nitrene-donor *p*-azido-phenylalanine has been described since the 1970s (e.g. [125], reviewed in [126]). For the same purpose more recently the carbendonor benzophenone derivatives, such *p*-benzoyl-phenylalanine found applications ([127], reviewed in [121]), the main advantage of the latter is that being an amino acid it can be easily built into the peptide chain.

An interesting family of photoaffinity labels is represented by compounds of heterobifunctional property capable of forming crosslinks between receptor functional groups. As an example the maleimido-benzophenone and its derivatives should be mentioned: these first react with sulfhydryl groups in the neighbourhood of the ligand binding site, and in a second step, upon activating by irradiation, in a less selective manner form a crosslink with other structural elements of the receptor protein. Application of the basic compound, *p*-maleimido-benzophenone, is described in the literature [128,129]. It would be worth trying to label opioid receptors by peptide ligands bearing both reactive groups.

Experiments aiming at the photoaffinity labelling of opioid receptors were reviewed by Simon and Hiller [17] and later by Schiller [19]. The pioneers of this field used an alkaloid-based affinity label [130], a tritiated levorphanol derivative, which as expected, upon irradiation bound irreversibly to the opioid receptors.

Of the numerous applications of opioid peptides as photoaffinity labels the experiment of Hazum *et al.* [131] should be mentioned first. They elongated the sequence of D-Ala^2-leucine enkephalin by a lysine residue, containing a nitro-azido-phenyl substituent on its ε-amino group. The iodinated derivative of this compound retained high binding affinity (K_d 2.1 nM). Following photoactivation covalent binding of this compound to a brain membrane preparation had been achieved.

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Methionine enkephalin served as the starting compound in the photoaffinity experiments of Lee *et al.* [132]. Here the nitro-azido-phenyl substituent was bound to the enkephalin via an ethylene diamine and a tyrosine residue on the *C*-terminus. After irradiation this compound irreversibly inactivated the δ-receptors of the NG108-15 cell line, inactivation was prevented by preliminary treatment with enkephalin. Similarly, Zioudrou *et al.* [133] built the 2-nitro-4-azidophenyl group through an ethylene diamine or β-alanine spacer into the carboxyl group of D-Ala^2,Leu^5-enkephalin. These peptides bind to opiate receptors with nanomolar affinities. Photolysis of these ligands bound to rat brain membranes results in the loss of 50% of the receptor binding sites. This decrease in receptor number is blocked by naloxone. In the presence of the nitro group, irradiation with visible light (540 nm) was sufficient to elicit irreversible binding diminishing the danger of inactivation of the receptor.

Smolarsky and Koshland [134] used for photolabelling D-Ala-enkephalin derivatives containing azido-phenylalanine residue in the Phe^4 position, or *p*-azido-phenyl propylamine in place of the *C*-terminal leucine residue. Irradiation with the full spectrum of the mercury lamp led to inactivation of opioid receptors, a process that did not occur after preincubation with normorphine.

p-Azido-phenylalanine and its *N*-methyl derivative were used by Roques and co-workers for the preparation of photoaffinity labels for the opioid receptor [135,136]. Azido-DAGO (Tyr-D-Ala-Gly-MePhe(N_3)-Gly-ol) and azido-DTLET (Tyr-D-Thr-Gly-Phe(N_3)-Leu-Thr) retained the selectivity — μ and δ respectively — of the parent compounds (DAGO and DTLET) and can be used for covalent labelling of the corresponding receptor subtypes. Azido-DTLET irradiated by a wavelength of 254 nm at a concentration of 1 nM irreversibly inhibited the electrically induced contractions of mouse vas deferens. This inhibition could be prevented by naloxone only when used in high concentration showing the expected δ-specificity of the label. Similar irreversible binding was observed on receptor fractions derived from rat brain. For the irreversible binding of azido-DAGO to the μ-receptors a concentration of greater by two orders of magnitude was necessary.

A few years later the radioiodinated derivative of azido-DTLET was also prepared [137]. As a consequence of iodine substitution the affinity of the compound was diminished while maintaining its original δ-selectivity. Both in rat brain membrane homogenate and NG108-15 hybrid cells, UV irradiation of the receptor–ligand complex resulted in the irreversible binding of the radiolabelled compound to membrane receptors. Separation of the labelled proteins by gel electrophoresis was successful, from the rat brain two fractions with molecular masses of 44 KDa and 34 KDa, and from the hybrid cells one fraction with a molecular mass of 44 KDa could be identified. As iodine-substituted azido-DTLET binds to the δ-receptor much better than to the μ-subtype, this compound in nanomolar concentration constitutes a highly selective tool for covalent labelling of δ-opioid receptors [138].

At about the same time another research team used similar compounds for affinity labelling. Yeung *et al.* [139,140] bound the δ-receptor specific ^{125}J-Tyr^1,D-Ala^2,Phe(N_3)^4,Met^5-enkephalin via irradiation to rat brain opioid receptors, and as proved by SDS gel electrophoresis, a 46 KDa protein was specifically photolabelled in this membrane preparation. As a concentration-dependent inhibition of photolabelling of this protein was observed in the presence of competing ligands specific for the μ-, δ- and κ-opioid receptors, this finding suggests that the labelled binding protein is common to all opioid receptor subtypes. In similar experiments in other mammalians this receptor protein possessed a molecular mass of 43 KDa [141].

p-Azido-phenylalanine was built into another opioid peptide by Landis *et al.* [142], by substituting the Phe^4 amino acid in DPDPE, a δ-opioid receptor specific, cyclic, conformationally constrained compound. The resultant photolabile peptide retained its excellent affinity and δ-selectivity, however, no data were reported on its application for receptor labelling.

The carbene-donor benzophenone derivative, *p*-benzoyl-phenylalanine was built into the morphiceptin molecule by Herblin *et al.* [143]. The resulting photoaffinity reagent, Tyr-Pro-Phe-Bpa-NH_2 had the same affinity to the receptor as morphiceptin and after irradiation of the reversible complex DAGO binding was fully suspended. Competition studies with DADLE and naloxone indicated selective inactivation of the μ-opioid receptor.

Photolysis of the benzoyl group made naloxone-benzoyl-hydrazone an irreversible label, a non-selective ligand of the opioid receptors. Short irradiation by UV light of the ligand-receptor complex led to 50% irreversible ligand binding [144].

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Site Directed Mutagenesis

Exchange of the individual amino acids in the receptor protein sequence affords valuable information for the elucidation of their role in the ligand binding. One has to keep in mind, however, that this exchange will not prove the direct participation of the respective amino acid in the binding process, conformation changes as a consequence of alteration of amino acids also have to be taken into consideration. Although for the exact determination of the structure of binding sites affinity labelling is the method of choice, the knowledge of the primary structure of cloned receptor point mutations in the receptor protein sequence greatly improved the chances of characterization of the ligand binding mechanism.

Since opioids are mostly cationic compounds and loss of the charge regularly leads to disappearance of the biological activity, the role of acidic side chains of the receptor in ligand binding became the primary subject of mutagenesis studies. In particular, aspartic acid residues were believed to form an ion pair with the cationic ligand. To localize this functional group Kong *et al.* [145] substituted Asp95 in the second transmembrane domain of the cloned mouse δ-opioid receptor with an asparagine. The mutant receptor had greatly reduced affinity for δ-selective peptide agonists, it did not bind these compounds even at micromolar concentrations. In contrast, the δ-selective antagonists or non-selective opioid agonists showed no difference in binding to the wild-type and mutant δ-receptor. These results indicate that Asp95 contributes to high affinity δ-selective agonist binding but agonists and antagonists bind differently to the δ-receptor.

One of the aspartic acid residues — Asp128 in the third putative transmembrane domain of the mouse δ-opioid receptor — is a highly conserved amino acid, it was therefore interesting to investigate its role in forming an ion pair with the opioid ligands [146]. Removal of this carboxylate group by substitution of alanine for aspartic acid in this position did not modify binding affinity of δ-specific opioid peptides or even that of naloxone, but affected the binding of agonists when the receptor was under a sodium-ion induced low affinity state. On the other hand, the aspartate to asparagine mutation strongly impaired the binding of all ligands. One can conclude that the conserved aspartate residue in the δ-opioid receptor is not the anionic counterpart for cationic opiate binding, but it is a constituent of the receptor binding site. Contrary to this conclusion, in the case of the μ-opioid receptor Li *et al.* [147] found Asp147, which corresponds to Asp128 in the δ-receptor, forming an ion pair with morphine and naltrexone. In that experiment mutation of Asp147 to Ala or Asn substantially reduced the affinity of the alkaloid ligands. Mutation of Asp147 to glutamic acid resulted, however, in a receptor with properties similar to those of the parent protein, indicating the significant role of a carboxylate group in ligand binding in this position.

Bot *et al.* [148] investigated the role of another conserved aspartic acid (Asp114) in the ligand binding of the μ-receptor, by converting Asp114 to Asn114. The binding of μ- and δ-specific full agonist ligands to the mutant receptor was greatly diminished, but not that of the antagonists and partial agonists. The overall conclusion is that the binding site of the opioid receptors is not a single strictly defined sequential part but a structural unit dependent on the specificity and agonist or antagonist character of the opioid ligands. Mutagenesis studies have shown that different classes of opiates may have distinct ligand binding domains in receptors. These binding differences may cause distinct functional actions of agonists.

To prove the existence of a hydrophilic binding pocket in the μ-receptor Surratt *et al.* [149] exchanged two aspartic acid residues — Asp114 in the transmembrane domain II and Asp147 in the transmembrane domain III — and His297 in the transmembrane domain VI with alanine in a receptor truncated by 64 amino acids on the *N*-terminus, resulting in a significant decrease of DAGO and naloxone binding, not affecting the signal transduction capability of the mutant receptor. It is worth mentioning that exchange of the same histidine residue with asparagine did not reduce the binding of DAGO [148]. It seems therefore that although asparagine and alanine have subtle structural differences, they can cause significant ligand binding variations.

Shahrestanifar and co-workers [78] found His223 in the second extracellular loop important in the binding of bremazocine, a ligand of the benzomorphan series with high affinity for μ- and δ-opioid receptors, substitution of His223 to Ser223 greatly diminished the ligand binding. It should be remembered that this binding was also decreased by the addition of *N*-ethylmaleimide making it conceivable that, corresponding to earlier observations [43, 44,150], in this case not an essential sulfhydryl group but a histidine residue had been alkylated. This statement was disputed later [151]. It is a

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fact, however, that opioid ligands could protect His297 from acylation (the authors called this reaction alkylation) by the histidine specific diethylpyrocarbonate indicating that this amino acid is positioned in or very near the opioid binding cavity [75].

By the mutation of the human μ-opioid receptor Gaibelet *et al.* [151] drew attention to the importance of the receptor conformation in the ligand binding. Exchange of the cysteine residues (positions 142 and 219) forming a disulfide bridge between the first and second extracellular loops with serine residues abolished the alkaloid or peptide binding to the receptor. Binding was also diminished by reducing agents, evidently because they split the conformation stabilizing disulfide bond. Further mutagenesis experiments combined with alkylation by *N*-ethylmaleimide showed the existence of sulfhydryl groups of different sensitivity (Cys81 in the first and Cys332 in the seventh transmembrane region).

As far as other receptor types are concerned, Ehrlich *et al.* [152] raised the question of which cysteines are essential for ligand binding of the δ-receptor expressed in Chinese hamster ovary cells. They used site-directed mutagenesis for the replacement of cysteine residues in the extracellular and transmembrane domains and changed them to serine or alanine, one at a time. Mutation of the extracellular cysteines produced a receptor devoid of binding activity, indicating the significance of the disulfide bond between the extracellular loops, while replacement of any one of the six transmembrane cysteines did not influence the binding of δ-specific ligands, these cysteines are therefore not essential for receptor binding. These observations seem to contradict former and later ones. A possible explanation is that ligand binding is inhibited by the introduction of bulky alkylating substituents rather than by elimination of the sulfhydryl groups. The role in the ligand binding of further sulfhydryl groups in μ-receptors expressed in Chinese hamster ovary cells was investigated by Deng *et al.* [153]. Using methanethiosulfonate ($CH_3SO_2SCH_2CH_2$-R) reagents they found Cys161,Cys192,Cys237 and Cys332, situated in the transmembrane domains III, IV, V and VII respectively, responsible for ligand binding, by gradually exchanging them with serine residues. In the light of the observations mentioned above cautious interpretation of these results is advisable.

The role of Lys108 in the binding of δ-ligands to the δ-opioid receptor was emphasized by Minami *et al.* [154]. Replacement of this amino acid with asparagine dramatically increased the affinity for μ-receptor selective peptide ligands.

In order to further characterize the receptor-ligand binding site Mansour *et al.* [155] performed a series of amino acid exchanges on the rat μ-opioid receptor: Asn150 to Ala150, His297 to Ala297, Tyr326 to Phe326, Ile198 to Val198 and Val202 to Ile202. These experiments showed again that no single amino acid can be made responsible for the ligand binding, the individual mutations exert different effects to the various ligands. The involvement in the ligand binding of further amino acids (Tyr148, Trp318, His319) was demonstrated by Xu *et al.* [156]. They pointed out that ligands bind to their receptors not only by potent electrostatic and hydrogen bonding reactions but by weak interactions as well.

Tritiated β-funaltrexamine was the first affinity label used for the exact localization of the binding site in the μ-opioid receptor [115]. The labelled receptor was solubilized, purified, cleaved by cyanogen bromide and the size of labelled receptor fragments determined. The label was found in the fragment comprising the Ala206–Met243 amino acid sequence. Mutation of Lys233 to a number of other amino acids completely eliminated covalent binding of the alkylating agent, although these mutants still bound funaltrexamine with high affinity. Thus Lys233 was supposed to be the site of alkylation, a surprising finding in the knowledge of the low nucleophilicity of the lysine ε-amino group at physiological pH values.

A survey of involvements of individual amino acids of different receptors in ligand binding is given in Table 7.

CONCLUDING REMARKS

If we survey the results of opioid receptor research in the past quarter of a century we can establish that a respectable quantity of scientific activity has been devoted to the field of the investigation of receptor specificity, the mechanism of ligand binding and the chemical characterization of binding sites. Following the identification of functional groups responsible for ligand binding, the determination of the amino acid sequence of cloned receptor proteins at the beginning of the 1990s was of revolutionary significance. However, even the most intensive research has not yielded results, which had been aimed at 20 years ago, namely the exploration of the

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Table 7 Involvement of Amino Acid Residues in Ligand Binding

Amino acid residue	Receptor	Reference
Asp95	δ	[145]
Asp114	μ	[148]
Asp128	δ	[146]
Asp147	μ	[147]
Asn150, His297, Tyr326, Ile198, Val202	μ	[155]
Cys81,Cys332	μ	[151]
Cys142-Cys219 disulfide bridge	μ	[151]
Cys101, Cys192, Cys237, Cys332	μ	[153]
Asp114, Asp147, His297	μ	[149]
His223	μ	[78]
Lys108	δ	[154]
Lys233	μ	[115]
Tyr148, Trp318, His319	μ	[156]

structures of the binding region and identification of the amino acid side chains playing a role in ligand binding, briefly, the mapping of the receptor binding sites. The reasons for this are partly methodological, inconsistencies between the results of different research teams can be explained by the different populations and origin of the receptors examined, and partly due to the fact that an absolutely selective ligand simply does not exist.

The only safe method of identification seems henceforward the covalent affinity labelling. It is somewhat surprising that nobody has yet reported its successful application, although many papers appeared on the synthesis of promising potential affinity labels.

It is remarkable that almost every single alteration of the receptor structure, such as amino acid exchange or chemical reaction is accompanied by modification of ligand binding. Because it is unlikely that all changes can be brought into immediate relation to ligand binding, it is obvious that in most cases an alteration of the receptor conformation, or a change of its arrangement in the membrane bilayer affecting the ligand binding is involved. The receptor is a dynamic and not a rigid system, which can be modulated by chemical reagents and even by the bound ligand as well. A binding site of a constant, steady structure, which does not depend on the ligand, may not exist at all, the exact chemical details of binding might perhaps never be described. In concrete cases the position of the bound ligand in the receptor protein sequence can certainly be established by affinity labelling, and even if in this way the molecular mechanism of binding is still not fully understood, affinity labelling will anyway greatly contribute to its knowledge and understanding.

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