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Affinity labels as tools for the identification of opioid receptor recognition sites☆

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Abstract

Affinity labels have proven to be useful tools in opioid research. We review experiments carried out with the μ opioid receptor affinity label, β-funaltrexamine (**2**), that support the concept of different recognition sites for μ opioid agonists and antagonists. The data are interpreted in the context of a dimeric receptor that contains two allosterically coupled binding sites: one that binds endogenous agonist, and the second that functions as an inhibitory modulator of agonism. It is proposed that exogenous antagonists bind selectively to the second site. The first of a new class of affinity labels, PGNA (**5**), that contains the phthaldehyde moiety attached to an opioid antagonist pharmacophore, is described. This class of ligands has been named 'reporter affinity labels' because covalent association leads to the formation of a fluorescent isoindole that is diagnostic for cross-linking of lysine and cysteine residues. PGNA binds opioid receptors covalently, as suggested by (a) irreversible binding to cloned opioid receptors, (b) irreversible opioid antagonism in the guinea pig ileum preparation, and (c) ultra-long opioid antagonism in mice. Since flow cytometry experiments revealed specific enhancement of fluorescence in cloned mu receptors after a 1 min exposure to **5**, it is concluded that covalent binding has occurred via the formation of an isoindole, presumably by cross-linking neighboring lysine and cysteine residues in the vicinity of the receptor recognition site.

1. Introduction

Electrophilic affinity labels have been used extensively to irreversibly block opioid receptors in vivo and in vitro [1]. For example, β-chlornaltrexamine (**1**) has been employed for the irreversible blockage of multiple opioid receptors, and β-funaltrexamine (**2**) is widely used as a selective, irreversible mu opioid receptor antagonist. With the cloning of the three major types of opioid receptors, electrophilic affinity labels have found an additional use as tools to assist in the identification of ligand binding loci on opioid receptors [2,3].

In this presentation we discuss the action of reported affinity labels in light of recent evidence for the existence of G protein-coupled receptor dimers [4–9]. We then disclose the results of recent studies on a new type of affinity label that becomes fluorescent as a consequence of covalent binding to opioid receptors. We have named such fluorogenic ligands 'reporter affinity labels', because the generation of fluoresence reports the cross-linking of neighboring lysine and cysteine residues on the receptor.

2. Affinity labels revisited

High selectivity of affinity labels for opioid receptors is dependent on a number of factors [1]. These include: (a) the residence time (affinity) of the ligand on the receptor recognition site, (b) the location of the electrophilic center in the ligand, and (c) the reactivity and chemical selectivity of the electrophilic group [1]. In considering each of these factors, it becomes apparent that covalent bonding to a receptor can be viewed to involve two consecutive recognition steps (Fig. 1). The first depends on the relative affinity of the ligand for the target site, and the second involves proper alignment

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of the electrophilic center with a compatible, receptor-based nucleophile. Because two recognition steps, rather than one, lead to covalent bonding, high selectivity is possible (recognition amplification) with a properly oriented, chemically selective electrophilic group.

Thus, the design of the μ-selective affinity label, β-funaltrexamine (**2**) involved the attachment of a chemically selective Michael acceptor group to a naltrexone-derived antagonist pharmacophore [10]. The participation of a selective electrophilic group in the second recognition step is in contrast to the effect of a highly reactive nitrogen mustard group (i.e. **1**), in that the latter promotes covalent bonding to different types of opioid receptors rather than a single type [11]. The promiscuous nature of the N-mustard group enables it to alkylate almost any opioid receptor-based nucleophile within covalent bonding distance. In such a case, covalent bonding is determined primarily by the binding mode and affinity of the ligand for the receptor (first recognition step).

Another illustration of this principle involved the attachment of an electrophilic moiety to an opioid agonist pharmacophore [12]. Modification with a highly reactive electrophilic group (N-mustard) afforded an irreversible agonist **3**, whereas attachment of a Michael acceptor group to the opioid agonist pharmacophore gave rise to a reversible agonist **4**. Given that the N-mustard affinity labels (**1**, **3**) are both irreversible, while only **2** was irreversible in the pair of Michael acceptor-containing ligands (**2**, **4**), it appears that the recognition sites for agonists and antagonists differ. One explanation for the retention of agonism upon modification of an agonist with an electrophilic group is that the receptor is covalently bound in an agonist state. Similarly, covalent binding by an antagonist locks the receptor in an antagonist state. Evidence for the existence of agonist and antagonist states, and the concept of 'inverse agonism' among G protein-coupled receptors, are consistent with this view [13]. The ability of both **1** and **3** to bind covalently to opioid receptors underscores the lower discrimination of the N-mustard group for receptor-based nucleophiles. The fact that **4** is reversible suggests that the proximal receptor-based nucleophiles may be different in agonist and antagonist states.

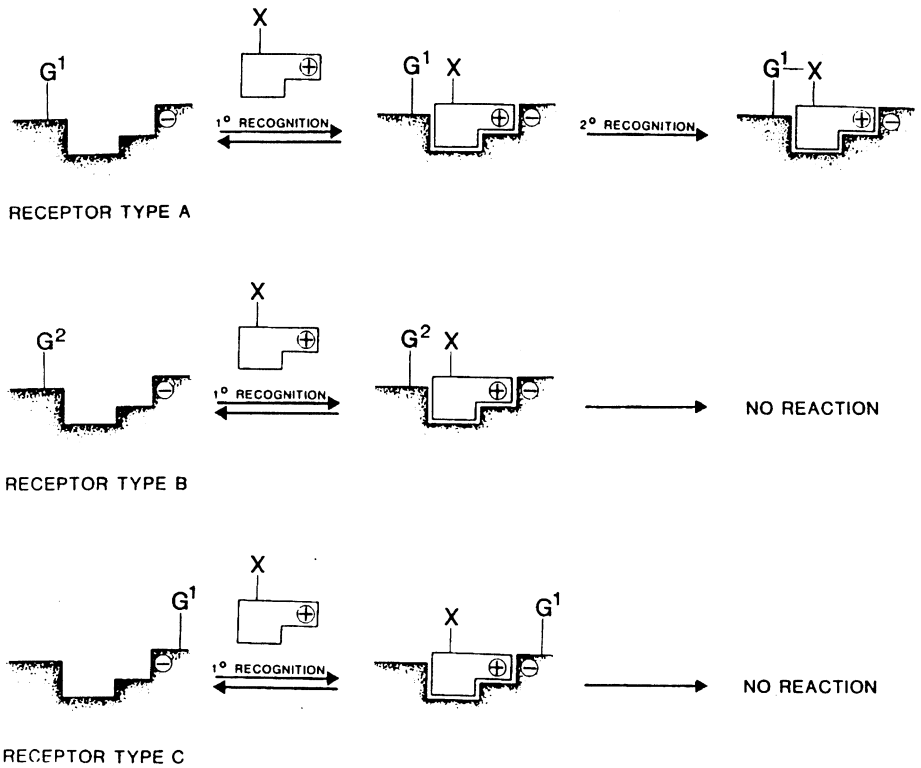


Fig. 1. A schematic illustration of the principle of two recognition steps leading to selective covalent binding of an electrophilic affinity label to a family of receptors A–C. Note that A–C have similar topographic features that contribute to reversible association of the ligand (first recognition step). When the electrophilic group X is in proximity to a compatible receptor-based nucleophile (G^1) in receptor type A, covalent bonding occurs. However, the reaction may not proceed readily, either due to low reactivity of the nucleophile (G^2) in B or to a distal relationship between X and G^1 in C. Another factor leading to selectivity is the differential affinity of the ligand (residence time on the receptor) for A–C.

Table 1
Protection against irreversible β-funaltrexamine (β-FNA) antagonism by μ-selective agonists and antagonists [a]

Protector	Concentration (nM)	Morphine IC_{50} ratio [b]
None		6.1
Morphine	1000	4.0
β-FOA	500	5.2
Naltrexone	2	2.4
	20	1.0
Naloxone	2	3.4
	20	1.9

[a] Data from Ref. [14].
[b] IC_{50} of morphine after 30 min incubation with β-FNA (20 nM) followed by washing, divided by the control IC_{50} of morphine.

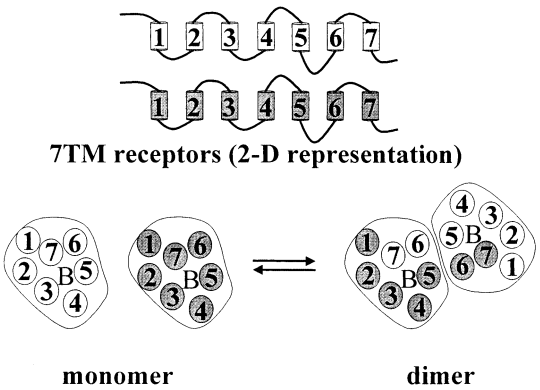
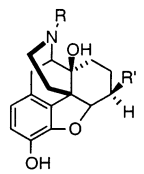


Fig. 2. Swapping of transmembrane domains in G protein-coupled receptors (7-TM). This can occur through dimerization or oligomerization, such that TM 6–7 of one monomer associate with TM 1–5 of a second monomer.



1 R = $CH_2CH(CH_2)_2$, R' = $N(CH_2CH_2Cl)_2$

2 R = $CH_2CH(CH_2)_2$, R' = $NHCOCH{=}CHCO_2CH_3$

3 R = CH_3, R' = $N(CH_2CH_2Cl)_2$

4 R = CH_3, R' = $NHCOCH{=}CHCO_2CH_3$

3. Separate agonist and antagonist sites on opioid receptor dimers

Based on the possible involvement of different recognition sites for agonists and antagonists, protection studies were carried out in the guinea pig ileum preparation using the irreversible μ opioid receptor antagonist, β-funaltrexamine (**2**), reversible μ agonists (morphine and **4**), and reversible antagonists (naltrexone and naloxone) [14]. The reversible antagonists were highly effective in protecting μ opioid receptors against alkylation by **2**. On the other hand, relatively high concentrations of μ agonists (**4** or morphine) were incapable of effectively protecting μ receptors from irreversible blockage. These data suggested that μ agonists and antagonists may bind to different recognition sites (Table 1). We proposed that two recognition sites on a dimer are negatively allosterically coupled in vectorial fashion, and that this may constitute a control mechanism for modulating the binding of endogenous ligands (Fig. 2). Accordingly, at low levels of opioid peptide, binding occurs preferentially at the agonist site, and at high levels occupation of the antagonist site reduces the affinity and activation by the opioid peptide at the agonist site. Additionally, the antagonist site was considered to have higher affinity for exogenous antagonists relative to agonists.

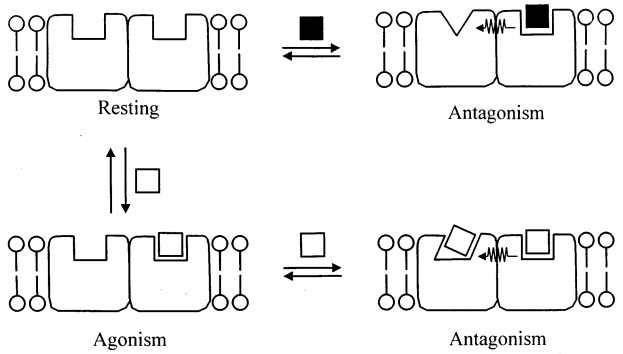


Fig. 3. A conceptual model of the interaction of a mu agonist and antagonist with allosterically coupled recognition sites on a dimeric mu opioid receptor [14]. An endogeneous agonist (□) at low concentration binds to one of the sites on the mu receptor dimer, and at higher concentration binds to the second site. The latter interaction triggers a vectorial decrease in the affinity of the ligand at the first site. The binding of an exogeneous antagonist (■) induces a greater loss of affinity with respect to its coupled receptor.

When this was proposed [14] in 1983, the only other evidence supporting this hypothesis came from the structure–activity analysis of bivalent ligands [15]. Data from these studies suggested that the pharmacophores of bivalent ligands with appropriate length spacers occupy vicinal recognition sites on μ receptors that are associated as dimers. In this regard, the relatively recent reports of dimers among opioid receptors has special relevance [8,9].

A combination of experimental data and modeling studies of G protein-coupled receptors have suggested that the 6- and 7-transmembrane (TM) helices of one monomer combine with TM 1–5 of the second monomer when associated as dimers [4–7] (Fig. 3). Consequently, the swapping of TM domains in a homodimer leads to a TM binding cavity that would be similar to that of its 7-TM monomer. The two recognition sites may become non-equivalent when bound, due to a ligand-induced conformational change of a ligand-

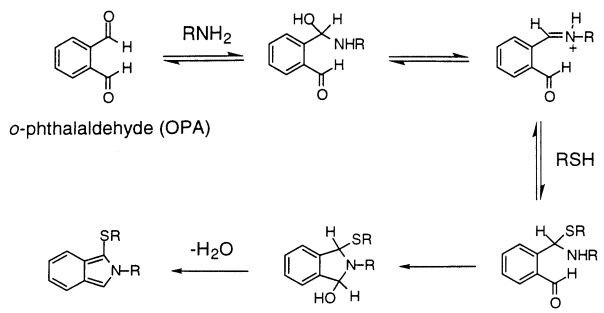
bound recognition site on the dimer and/or in the presence of other associated membrane-bound proteins. Such neighboring receptors could account for the inability of **4** both to alkylate the μ receptor and effectively protect it from alkylation by **2**. The bell-shaped dose–response curve for morphine in the upper concentration range that we commonly have observed in the guinea pig ileum preparation is consistent with such a model.

The receptor-based nucleophile that reacts with the electrophilic moiety of β-funaltrexamine (**2**) has been reported to be the ε-amino group of Lys233 located at the top of TM 5 of the μ receptor [2]. The identity of this residue was determined through site-directed mutagenesis and peptide mapping studies. The fact that this residue is conserved among the three types of opioid receptors suggests that the reversible binding mode of **2** is more critical for covalent bonding to a specific neighboring receptor-based nucleophile when the electrophilic group is selective.

4. A new approach: reporter affinity labels

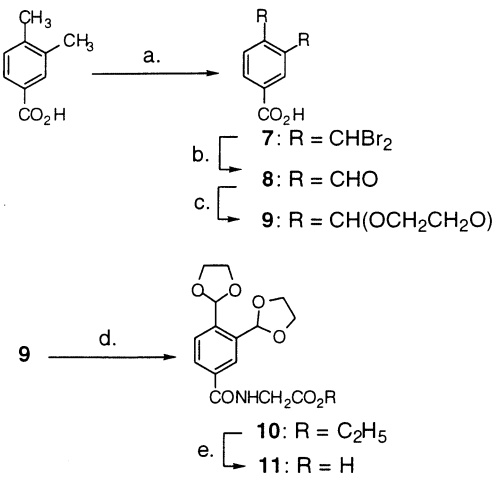
Although the β-funaltrexamine-alkylated residue of the mu receptor has been identified as Lys233, the conformational mobility of the bound lysine side-chain and the unknown regio- and stereospecificity of the alkylation reaction affords only minimal constraints with regard to the binding mode of the pharmacophore at the recognition site. However, if two neighboring residues can be bound covalently by a chemically selective affinity label, and the residues identified, it should be possible to model the bound receptor complex with greater confidence because of the greatly restricted translational mobility of the tethered ligand.

In an effort to accomplish this we have employed the *ortho*-phthalaldehyde (OPA) moiety as a group-specific, bifunctional electrophile. OPA has been employed as a fluorogenic reagent for the identification and quantitation of amino acids when the reaction is conducted in the presence of a thiol [16]. The fluorescence arises from the formation of an isoindole through a well established mechanism [17] (Scheme 1). In this regard, primary amines rapidly form a Schiff base with OPA, followed by attack by a thiol reagent to afford a cyclized intermediate that then undergoes dehydration to a fluorescent isoindole. The fact that the efficient formation of an isoindole from OPA requires both a primary amino and a thiol group has been utilized to cross-link neighboring lysine and cysteine residues in enzymes [18]. However, there were no reports of the incorporation of an OPA group in the design of affinity labels.

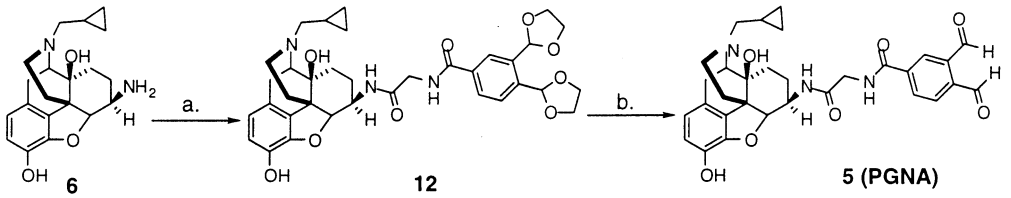


Scheme 1. Mechanism of isoindole formation upon reaction of primary amines and thiols with *o*-phthalaldehyde (OPA).

Since there are multiple conserved and non-conserved lysine and cysteine residues within the putative recognition loci on μ, δ, and κ opioid receptors [19], we have synthesized opiate-derived affinity labels that contain an OPA moiety, as exemplified by PGNA (**5**). The synthesis of **5** is outlined in Schemes 2 and 3. Compound **5** has been synthesized in seven steps from 3,4-dimethylbenzoic acid and β-naltrexamine (**6**). Synthon **11**, which was required for coupling to **6**, was prepared as follows (Scheme 2). 3,4-Dimethylbenzoic acid was converted to its tetrabromo derivative **7** in the presence of *N*-bromosuccinimide and benzoyl peroxide. Treatment of **7** with a hot aqueous solution of sodium carbonate followed by acidic hydrolysis gave 3,4-diformylbenzoic acid (**8**). Bis-acetalization of **8** with ethylene glycol under Dean–Stark conditions afforded the carboxylic acid **9**. Coupling of **9** with glycine ethyl ester in the presence of 1-hydroxybenzotriazole and dicyclohexylcarbodiimide gave the amido ester **10** which was saponified to afford the key intermediate **11**. Coupling of β-naltrexamine **6** with **11** afforded the corresponding amide **12** (Scheme 3). Hydrolysis of **12** under acidic conditions gave the hydrochloric salt of **5** as the hydrated dihydroxyphthalan.



Scheme 2. Reagents and conditions: (a) *N*-bromosuccinimide, benzoylperoxide, CCl_4, reflux, 10 h, 48%; (b) Na_2CO_3, H_2O, 60°C, 4 h, 80%; (c) $HO(CH_2)_2OH$, *p*-TsOH, benzene, Dean Stark, 15 h, 67%; (d) $HCl{\cdot}H_2NCH_2CO_2C_2H_5$, $N(C_2H_5)_3$, DCC, HOBt, THF, r.t., 10 h, 90%; (e) (1) NaOH, H_2O, r.t., 5 h; (2) HCl 1 N, 75%.



Scheme 3. Reagents and conditions: (a) **11**, DCC, HOBt, THF, r.t., 12 h, 64%; (b) HCl 1 N, N_2 acetone, r.t., 7 days, 38%.

If the reversibly bound **5** directs the OPA moiety within covalent bonding distance of both the lysine and cysteine residues, isoindole formation associated with irreversible, specific binding should produce fluorescence that is above that of background. It should then be possible to identify the positions of the two receptor-based residues through site-directed mutagenesis studies and peptide mapping.

Binding studies have demonstrated that PGNA (**5**) bound irreversibly to cloned μ, δ, and κ opioid receptors, with apparent K_i values in the range of 0.6–3 nM after incubation for 1 h followed by washing. At a concentration of 100 nM, PGNA behaved as an irreversible antagonist (20 min incubation time) of standard μ and κ agonists on the electrically stimulated guinea pig ileum preparation (GPI). Mice pretreated with 1.2 nmol-icv of PGNA exhibited long-lasting antagonism of morphine (μ), U50488 (κ), and DPDPE (δ), whose duration was greater than 5 days.

These results suggested that PGNA reacted covalently with all three opioid receptors to irreversibly block receptor activation by opioid agonists. However, the data did not reveal whether the ligand was linked to the receptors through an isoindole heterocycle formed from the reaction of the OPA moiety with neighboring lysine and cysteine residues (Fig. 4). This was verified by comparing the fluorescence of PGNA-treated CHO cells containing transfected μ receptors with CHO cells devoid of opioid receptors. By conducting flow cytometry on both sets of CHO cells, we found that the opioid receptor-containing cells fluoresced to a greater degree than the control cells when measured at an emission wavelength (530 nm) in the same range as the isoindole fluorophore heterocycle (Fig. 5). Site-directed mutagenesis studies presently are in progress in order to determine the position of the residues involved in isoindole formation.

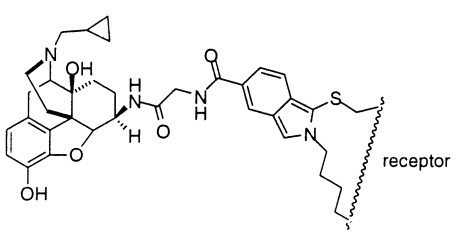


Fig. 4. Proposed structure of covalently bound **5** to opioid receptors.

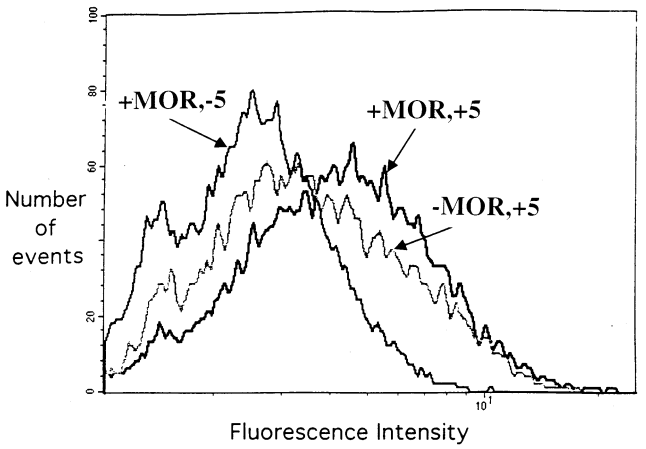


Fig. 5. Representative flow cytometric analysis of fluorescent opioid labeling of CHO cells. Untransfected CHO cells (− MOR) or transfected with μ opioid receptor (+ MOR) were incubated with (+ **5**) or without (− **5**) PGNA (1 μM) at 25°C for 1 min.

5. Summary and conclusions

Recent evidence for homodimeric and heterodimeric opioid receptors, together with the results of previously reported protection studies of μ opioid receptors against alkylation by β-funaltrexamine (**2**), have provided a structural basis for the original proposal that μ agonist and antagonist ligands bind to separate recognition sites on the monomeric subunits of the dimer. We have proposed that the site on each subunit in the dimer is allosterically coupled with each other to negatively modulate the effect of endogenous agonist at high concentrations. The site that promotes the modulation is postulated to possess high affinity for exogenous antagonists.

An approach to designing flurorogenic affinity labels has led to the synthesis of PGNA (**5**) which specifically cross-links neighboring lysine and cysteine residues of opioid receptors via the generation of an isoindole fluorophore. The fluorescence enhancement was detected in μ opioid receptor-transfected CHO cells. This new class of affinity labels offers an advantage over

conventional affinity labels, in that it is chemically selective and reports covalent binding through the formation of a fluorescent compound; hence the term 'reporter affinity label'. PGNA (**5**) bound irreversibly to μ, κ and δ cloned opioid receptors and was an irreversible antagonist in the GPI. In mice it was a potent, long-lasting opioid antagonist. Reporter affinity labels offer a novel approach to localizing the ligand recognition locus on opioid receptors and other receptor systems.

Acknowledgements

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