Specific Cross-Linking of Lys233 and Cys235 in the Mu Opioid Receptor by a Reporter Affinity Label†

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ABSTRACT: The first example of the use of a reporter affinity label (NNA) that contains a fluorogenic naphthalene dialdehyde moiety to identify neighboring lysine and cysteine residues at a recognition site is described. The opioid receptors have served as the proof-of-concept because they contain multiple lysine and cysteine residues. The kinetics of isoindole formation resulting from covalent binding of NNA to wild-type and mutant opioid receptors were followed in cultured cells using flow cytometry. The finding that NNA bound to mutant mu opioid receptors (K233R and C235S) without producing specific fluorescence enhancement suggested that covalent bonding occurred at these positions to produce an isoindole fluorophore in the wild-type mu receptor. The similar kinetics of fluorophore formation for wild-type mu, delta, and kappa opioid receptors suggest that these conserved residues are the cross-linking sites in all three types of opioid receptors. The combined utilization of a reporter affinity label and site-directed mutagenesis offers a more expeditious method of identifying cross-linking at a recognition site when compared to classical procedures.

Aromatic ortho-dialdehydes have been employed in the detection and analysis of amino acids (*1, 2*). In the presence of a mercaptan, the dialdehyde forms a fluoresent isoindole derivative of the amino acid (*3*). In proteins, the formation of an isoindole fluorophore involves the selective cross-linking of the thiol group of cysteine and the ϵ-amino group of a neighboring lysine (*4*). The fact that this reaction occurs rapidly at ambient temperatures has led to the design of affinity labels that contain this electrophilic, fluorogenic moiety (*5*−*7*). Such ligands have been named reporter affinity labels because they offer the opportunity to monitor covalent cross-linking at the recognition site of a receptor or enzyme by measuring the formation of specific fluorescence (*8*). In this connection, the kinetics of isoindole formation have been determined for cloned opioid receptors in cultured cells through use of flow cytometry. Here we present the first example of the use of a reporter affinity label for the identification of specific neighboring lysine and cysteine residues on a receptor recognition site.

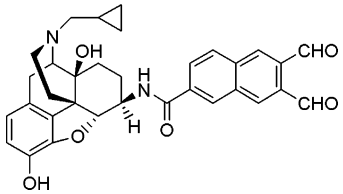

FIGURE 1: Reporter affinity label NNA.

Reporter affinity label NNA (Figure 1) is known to bind irreversibly to mu opioid receptors (MOR)[1] with the concomitant formation of a benzoisoindole fluorophore. (*8*) Kinetic studies have demonstrated the specificity of fluorescence generation, and molecular modeling has pointed to conserved Lys233 and Cys235 residues as the sites of cross-linking at the recognition site. The proposed mechanism of cross-linking by the naphthalenedialdehyde moiety of NNA is outlined in Figure 2. To establish the position of these residues, we have studied the interaction of NNA with mutant MOR whose Lys233 or Cys235 residues have been replaced with arginine or serine, respectively. In addition, we have

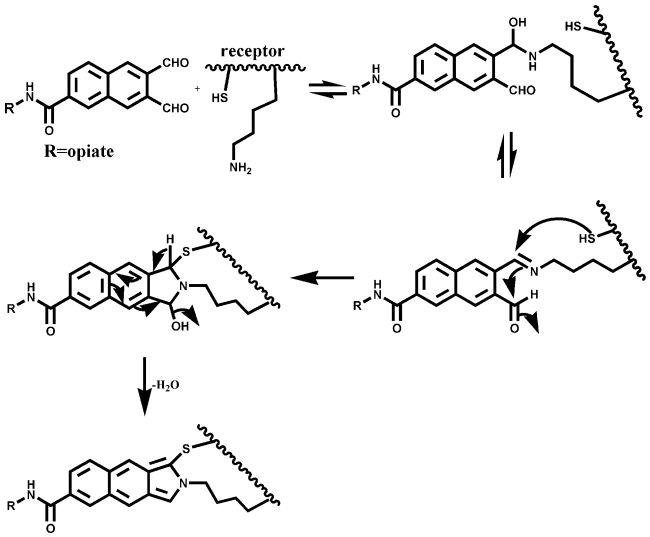

FIGURE 2: Mechanism of NNA cross-linking of lysine and cysteine residues in the receptor binding pocket.

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[1] Abbreviations: MOR, mu opioid receptor; DOR, delta opioid receptor; KOR, kappa opioid receptor; HEK, human embryo kidney; GPCRs, G-protein-coupled receptors.

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Table 1: Irreversible Binding of NNA or Naloxone to Opioid Receptors Expressed in HEK Cells

	[^{3}H]diprenorphine binding (% of total binding ± SEM[a])					
	MOR		DOR		KOR	
compd	prewash	postwash	prewash	postwash	prewash	postwash
NNA	18.1 ± 7.1	19.4 ± 4.2	4.3 ± 2.7	11.6 ± 4.0	30.8 ± 14.8	31.1 ± 13.5
naloxone (control)	2.7 ± 1.0	84.4 ± 8.6	20.4 ± 5.9	100.0 ± 0.0	10.1 ± 3.8	85.5 ± 11.1

[a] SEM: standard error of the mean ($n = 3$).

investigated the binding and kinetics of cross-linking of NNA to delta (DOR) and kappa (KOR) opioid receptors.

EXPERIMENTAL METHODS

Site-Directed Mutagenesis and Transient Transfections. Point mutations were produced by site-directed PCR mutagnesis and confirmed by restriction endonuclease digestion and direct sequencing. HEK-293 cells (ATCC, Manassas, VA 20108) were used for transfections. The methods involved were established and described previously (*9*).

Competition Binding Procedure. Competition binding experiments were performed using HEK 293 cells genetically modified to produce wild-type mu, delta, and kappa opioid receptors. Ten concentrations of the tested compounds (50 μL) were added to test tubes containing 0.1 nM [^{3}H]-diprenorphine ($\approx$(0.5−1.0)K_D) (50 μL) and whole cells (75 mm^2 plate, 80−90% confluent) suspended in 12.5 mL HEPES buffer (25 mM, pH = 7.4) (400 μL). The final volume was 500 μL. Nonspecific binding was measured using 1 μM naloxone. Assays were incubated at room temperature for 90 min and then filtered using a Brandel M-48 tissue harvester through Whatman GF/C filter paper that was presoaked in 0.25% poly(ethyleniminie). Filters were washed three times with ice cold HEPES buffer (see above), and the radioactivity was counted using a Beckmann LS 3801 liquid scintillation counter. All measurements were performed in duplicate. IC_{50} values were calculated using LIGAND, and K_i values were determined from the Cheng−Prusoff equation assuming a single-site binding model.

Irreversible Binding Procedure. Aliquots of cells expressing wild-type mu, delta, and kappa opioid receptors or mutant mu opioid receptors (C235S or K233R) (2.0−9.6 mg protein) were used untreated (control) or incubated with compounds at a concentration of 1000 nM at room temperature for 60 min. Aliquots of each were taken and tested for displacement binding (see prewash procedure below). The remaining mixture was centrifuged at 2000*g* (3000 rpm for 15 min in an IEC Centra C13R centrifuge), and the pellet was resuspended with 1 mL of HEPES buffer. This procedure was repeated two more times for a total of three washes. Aliquots (2.0−5.0 mg of protein added) were then tested for displacement binding (see postwash procedure below).

Binding to cells was measured by adding [^{3}H]-diprenorphine (0.1 nM) to aliquots taken from untreated (control), prewash, and postwash samples and incubating for 90 min at room temperature. Nonspecific binding was measured using 1000 nM naloxone. Specific binding was calculated by subtracting nonspecific binding from total binding and was corrected for protein added for each of these groups (control, prewash, and postwash).

Fluorescence Experiments. A Varian Cary Eclipse fluorometer was used to characterize the fluorescence of the isoindole system generated from NNA in the presence of a suspension of HEK cells in HEPES buffer. The concentration of NNA was identical to those employed in flow cytometry experiments.

Flow Cytometry. Kinetic studies were carried out with a Becton-Dickinson FACS Vantage equipped with an argon laser (excitation wavelength 488 nm) and a band-pass filter of a 530 ± 15 nm detector. The experimental conditions are the same as those reported previously (*4*) except the median values of fluorescence intensity from only viable cells were recorded. Propidium iodide was employed to identify viable cells (target cells) versus nonviable cells. The increase in fluorescence intensity after addition of NNA to viable HEK cells expressed with MOR was recorded over a 15 min period. The specific fluorescence represents the difference between the fluorescence intensity arising from the interaction of NNA with opioid receptors in HEK cells in the absence and presence of protection by naltrexone (NTX).

RESULTS AND DISCUSSION

Receptor binding experiments of NNA conducted on opioid receptors stably expressed in human embryonic kidney (HEK) cells afforded prewash apparent K_i values of 0.74 ± 0.31, 3.30 ± 0.06, and 4.90 ± 1.47 nM for MOR, DOR, and KOR, respectively. As the pre- and postwash (irreversible binding) percentages of total binding exhibited only minor differences, it appears that irreversible binding of NNA to all three types of opioid receptors occurred during the incubation period (Table 1). This was in contrast to the near-complete removal of reversibly bound naloxone upon washing.

Since it has been reported that NNA covalently binds to MOR with well-defined pseudo-first-order kinetics (*8*), we wished to determine if DOR and KOR have similar kinetic profiles, as this would suggest that identical conserved residues are cross-linked in all three types of opioid receptors. This was carried out by determining the maximum excitation and emission wavelengths of the benzoisoindole fluorophore upon the treatment of HEK cells expressing MOR, DOR, or KOR with NNA (1 μM). The excitation spectra for all three systems contain broad bands, with maximum excitation wavelengths ranging from 460 to 490 nm. The emission spectra are sharper with maximum emission wavelengths at 545, 546, and 533 nm for MOR, DOR, and KOR receptors, respectively. The finding that these spectral data are close to the reported values [λ(exc max) = 472 nm, λ(em max) = 528 nm] (*1b*) for the emission and excitation wavelengths suggested that the benzoisoindole fluorophore was formed in the interaction of NNA with the three types of receptors.

To ensure that all three opioid receptor types undergo pseudo-first-order cross-linking, the concentration of NNA (1 μM) in the kinetic flow cytometry studies was 2−3 orders

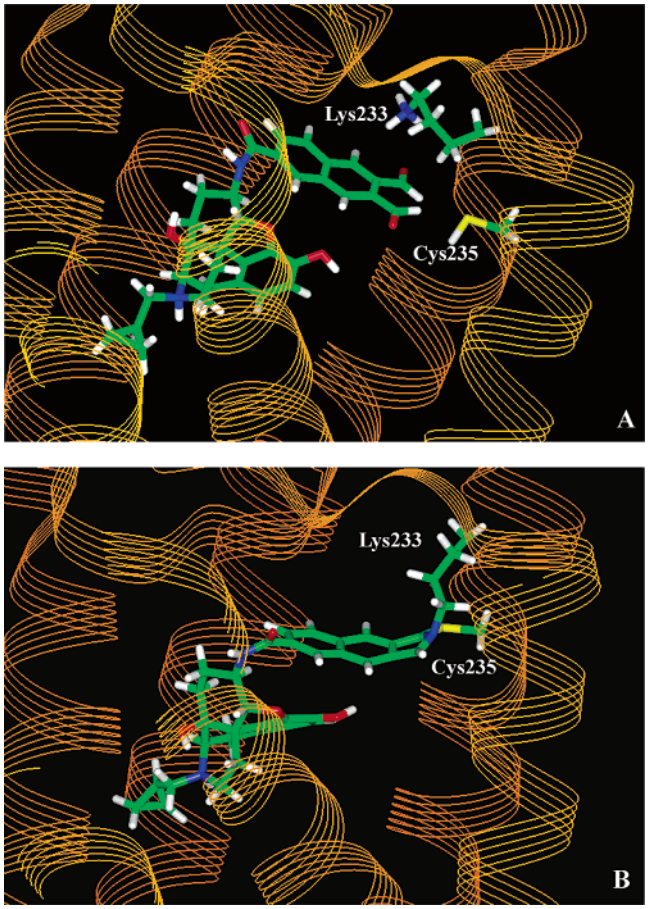

FIGURE 3: Interaction of NNA with MOR before (A) and after (B) the covalent bonding. Note that the naphthalenedialdehyde moiety of docked NNA is in the vicinity of K233 and C235 in the reversibly bound state (A). The reversibly bound complex undergoes cross-linking with these residues to form the benzo[*f*]isoindole fluorophore (B).

of magnitude higher than the apparent K_i values. This was based on the assumption that reversible association of NNA with the receptors is very fast relative to the covalent cross-linking step (*10*). The observation that the rate constants for the generation of fluorescence arising from interaction of NNA with the three receptors were similar (0.21 ± 0.05, 0.23 ± 0.03, and 0.32 ± 0.09 s^{-1} for MOR, DOR, and KOR, respectively), suggested that the reversible ligand−receptor complex places its aromatic dialdehyde moiety in the vicinity of identical conserved neighboring lysine and cysteine residues in all three opioid receptors. Moreover, this similarity is consistent with the high homology (~70% with respect to amino acid identity) in the transmembrane domain of the three opioid receptors.

Since there are multiple conserved lysine and cysteine residues among the three types of opioid receptors, we wished to determine the location of the residues involved in covalent cross-linking by NNA. Modeling of NNA docked to MOR (see Figure 3) suggested that the aromatic dialdehyde moiety was in the vicinity of Lys233 and Cys235 located at the upper half of transmembrane helix 5 (TM5) (*11*). Similar results were observed for the modeling of NNA complexed to DOR and KOR.

Based upon the modeling studies, point mutants of MOR in which lysine was replaced by arginine (K233R) or cysteine was substituted by serine (C235S) were prepared. Arginine and serine were selected as replacements because we have found that these amino acids do not react with aromatic ortho-dicarboxaldehydes to form a fluorescent isoindole. Also, as these are conservative replacements, the conformation and recognition properties of the mutant receptors should be similar to those of the corresponding wild-type receptor. Indeed, the comparable apparent K_i values of the ligand NNA for K233R (0.28 ± 0.04 nM) and C235S (0.27 ± 0.06 nM) mutants and their similarity to wild-type MOR supported this idea (*12*).

The kinetic profiles for the generation of specific fluorescence upon incubation of NNA with wild-type and mutant receptors are illustrated in Figure 4. To distinguish between specific and nonspecific fluorescence, NNA was incubated with the HEK cells expressed with wild-type and mutant MORs in the presence or absence of the opioid antagonist, naltrexone (NTX). The difference between the fluorescence intensities under these conditions is the specific fluorescence arising from fluorophore formation at the recognition site. As illustrated in Figure 4a, the specific fluorescence in wild-type MOR rose rapidly and plateaued after 10 min. On the other hand, the K233R and C235S mutants displayed no specific fluorescence (Figure 4b and c), which was very similar to the case of the HEK cells without opioid receptor expressed on the membrane (Figure 4d). These data strongly suggested that these neighboring residues are involved in the formation of an isoindole fluorophore when NNA interacts with the recognition site of wild-type MOR.

Interestingly, washing experiments with K233R or C235S MOR mutants showed that the blockage of the recognition site was irreversible even though no isoindole fluorophore was formed (Table 2). This suggests either an alternate covalent bonding pathway that does not lead to a fluorophore, or the possibility that very high affinity noncovalent binding occurred (*13*).

Taken together, the data provide strong evidence for the participation of neighboring residues Lys233 and Cys235 on transmembrane helix 5 (TM5) in isoindole formation. These residues are located in the upper half of TM5 and within the putative ligand recognition locus of the receptor. As such, they are readily accessible to the carboxaldehyde groups of the reversibly bound ligand, thereby permitting facile Schiff base formation with the ϵ-amino group of Lys233, followed by thiol-promoted (Cys235) cyclization and dehydration to form the benzoisoindole fluorophore (Figure 2). Inasmuch

Table 2: Irreversible Binding of NNA to Wild-Type and Mutant MOR

	[^{3}H]diprenorphine binding (% of total binding ± SD)					
	wild-type		K233R		C235S	
compd	prewash	postwash	prewash	postwash	prewash	postwash
NNA	18.1 ± 7.1	19.4 ± 4.2	1.5 ± 0.7	5.9 ± 3.0	1.0 ± 0.8	13.1 ± 1.0
naloxone (control)	2.7 ± 1.0	84.4 ± 8.6	4 ± 1.3	73.3 ± 8.5	2 ± 1.3	87.8 ± 9.9

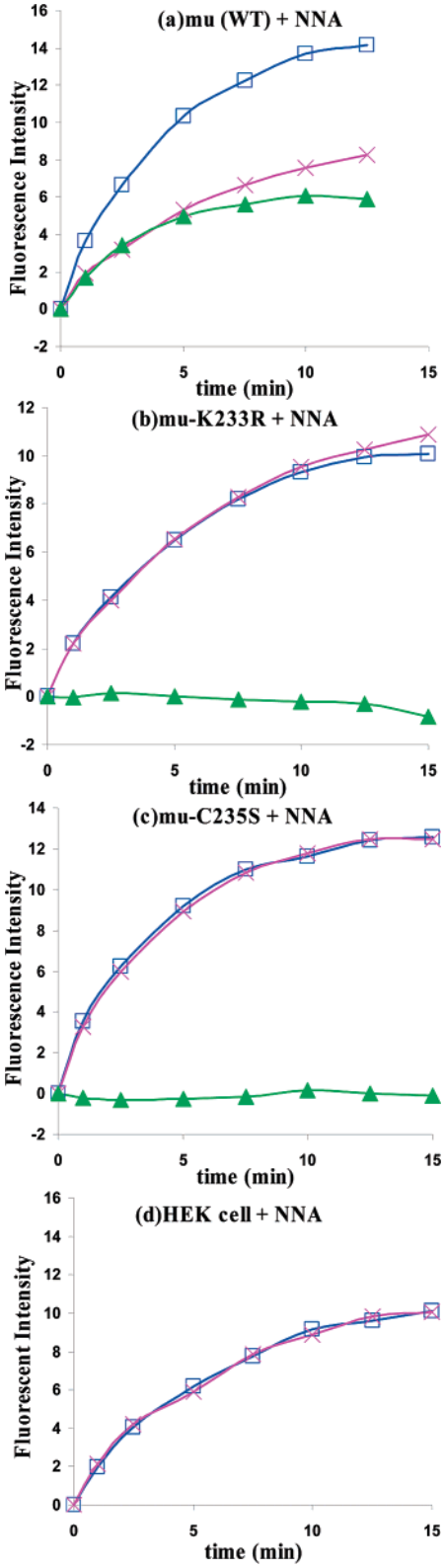

FIGURE 4: Comparison of the change in specific fluorescence intensity of wild-type and mutant opioid receptors expressed in HEK cells upon incubation of NNA (1 μM) in the presence and absence of the opioid antagonist NTX (10 μM). Note that wild-type MOR (a) exhibits a difference in fluorescence intensity (green) between NTX-treated (magenta) and untreated (blue) cells, whereas the mutants (b and c) show no difference upon identical treatment. Part d represents the fluorescence intensities of HEK cells (no opioid receptor expressed) upon incubation of NNA (1 μM) in the presence and absence of the opioid antagonist NTX (10 μM). Experiments were performed in duplicate for each receptor and cell line. Variations between experiments were less than 5%.

as the TM domains of MOR, DOR, and KOR possess ~75% homology with respect to amino acid identity, and given the similar binding and kinetic rate constants for isoindole formation, it is possible that the conserved lysine and cysteine residues on TM5 are involved in covalent cross-linking homologous recognition sites in the three types of opioid receptors.

In conclusion, the proof-of-concept for use of reporter affinity labels as a tool to identify neighboring lysine and cysteine residues at a recognition site has been verified through use of reporter affinity label NNA to cross-link neighboring lysine and cysteine residues in the binding cleft of opioid receptors. The finding that the MOR mutants (K233R and C235S) in this study exhibited no specific fluorescence intensity increase upon treatment with NNA implicates K233 and C235 in TM5 as the cross-linking sites. Moreover, the similar kinetic rate constants for the specific fluorescence arising from the covalent interaction of NNA with DOR and KOR suggest that the same conserved residues are involved in fluorophore formation. The combined utilization of a reporter affinity label and site-directed mutagenesis offers the advantage of being more expeditious than classical methods in identifying covalently modified amino acid residues at recognition sites of proteins. Also, the fact that NNA becomes covalently bound through two sites provides greater assurance of the validity of ligand-docked receptor models.

The present study suggests that properly constituted reporter affinity labels may be useful for cross-linking other GPCRs whose recognition sites possess lysine and cysteine residues. For example, chemokine CCR9 and neuropeptide Y type 9 receptors contain neighboring lysine and cysteine residues (*14*).

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10. If R refers to receptor, L ligand, RL the binding complex of receptor with ligand, and RL* the benzoisoindole fluorophore, then we may have

$$R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL \xrightarrow{k} RL^*$$

Since [compound **1**] = 1 μM $\gg K_i$, $k_1/k_2 \gg k$. Therefore, the second step is considered to be the rate-determining step when [L] $\gg$ [R]. For this reason, the fluorescence formation should follow pseudo-first-order kinetics. Therefore, we will have

$$\ln\{([RL^*]_{max} - [RL^*])/[RL^*]_{max}\} = -kt$$

Here [RL*] max may be substituted by the highest fluorescence intensity during a certain time course, while [RL*] may be the fluorescence intensity at a certain point of time.
11. Accelrys InsightII, a 3D graphical environment for molecular modeling, was used for the modeling. The homology model of MOR, DOR, and KOR was constructed based on the bovine rhodopsin X-ray structure.
12. The K_d values of K233R and C235S for [3H]diprenorphine are 416 ± 76 pM and 237 ± 82 pM while the B_{max} values are 367 ± 163 pM and 139 ± 24 pM, respectively.
13. An analogue bearing a naphthalene in the place of the naphthalenedialdehyde moiety in NNA exhibited wash resistance in smooth muscle preparations (mouse vas deferens and guinea pig ileum) (unpublished data). This suggests that very strong noncovalent binding of NNA with the mutant receptors was responsible for the observed wash resistance.
14. GPCRDB: Information system for G-protein-coupled receptors (GPCRs). http://www.gpcr.org/7tm/.

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