Identification in the μ-opioid receptor of cysteine residues responsible for inactivation of ligand binding by thiol alkylating and reducing agents

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Abstract Inactivation by thiol reducing and alkylating agents of ligand binding to the human μ-opioid receptor was examined. Dithiothreitol reduced the number of [^{3}H]diprenorphine binding sites. Replacement by seryl residues of either C142 or C219 in extracellular loops 1 and 2 of the μ receptor resulted in a complete loss of opioid binding. A disulfide bound linking C142 to C219 may thus be essential to maintain a functional conformation of the receptor. We also demonstrated that inactivation of ligand binding upon alkylation by *N*-ethylmaleimide occurred at two sites. Alteration of the more sensitive (IC_{50} = 20 μM) did not modify antagonists binding but decreased agonist affinity almost 10-fold. Modification of the less reactive site (IC_{50} = 2 mM) decreased the number of both agonist and antagonist binding sites. The alkylation site of higher sensitivity to *N*-ethylmaleimide was shown by mutagenesis experiments to be constituted of both C81 and C332 in transmembrane domains 1 and 7 of the μ-opioid receptor.

Key words: μ-Opioid receptor; Sulfhydryl group; Disulfide bond; Agonist binding site; G-protein

1. Introduction

Opioid receptors are the primary site of action of morphine and opiate alkaloids used in the treatment of pain. Three classes of opioid receptors, μ, ∂ and κ have been identify on the basis of their distinct pharmacological profiles, anatomical distributions and functions [1,2]. The cDNAs coding for these three opioid receptors have recently been cloned [3–10]. The deduced amino-acid sequences confirmed that each receptor displays structural characteristics of G-protein-coupled receptors including seven hydrophobic α-helical segments thought to constitute membrane spanning domains.

Morphine analgesia as well as side effects such as addiction, euphoria and respiratory depression seem mostly mediated by the μ receptor [11] possibly through coupling to multiple G-protein isoforms [12,13]. Analysis of the structure/function relationships of the μ receptor is thus of particular importance in view of the emerging concept of signalling-specific drugs [14–16].

In the absence of direct structural information, the engineering of receptor genes and the chemical modification of functional groups constitute alternative approaches to investigate the role of particular residues in ligand recognition and/or receptor structure. It has long been known that opioid binding is inhibited by sulfhydryl alkylating [17,18] and reducing [19] agents such as *N*-ethylmaleimide (NEM) and dithiothreitol (DTT). The effects of NEM on the three opioid receptors are not identical. Their order of sensitivity is μ > ∂ > κ [20]. Moreover, upon alkylation by NEM the number of binding sites is reduced for all three μ, ∂ and κ receptors but only the residual μ and ∂ receptors, and not κ, display a reduced affinity for agonists [21–23]. Because NEM is able to shift μ- and ∂-opioid receptors into a low-affinity state for agonists and because this effect is modulated by Na^+ ions and GTP analogues [21,22], it remains unclear whether the -SH group(s) alkylated by NEM is located on the opioid receptors or on associated G-proteins.

Previous studies on μ-opioid receptors [20,22] were performed on brain membranes which harbour the three classes of receptor. To precise the effects of sulfhydryl reducing or alkylating agents we used mammalian cells transfected with the human μ-opioid receptor cDNA and thus expressing a single class of receptor. Mutagenesis experiments were also performed to identify the molecular determinants at the basis of DTT and NEM actions on μ-opioid receptor.

The inhibitory effect of DTT on ligand binding was attributed to the reduction of a disulfide bridge linking C142 and C219 in extracellular loops 1 and 2 of the μ-opioid receptor. Alkylation of C81 and C332 occurred at low concentrations of NEM and decreased agonists but not antagonists binding; this effect is suggested to result from alteration of receptor interactions with agonists or with G-proteins. Modification of a second site of lesser sensitivity to NEM and which remains to be identify, reduced the number of binding sites for both agonists and antagonists.

2. Materials and methods

2.1. Chemicals and reagents

Tissue culture reagents and dithiothreitol were from GIBCO-BRL (Cergy-Pontoise, France). DEAE dextran was purchased from Pharmacia (Saint-Quentin en Yvelines, France) and polyethylenimine, *N*-ethylmaleimide, chloroquin and [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAGO) from Sigma (St. Louis, MO). Morphine and naloxone were from Francopia (Paris France) and diprenorphine from Reckitt and Colman (Kingston, UK). [^{3}H]Diprenorphine ([^{3}H]DPN; 40 Ci/mmol) and [^{3}H]DAGO (55 Ci/mmol) were from Amersham (Little Chalfont, UK). Synthetic oligonucleotides (Genosys, Cambridge, UK) were purified by SDS-PAGE electrophoresis. DNA modifying and restriction enzymes were from New England Biolabs (Beverly, MA).

2.2. Cloning and mutagenesis of the μ-opioid receptor cDNA

A cDNA encoding the human μ-opioid receptor was amplified from SH-SY-5Y human neuroblastoma mRNA by RT-PCR using 5′-cgtctagaggcagaggagaatgtc-3′ sense and 5′-cttctagacttggtgaaggtgga-3′ antisense primers. These were derived from published sequences [24,25] modified to create *Xba*I sites (underlined) at the 5′-end of

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each primer. The amplified fragment (1.5 kb) was cloned into the *Xba*I sites of pGEM4z and both strands entirely sequenced (Sequenase version 2.0; USB, Cleveland, OH) using 14 sense and antisense internal primers.

Silent substitutions were introduced into the μ-opioid receptor cDNA to facilitate subsequent mutagenesis. *Sal*I and *Bsu*36I restriction sites were created in the sequences coding for TST (positions 120–122 in TM 2) and ALG (positions 325–327 in TM7). Using these and naturally occurring sites, short sequences were replaced by synthetic oligonucleotides coding for the desired mutations and containing a restriction site convenient for mutant screening. Oligonucleotides and restriction sites were: a 50 bp *Bcl*I to *Ban*II fragment introducing a *Bsr*GI site for C81S; a 69 bp *Sal*I to *Bsa*BI fragment introducing an *Acc*65I site for C142S; a 68 bp *Bsa*BI to *Bsp*DI fragment introducing a *Bst*XI site for C161S; a 74 bp *Bam*HI to *Bsr*GI fragment introducing a *Sac*I site for C219S; a 39 bp *Bsu*36I to *Nsi*I fragment introducing an *Acc*I site for C332S. Each construct was verified by restriction enzyme analysis and sequencing throughout the modified regions. A 1.3 kb *Nco*I to *Xba*I fragment containing the entire receptors coding region was then subcloned into the *Bam*HI and *Xba*I sites of the pcDNA/Amp eukaryotic expression vector (Invitrogen, San Diego, CA).

2.3. Receptor expression and binding assays

Cos-M6 cells (5.10^6) were transfected with plasmid DNA (15 μg) by the DEAE-dextran method and grown for 64 h at 37°C in a 5% CO_2 atmosphere in DMEM supplemented with sodium glutamate (862 mg/l), sodium pyruvate (110 mg/ml), foetal calf serum (10%), penicillin (50 U/ml) and streptomycin (50 μg/ml). After washing and harvesting, cell pellets were frozen at −80°C, thawed and resuspended in 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA. After Potter homogenization (20 strokes, 4°C) and centrifugation (10 min, $1000\times g$, 4°C), the pellet was re-homogenized in the same buffer and centrifuged as above. Supernatants were pooled and crude membranes were pelleted ($100\,000\times g$ and 4°C for 30 min) and resuspended in 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA to be aliquoted and stored at −80°C. Protein content was measured as described [26].

Saturation experiments were performed on membranes aliquots (20–100 μg of protein) in 0.5 ml of 50 mM Tris-HCl (pH 7.4), using 13 concentrations (0.1–3 nM) of [^{3}H]DPN, a non-selective opioid antagonist or [^{3}H]DAGO (0.1–5 nM), a μ-selective agonist. Inhibition of [^{3}H]DPN (1 nM) binding was realized with 12 concentrations of competitors (10 pM to 20 or 200 μM according to the selectivity of the inhibitor used). Non-specific binding was determined in the presence of 1 μM unlabelled diprenorphine. Following a 1 h incubation period at 25°C, free ligand was removed by filtration on to Whatman GF/B filters and bound radioactivity was measured. Data were analyzed with the Inplot program (Graphpad Software Inc., San Diego, CA).

2.4. Treatment with thiol reagents

Membrane aliquots of COS-M6 cells transfected with μ-opioid receptor cDNA were maintained for 10 min at 25°C in 50 mM Tris-HCl (pH 7.4), containing varying concentrations of either DTT (10–500 mM) or NEM (0.05–8 mM). Alkylation by NEM was stopped by adding DTT in a 2-fold molar excess and further incubating for 10 min at 25°C. Reactions were diluted 5-fold in ice-cold 50 mM Tris-HCl (pH 7.4), and binding assays were performed.

3. Results

3.1. Cloning of the human μ-opioid receptor cDNA and expression in COS-M6 cells

The coding region of the cDNA isolated from SH-SY-5Y neuroblastomas had a sequence identical to that of Mestek et al. [24] but differed from that of Wang et al. [25] at three positions, resulting in D51N (G/A) and L234V (GC/CG) substitutions. Transfection of this cDNA into COS-M6 cells resulted in the expression of binding sites with K_d values for [^{3}H]DPN and [^{3}H]DAGO, and K_i values for DAGO, morphine and naloxone (Tables 1 and 2) which closely agreed with those previously reported for μ receptors expressed in mammalian tissues or transfected cells [24,27–30].

3.2. Inactivation by dithiothreitol and N-ethylmaleimide of ligand binding to μ-opioid receptors

The sensitivity of the μ receptor to thiol reducing and alkylating agents was investigated by measuring [^{3}H]DPN binding following treatment of membranes with varying concentrations of DTT or NEM. Both compounds completely inhibited [^{3}H]DPN binding with half-effective concentrations (IC_{50}) of 250 mM for DTT and 1 mM for NEM (Fig. 1). The lack of action of DTT at concentrations up to 20 mM ruled out the possibility that the decreased [^{3}H]DPN binding observed upon NEM treatment may be attributed to the DTT added to stop alkylation.

The effect of NEM on [^{3}H]DAGO binding was also analyzed since it has been reported that binding of agonists was more sensitive than that of antagonists to NEM inactivation [20]. The biphasic nature of the curve (Fig. 1) suggested that two distinct sites of alkylation were involved in inhibition by NEM of [^{3}H]DAGO binding to the μ-opioid receptor. The first one with an IC_{50} value of 20 μM seemed specific of agonists binding whereas the second one, the IC_{50} of which (2 mM) was in the same range as that for the inhibition of [^{3}H]DPN binding, could be involved in inactivation of both agonist and antagonist binding.

3.3. Consequences of DTT and NEM treatment on the number of μ-opioid binding sites and their affinity for agonists and antagonists

Inhibition of opioid binding by DTT and NEM was further characterized by performing saturation experiments following reduction or alkylation of μ-opioid receptors. Scatchard's analysis [31] of [^{3}H]DPN binding isotherms revealed that 250 mM DTT reduced B_{max} values from 1.7 to 0.7 pmol/mg

Table 1
Two alkylation sites of different sensitivity to NEM differentially affect [^{3}H]DPN and [^{3}H]DAGO binding to the μ-opioid receptor

(NEM)	[3H]DPN		[3H]DAGO	
	K_d (nM)	B_{max} (% of control)	K_d (nM)	B_{max} (% of control)
None	0.21 ± 0.03	100	0.95 ± 0.16	100
50 μM	N.D.	N.D.	6.0 ± 0.7*	111 ± 15
1 mM	0.39 ± 0.04	42 ± 5*	5.6 ± 0.4*	60 ± 5*

Dissociation constants (K_d) and receptor densities (B_{max}) were calculated by Scatchard's transformations of saturation isotherms following treatment of COS-M6 membranes expressing the wild-type μ-opioid receptor with the indicated concentrations of NEM. B_{max} values varied from one transfection to another (1–4 pmol/mg of proteins) and are represented as the mean percentage of that occurring in the absence of NEM.* $P < 0.05$ treated *versus* untreated.

Table 2
Effects of NEM on antagonists and agonists binding to wild-type and mutant μ-opioid receptors

Receptors	NEM	K_d (nM)		K_i (nM)	
		[3H]DPN	Naloxone	Morphine	DAGO
μ	−	0.21 ± 0.03	3.7 ± 0.3	9.5 ± 2.5	7.5 ± 1.5
	+	0.39 ± 0.04	4.5 ± 1.0	82 ± 4[a]	55 ± 5[a]
μC161S	−	0.20 ± 0.03	1.8 ± 0.8	5.4 ± 1.0	1.8 ± 0.3[b]
	+	0.94 ± 0.05[b]	7.3 ± 0.3[b]	140 ± 30[b]	100 ± 20[b]
μC81S	−	0.17 ± 0.03	2.6 ± 1.0	9.5 ± 2.7	4.3 ± 1.0
	+	0.30 ± 0.04	3.5 ± 0.7	30 ± 3[a]	18 ± 4[a]
μC332S	−	0.18 ± 0.03	1.8 ± 0.4	9.8 ± 1.1	5.4 ± 0.3
	+	0.20 ± 0.03	1.9 ± 0.1	28 ± 3[a]	18 ± 7
μC81S/C332S	−	0.28 ± 0.08	4.9 ± 0.9	49 ± 11[b]	14 ± 1
	+	0.21 ± 0.06	2.5 ± 0.3	48 ± 8	15 ± 1

Dissociation (K_d) and inhibition (K_i) constants were calculated from direct and inhibition assays of [^{3}H]DPN binding to membranes of COS-M6 cells expressing the wild-type (μ) or the indicated mutant μ-opioid receptors before (−) and after (+) treatment with 1 mM NEM for 15 min at 25°C. Scatchard's analysis of [^{3}H]DPN binding showed a mean reduction in the number of binding sites of 48% for the μ receptor, 61% for μC161S, 62% for μC81S, 54% for μC332S and 53% for μC81S/C332S. Significant differences ($P < 0.05$) between K_d or K_i values of treated *versus* untreated receptors (a) and mutant *versus* wild-type receptors (b) are indicated.

of membrane protein with no significant effect on the affinity of the residual sites (not shown).

The affinity of [^{3}H]DPN was not affected by 1 mM NEM which only reduced the number of binding sites by 50% (Fig. 2 and Table 1). The density of [^{3}H]DAGO binding sites was similarly decreased by 1 mM NEM but K_d value was increased from 1 to 6 nM (Fig. 2 and Table 1). This differential effect on [^{3}H]DAGO and [^{3}H]DPN binding and the possible existence of two alkylation sites (see above) suggested that modification of the first site ($IC_{50} = 20$ μM) could have accounted for diminished agonist affinity whereas alkylation of the second site ($IC_{50} = 2$ mM) could have resulted in decreased B_{max} values. Indeed, upon exposure to 50 μM NEM, the number of [^{3}H]DAGO binding sites was not modified whereas the affinity of the ligand was decreased to the same extent as with 1 mM NEM (Fig. 2 and Table 1).

The apparent affinities of other opioid ligands following NEM treatment were analyzed by competition binding assays [32]. These indicated that the inhibition constants (K_i) of naloxone, another opioid antagonist, was indeed unaffected by NEM and confirmed that K_i of agonists DAGO and morphine were increased about 10-fold (Table 2).

3.4. *Identification in the μ-opioid receptor of the cysteyl residues involved in inactivation by DTT and NEM*

Inactivation of other G-protein-coupled receptors by DTT has been attributed to the reduction of a disulfide bond linking conserved cystein residues in extracellular loops 1 and 2 [33–35]. To examine whether this was also the case here, the corresponding C142 and C219 of the μ-opioid receptor were replaced by serine residues. COS-M6 cells transfected with cDNA coding for mutants μC142S or μC219S receptors did not exhibit any specific binding to agonist [^{3}H]DAGO or to antagonists [^{3}H]DPN and [^{3}H]naloxone (not shown).

The μ-opioid receptor has been suggested to be more sensitive to inactivation by NEM than δ or κ receptors [20]. To identify the molecular determinants of NEM action on the μ-opioid receptor, C81, C161 and C332 which are specifically present in transmembrane domains 1, 3 and 7 (C81 is also found in the δ receptor), were substituted by serine.

The apparent affinities of [^{3}H]DPN, naloxone and morphine for the μC161S receptor were almost identical to those for wild-type receptor whereas that of DAGO was slightly increased (Table 2). Surprisingly, the effect of NEM treatment on binding affinities was more pronounced for this mutant receptor than for the wild-type receptor (Table 2). As for

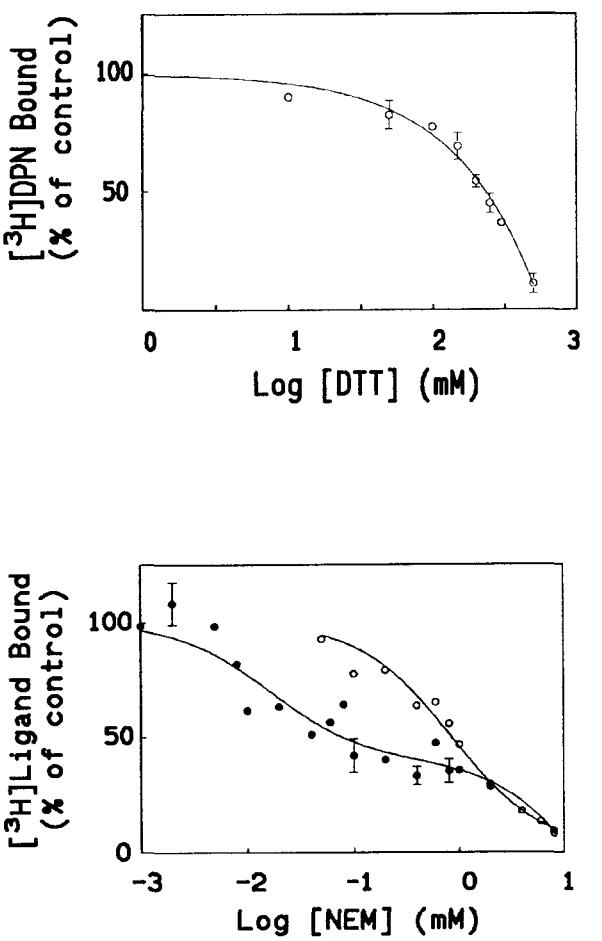

Fig. 1. Dithiothreitol and *N*-ethylmaleimide inhibit ligand binding to the μ-opioid receptor. Residual binding of 1 mM [^{3}H]DPN (○) and [^{3}H]DAGO (●) was measured after incubation of membranes with increasing concentrations of DTT (upper panel) or NEM (lower panel). Each curve is representative of 2 to 3 experiments performed in duplicate.

wild-type receptors, a 60% decrease in the number of binding sites was obtained.

The binding profiles of μC81S and μC332S receptors were not modified as compared to that of the native receptor (Table 2). For both mutants, the number of [^{3}H]DPN binding sites was decreased by about 50% upon receptor alkylation by NEM. The K_i values of agonists were also increased by NEM but to a lower extent than for the μ receptor. This suggested that alkylation of C81 and C332 may be responsible for the inhibition by NEM of agonist binding to the μ receptor. A receptor carrying both substitutions was thus constructed. Compared to the wild-type μ-opioid receptor, the μC81S/C332S receptor had unmodified affinities for antagonists and its affinity for agonists was decreased for morphine and to a lesser extent for DAGO (Table 2). Affinities of the double mutant for DAGO and morphine were no longer sensitive to NEM treatment (Table 2) whereas the number of binding sites was still decreased by 50% (Table 2). This loss of modulation of agonist binding was further evidenced by the disappearance from the inhibition curve by NEM of [^{3}H]DAGO binding to the μC81S/C332S receptor of the component of higher sensitivity to NEM which was observed for the wild-type receptor (Fig. 3).

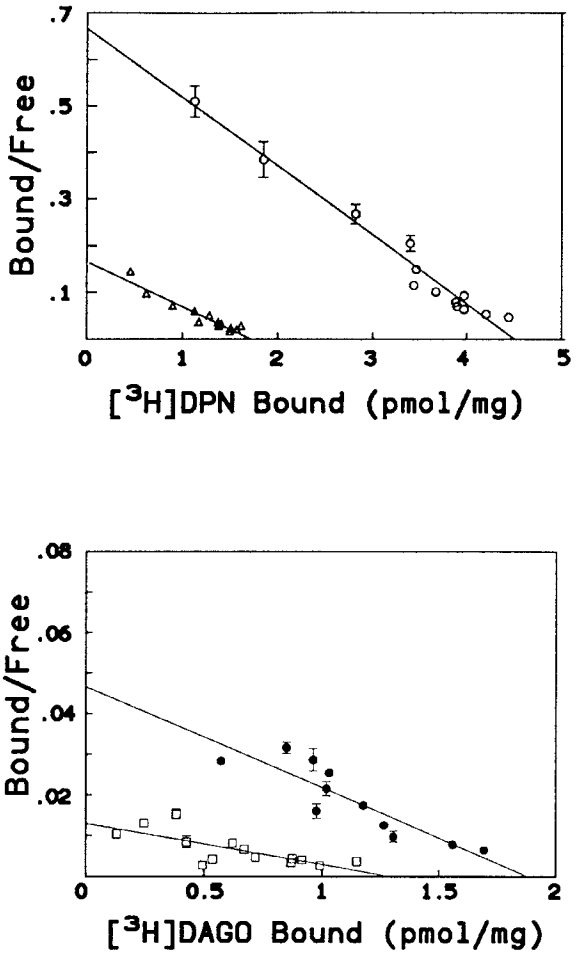

Fig. 2. Differential effects of NEM on [^{3}H]DPN and [^{3}H]DAGO binding to μ-opioid receptors. Upper panel: Scatchard's plots of [^{3}H]DPN binding isotherms for control (○) COS-M6 membranes and membranes treated with 1 mM NEM (△). Lower panel: Scatchard's plots of [^{3}H]DAGO binding isotherms for COS-M6 membranes treated (□) or not (●) with 1 mM NEM. Curves are representative of 3 to 4 experiments performed in duplicate; the corresponding mean K_d and B_{max} values are given in Table 1.

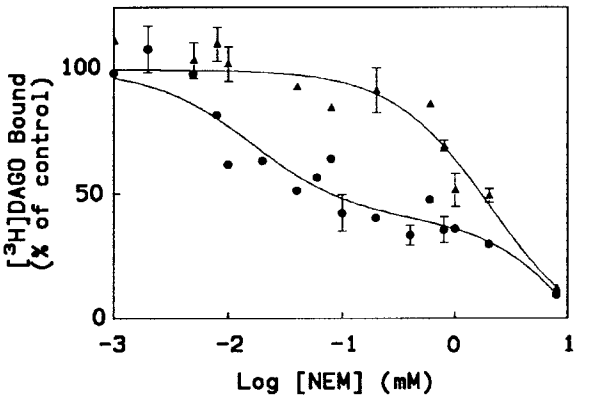

Fig. 3. Comparison of NEM effects on [^{3}H]DAGO binding to wild-type and C81S/C332S μ-opioid receptors. Residual binding of 1 nM [^{3}H]DAGO to wild-type (●) and C81S/C332S (▲) μ-opioid receptors was measured following incubation with the indicated concentrations of NEM. Curves are representative of two experiments performed in duplicate.

4. Discussion

4.1. *A disulfide bridge connects extracellular loops 1 and 2 of the μ-opioid receptor*

Treatment of cell membranes expressing μ-opioid receptors with increasing concentrations of the thiol reducing agent DTT decreased the number of [^{3}H]DPN binding sites. The high concentrations of DTT required to inactivate ligand binding may have resulted in non-specific deterioration of the cell membrane. Such an effect is, however, expected to be gradual and accompanied by a parallel loss of receptor affinity and that was not observed here. Alternatively, inactivation of ligand binding could have arisen from the reduction within the μ-opioid receptor of a disulfide bond barely accessible to the solvent or of low reactivity to NEM. Indeed, mutant μ-opioid receptors in which C142 and C219 in extracellular loops 1 and 2 were changed to serine both failed to bind agonists and antagonists. Since it is possible that μC142S and μC219S receptors were not expressed at the cell membrane, our results suggest that the formation of a disulfide bound between extracellular loops 1 and 2 is necessary to constrain the μ-opioid receptor in a proper conformation for ligand binding and/or for membrane integration.

4.2. *Differential effects of NEM on agonist and antagonist binding*

The inhibitory effect of NEM on ligand binding to the μ-opioid receptor [20,22] was precised here using transfected COS-M6 cells expressing a homogenous receptor population. The EC_{50} of NEM for inhibition of [^{3}H]DPN binding (1 mM) agreed with that measured for [^{3}H]bremazocine [20], another opioid antagonist. We showed that the effects of NEM on [^{3}H]DPN binding were mainly attributed to diminished binding capacity of cell membranes; the affinities of antagonists for μ-opioid receptors were almost unaffected. In contrast but in agreement with other reports [22], 1 mM NEM reduced both receptor density and affinity for agonists. Such a differ-

ential effect of NEM on agonist versus antagonist binding has also been reported for δ-opioid receptors [21,36].

At lower concentrations of NEM (50 μM) only the affinity for the agonist [^{3}H]DAGO was modified. This suggested that NEM is acting at two sulfhyldryl groups with alkylation of the more sensitive (IC_{50} = 20 μM) leading to altered agonists affinities and modification of the less reactive (IC_{50} = 1–2 mM) to decreased B_{max} values. Similarly to our results, it has recently been shown that inhibition by NEM of substance P binding to the NK-1 receptor occurred in a biphasic manner and that alkylation of the more sensitive site decreased affinity for the neuropeptide [37].

4.3. *Alkylation by NEM of cysteine residues 81 and 332 in the μ-opioid receptor is responsible for decreased agonists affinity*

Cystein residues C161 and C332 which are specifically found in the μ-opioid receptor, and C81 which also occurs in the δ receptor, were replaced by serine residues to identify the molecular determinants of the inhibition by NEM of agonists binding to the μ-opioid receptor. Each single mutation had no or little intrinsic effect on ligand binding.

Antagonist binding to μC161S receptors became sensitive to NEM and the loss in agonist affinity was even more pronounced than for wild-type receptors. This could have reflected alkylation of a cysteine residue not exposed to NEM in wild-type receptor but accessible in μC161S, either as a result of a conformational change of the receptor structure or because it is normally forming a disulfide bridge with C161.

Affinities of agonists for μC81S and μC332S receptors were partially protected from NEM action; total protection was obtained for the μC81S/C332S receptor. The 50% decrease in receptor density was still observed for the three mutant receptors. In agreement with this result, the NEM dose–response curve for inhibition of [^{3}H]DAGO binding to μC81S/C332S receptors did not any longer present the component of high sensitivity to NEM which was shown to be responsible for modulation of agonists affinities for the native μ-opioid receptor. Alkylation by NEM of the μ-opioid receptor at both C81 and C332 was thus certainly responsible for decreased agonists affinities. This correlates with previous studies which showed that μ receptors were more sensitive to NEM than δ and κ [20], and that both B_{max} and K_d values were affected for μ and δ receptors [21,22,36], whereas only the number of binding sites was decreased for κ receptors [23]. Indeed the μ receptor contains two cysteins the alkylation of which results in decreased agonists affinities whereas the δ receptor has only one of these (C81) and the κ receptor none.

In contrast with these results, it has recently been reported that H223 was critical for μ-opioid receptor inactivation by NEM, and that cysteine residues were not involved [38]. The C332S substitution was, however, not investigated in this study [38]. Moreover, the effect of NEM on the binding of agonists could not be addressed since only the antagonist [^{3}H]bremazocine was used. Therefore, as suggested [38], the effect of the μH223S substitution on NEM sensitivity could have resulted from a conformational change in the receptor structure.

Because NEM is able to shift μ- and δ-opioid receptors into a low-affinity state for agonists, but not for antagonists, and because this effect is modulated by sodium and to a lesser extent by lithium, it has been suggested that the cysteine residues alkylated by NEM could either belong to the opioid receptor and be involved in coupling to G-proteins or lie within the G-protein itself [22,36]. Our results with the μC81S/C332S receptor clearly argue against the latter possibility. In the former case, they suggest that coupling to G-proteins still occurs for μC81S and μC332S receptors since these display affinities for agonists which are identical to those of wild-type receptors and remain sensitive to NEM. Such substitutions may indeed be neutral in terms of coupling because the position corresponding to μC332 is naturally occupied by a serine in δ and κ receptors.

Although affinities of the double-mutant μC81S/C332S for [^{3}H]DPN, naloxone and DAGO were unaffected, that for morphine was slightly decreased. The C81 and C332 residues of the μ receptor may thus be important for high-affinity morphine binding, either because they are, or lie in the close vicinity of, agonists contact points or because their simultaneous replacement induces discrete modifications of receptor interactions with morphine or G-proteins. In this respect it is interesting to note that DAGO and morphine have already been shown to induce different intracellular trafficking of μ-opioid receptors [39,40], possibly by coupling to different G-proteins and intracellular effectors.

Altogether, ours results support the conclusion that in mammalian cells alkylation of μ-opioid receptors by NEM at C81 and C332 accounts for decreased agonist affinities. They also suggest that such an effect is due to modifications of receptor interactions with agonists or with G-proteins. Alkylation of other cystein residue(s) which still have to be identified but are expected to be common to μ, δ and κ receptors, may be responsible for the diminished B_{max} values observed for all three receptors upon NEM treatment.

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