

Construction and characterization of a kappa opioid receptor devoid of all free cysteines

David Ott, Renato Frischknecht and Andreas Plückthun¹

Department of Biochemistry, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

¹To whom correspondence should be addressed.
E-mail: plueckthun@bioc.unizh.ch

We have constructed an optimized mutant of the kappa opioid receptor (KOR), which is devoid of its 10 free cysteines. It was necessary to test different amino acid replacements at various positions and we used a structural model and homology with other receptor family members as a guide. This mutant binds ligands and couples to the cognate G-proteins in a very similar fashion to wild-type KOR. The addition of the antagonist naloxone during cell growth greatly enhances heterogeneous expression of the mutant in mammalian cells, such that amounts similar to wild-type could be produced. We showed by fluorescence microscopy that naloxone stabilizes the mutant in the plasma membrane. This mutant, which now permits the insertion of single cysteines, was designed for use in spectroscopic studies of ligand-induced receptor conformational changes as well as to simplify folding studies.

Keywords: cysteine replacement/G protein-coupled receptor/membrane proteins/protein design

Introduction

The kappa opioid receptor (KOR) is a member of the peptide-binding subfamily of class A of G protein-coupled receptors (GPCRs) and couples to G_i, G_o and G_z proteins (Prather *et al.*, 1995; Tso and Wong, 2000). Major cellular manifestations of receptor-mediated signaling are the inhibition of adenylyl cyclase activity, the inhibition of voltage-dependent Ca²⁺ channels and the increase in K⁺ conductance (Satoh and Minami, 1995; Standifer and Pasternak, 1997). The receptor mediates many important physiological functions, such as modulation of pain, drinking, water balance, food intake, gut motility, temperature control and various endocrine functions. Furthermore, in humans, activation of KOR has been found to produce dysphoria and psychotomimesis (Meng *et al.*, 1993). Agonists selective for KOR produce an effective analgesia without the substantial side effects (constipation, respiratory depression, vomiting and physical dependence) associated with mu opioid receptor agonists such as morphine (Reece *et al.*, 1994).

Despite extensive studies on structure–function relationships using receptor chimeras of the related kappa, mu and delta opioid receptors (Kong *et al.*, 1994; Wang *et al.*, 1994; Xue *et al.*, 1994, 1995; Chen *et al.*, 1995; Liu *et al.*, 1995; Meng *et al.*, 1995, 1996; Minami *et al.*, 1995; Hjorth *et al.*, 1996; Zhu *et al.*, 1996; Coward *et al.*, 1998; Lu *et al.*, 1998; Seki *et al.*, 1998; Mollereau *et al.*, 1999) and ample work on KOR mutagenesis, focused on receptor desensitization, internaliza-

tion and down-regulation (Appleyard *et al.*, 1999; Li, J. *et al.*, 2002; Li, J. G. *et al.*, 2002; Schulz *et al.*, 2002; Zhang *et al.*, 2002), activation (Thirstrup *et al.*, 1996; Standifer and Pasternak, 1997) and the location of the ligand-binding pocket (Hjorth *et al.*, 1995; Jones *et al.*, 1998; Xu *et al.*, 2001), detailed structural understanding of signal transduction mediated by KOR remains elusive. Clearly, this is primarily because a direct investigation of the opioid receptor tertiary structure by crystallography or NMR has not been achieved. It has been reported in numerous studies (Ruegg *et al.*, 1982; Itzhak *et al.*, 1984; Giannini *et al.*, 1985; Demoliou-Mason and Barnard, 1986; Simon *et al.*, 1985, 1986, 1987) that this receptor forms higher order aggregates when extracted from the membrane-embedded form using detergents, at least under the described experimental conditions. This behavior makes even 2-D crystallization difficult. Recently, it was shown by bioluminescence resonance energy transfer *in vivo* that KOR constitutively forms oligomers in HEK293 cells under conditions where the receptor is overexpressed (Ramsay *et al.*, 2002). All of these studies suggest that the receptor is prone to aggregation.

For these reasons, the agonist-induced conformational changes of the opioid receptors might best be studied by fluorescence spectroscopy *in vitro* using artificial bilayers with immobilized receptor. In particular, fluorescent probes are known (Gether *et al.*, 1995) which change their fluorescent properties depending on the hydrophobicity of the environment. One of the most convenient site-specific modifications is a coupling to cysteine. This approach was pioneered on the β 2-adrenergic receptor (Gether *et al.*, 1997; Ghanouni *et al.*, 2001; Neumann *et al.*, 2002) by exploiting the distinct reactivity of the endogenous sulfhydryl groups. Another approach for studying agonist-induced conformational changes might be the use of an *in situ* disulfide cross-linking strategy, which was employed in the case of the M3 muscarinic acetylcholine receptor (Zeng *et al.*, 1999; Ward *et al.*, 2002).

To make these approaches fully general, one needs to be able to introduce free cysteines at desired positions in the sequence. Most GPCRs have a large number of endogenous cysteines, which would preclude selective labeling at a newly engineered cysteine. Therefore, a prerequisite for the designed introduction of a unique free cysteine into a receptor at a specific position is the creation of a cysteine-free receptor or else the simultaneous labeling of endogenous free cysteines may cloud the interpretation of the experimental data (Javitch *et al.*, 2002). However, a cysteine-free GPCR has not yet been reported.

Almost all GPCRs are thought to contain a conserved disulfide bond, connecting the transmembrane helix 3 and the second extracellular loop. The sensitivity of the opioid receptors to DTT in neuronal membrane preparations was reported to decrease in the order $\mu > \delta \gg \kappa$, which the authors interpreted to mean that the disulfide bridge may not be crucial

for the function of KOR (Gioannini *et al.*, 1989). However, it was not directly demonstrated in these studies that the putative disulfide bond was actually reduced. Zhang *et al.* mutated highly conserved cysteines, believed to form a disulfide bond, in human mu opioid receptor (MOR) to alanine and serine (Zhang *et al.*, 1999). Ligand binding was lost, but the receptor was still expressed in the cell. Xu *et al.* successfully replaced individual transmembrane cysteines of human delta opioid receptor (DOR), human KOR and rat MOR with serine and one additional cysteine, present in rat MOR only, with methionine (Xu *et al.*, 2000).

An initial experiment in which we replaced all free endogenous cysteines of KOR at once with alanine indicated a total loss of activity, even when keeping the potential disulfide bond (see below). However, we report here a strategy for finding optimized replacements for all free cysteine residues and we combined them to generate a final free-cysteine-less (FCL) mutant. Its expression level was enhanced by exploiting ligand-mediated receptor up-regulation, described recently (Li *et al.*, 2001a,b). The ligand binding and G-protein coupling characteristics of the wild-type (WT) and the FCL receptors were compared. We also studied the cellular localization of the receptor by means of cytoimmunochemistry.

Materials and methods

Materials

Reagents were obtained from Sigma, Fluka or Merck, unless stated otherwise. Tris was from Serva and forskolin and IBMX (3-isobutyl-1-methylxanthine) were from Calbiochem. Dynorphin A(1–13) (Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys) was from Bachem and a stock solution was prepared in 50% (v/v) aqueous methanol containing HCl (50 mM) (Goldstein, 1984). Stock solutions of the MOR agonist DAMGO (Tyr–D–Ala–Gly–N-methyl–Phe–Gly–ol), the KOR agonist (–)-*trans*-1*S*,2*S*-U-50488 hydrochloride (hereafter U-50488) and the opioid antagonists naloxone hydrochloride (hereafter naloxone) (Blumberg *et al.*, 1961; Foldes *et al.*, 1963) and naltrexone were prepared in water. An inverse agonist of KOR, annotated in the original paper (Becker *et al.*, 1999) as Peptide I (structure in figure 8 of Becker *et al.*, 1999) was kindly provided by Dr A.Pessi (IRBM P. Angeletti, Rome, Italy); a stock solution was prepared in DMSO.

Construction of receptor variants

The cDNA of rat KOR-subtype 1 (NCB accession No. P34975) (Minami *et al.*, 1993) was a gift from Dr M.Satoh (Kyoto University, Kyoto, Japan). The internal *Hind*III site was removed by overlapping PCR and the gene was first cloned into the mammalian expression vector pcDNA3.1 (Invitrogen) with a C-terminal hexahistidine tag. This construct was used as a template for further manipulation and mutagenesis. Every cysteine substitution was introduced using a set of two complementary primers, each about 40 nucleotides long with a mutation in the middle. The mutated gene was assembled by overlapping PCR and used to replace the original gene in the template using two internal restriction sites flanking the mutated part of the template. Other tagged constructs were prepared as follows. First, the receptor was recloned into the pcDNA3.1a(–)/myc/His vector in frame with C-terminal myc and His tags, both encoded in the vector. For the WT and FCL receptors, the first three N-terminal amino acids were replaced by a FLAG tag (MDYKDDDDK), followed by a glycine. This

N-terminal FLAG tag was previously shown not to affect the signaling of KOR (Li, *et al.*, 2002). *Hind*III sites were introduced at both ends of the enhanced green fluorescent protein gene (eGFP from vector pEGFP-N3; Clontech) using PCR, and the eGFP gene was fused to the receptor gene at the *Hind*III site, present on the vector. Therefore, the residues Lys-Leu were inserted between KOR and eGFP and also between eGFP and the C-terminal myc/His tag. For use with the Flp-In™ System (see below), the receptor with the FLAG tag on its N-terminus and a decahistidine tail on its C-terminus was subcloned into the pcDNA5/FRT vector (Invitrogen). The *Escherichia coli* strain XL1-Blue (Stratagene) was used for the amplification of DNA. All constructs were confirmed by DNA sequencing.

Cell culture

Cell lines HEK293T (a clonal line of HEK 293 cells stably expressing the SV40 large T antigen) and COS-1 (both ATCC) were grown in Dulbecco's modified Eagle medium (DMEM) with high glucose (Sigma, Cat. No. D 6429) in the presence of 10% (v/v) heat-inactivated fetal calf serum (EC approved; Invitrogen) and 5% CO₂ at 37°C in a humidified incubator. Cells were routinely seeded into 24-well cell culture plates in 0.5 ml of medium and grown for 24 h, reaching 80–95% confluence, prior to transfection. Flp-In™-293 cells (Invitrogen) were grown in the presence of selection antibiotics under the same conditions.

Transient transfection

Cells were transfected with DNA using calcium phosphate precipitation based on the original protocol (Graham and van der Eb, 1973) with a small modification as follows. First, 25 µl of CaCl₂ (500 mM) was mixed with 1 µg of DNA. To this solution, 25 µl of a buffer containing 1.5 mM Na₂HPO₄, 140 mM NaCl, 50 mM HEPES, pH 7.05, were added. The mixture was then incubated for 60 s at room temperature and added dropwise to a well with cells overlaid with 0.5 ml of medium. Four hours later, the medium was exchanged and cells were further grown for 20 h in the absence or presence of naloxone (30 µM). Cells were harvested, washed three times with 0.5 ml of PBS and frozen in liquid nitrogen. Cell pellets were stored at –80°C.

Isogenic stable cell line generation based on polyclonal selection

The Flp-In™ System based on Flp recombinase-mediated gene site-specific integration [O'Gorman *et al.* (1991) and www.invitrogen.com] was used as follows. The Flp-In™-293 cell line with a single chromosomal Flp recombination target site was expanded in medium containing Zeocin (100 µg/ml). One day prior to transfection, 5 × 10⁵ cells were seeded per well in a six-well plate in 2 ml of medium without Zeocin. Co-transfection using Lipofectamine-2000 (Invitrogen) was performed as follows. The plasmid pcDNA5/FRT encoding the WT or FCL receptor (200 ng) and the plasmid pOG44 encoding Flp recombinase (1.8 µg) were resuspended in OptiMEM® I Reduced Serum Medium (250 µl). Lipofectamine-2000 (6 µl) was resuspended in a separate aliquot of OptiMEM® I Reduced Serum Medium (250 µl) and incubated for 5 min at room temperature. Both aliquots were mixed, incubated for 20 min at room temperature and added dropwise to the growth medium (1.5 ml). The medium was exchanged 24 h later. After a further 24 h, cells were trypsinized, transferred to a 10 cm dish and grown for 1 week

in the presence of the selection antibiotic Hygromycin B (100 µg/ml). The selection medium was exchanged every second day. All remaining cells were trypsinized and transferred into one 22 mm well and grown for 14 days until a few large colonies were identified. Cells were trypsinized and transferred as a pool into one 35 mm well. Cells were grown to 95% confluence, then trypsinized and transferred to a 75 cm² tissue culture flask and further expanded.

Binding assays with radioligand on whole cells

Binding assays were carried out essentially as described previously (Ruegg *et al.*, 1982) with a small alteration in the filtration step. Cell pellets were resuspended in 500 µl of Tris-HCl buffer (50 mM), pH 7.4, with 1 mM EDTA (TE buffer) and 1 nM [³H]diprenorphine (58 Ci/mmol) (Amersham Pharmacia Biotech). Non-specific binding was determined in the presence of 10 µM naloxone. The suspension (200 µl) was routinely incubated at room temperature for 45 min. Where indicated, the suspension was incubated at 4°C for 4 h. Separation of free and bound ligand was performed on a filtration vacuum manifold (Millipore) using 96-well filtration plates with glass-fiber filters (MAFBNOB plate from Millipore) pretreated with 0.5% (v/v) polyethylenimine (Bruns *et al.*, 1983). The filters were quickly washed with 1 ml of ice-cold TE buffer. OptiPhase HiSafe 2 scintillation cocktail (Wallac) was added directly to the wells (100 µl/well). The plate was shaken overnight and radioactivity was counted in a liquid scintillation counter (1450 Microbeta Plus, Wallac). Non-specific binding in the case of WT was <5% of total binding.

Membrane preparation

A rinsed pellet of transfected cells (from a confluent 75 cm² cell culture dish) grown in the presence of naloxone was resuspended in 3 ml of homogenization buffer (HB) containing 11% (w/v) sucrose, 10 mM MgCl₂ and 0.3% (v/v) protease inhibitor cocktail (Sigma, Cat. No. P-8340) in TE buffer. Cells were lysed using a 7 ml Dounce tissue grinder, with a tight piston (Wheaton) for 30 strokes. The suspension was centrifuged for 10 min at 4°C at 2300 r.p.m. (1100 g) in a Sigma 4K15 centrifuge. The pellet was resuspended in 3 ml of HB and the suspension was again treated with the Dounce tissue grinder and centrifuged for 10 min at 2300 r.p.m. The supernatants from the first and the second centrifugation steps were pooled and centrifuged for 1 h at 4°C at 31 000 r.p.m. (40 000 g) in a Beckman Optima TLX ultracentrifuge. The pellet was rinsed with 2 ml of HB, resuspended in 750 µl of HB using a Dounce tissue grinder, frozen in liquid nitrogen and stored at -80°C. Total membrane protein concentrations were determined using the Bio-Rad DC protein assay with bovine serum albumin as a standard.

Binding assays with radioligand on membrane preparations

Membrane proteins were diluted to 5 µg/ml in TE buffer with various concentrations of [³H]diprenorphine. Non-specific binding was determined in the presence of 10 µM naloxone. For calculating the receptor level and the apparent equilibrium dissociation constant (K_D) from the saturation binding data, we used a single-site binding model. In inhibition binding assays, concentrations of 0.25 nM [³H]diprenorphine and 5 µg/ml total membrane protein were used. Typically, samples of 800 µl were used for filtration, as in the binding assay with whole cells described above. Inhibition data were fitted to a single-site binding model with reversible inhibition by using non-linear

least-squares regression in order to obtain best estimates for R_T (total receptor concentration) and K_I (equilibrium inhibition constant of the non-radioactive ligand to be tested). Data were fitted by solving the following equation for C (equilibrium concentration of receptor-radioligand complex) by using the program MATLAB:

$$K_D C = L_F \left[R_T - C - \frac{K_D C I_T}{K_I L_T + (K_D - K_I) C} \right] \quad (1)$$

The parameter L_F (free equilibrium radioligand concentration) can be approximated with L_T (total radioligand concentration), since in our radioligand inhibition assays the concentrations L_T and L_F did not vary by more than 5%. K_D (equilibrium dissociation constant of the radioligand) was determined independently in the radioligand saturation binding assay and then kept constant. I_T (total inhibitor concentration) is an independent variable.

SDS-PAGE and western blotting

Pellets of 3×10⁵ cells from confluent wells in 24-well plates were resuspended in 50 µl of ice-cold radioimmunoprecipitation assay (RIPA) buffer (Brugge and Erikson, 1977) [our modified RIPA buffer contained 1% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate and 0.1% (w/v) SDS in PBS, pH 7.4] and incubated for 10 min on ice. Cellular debris was removed by centrifugation at 3300 r.p.m. (1100 g) in a 5417R centrifuge (Eppendorf) at 4°C for 10 min and the supernatant was mixed 1:1 (v/v) with 2.5-fold concentrated SDS-PAGE loading buffer (Sambrook and Russell, 2001) containing 4 M urea (Soulie *et al.*, 1996) and incubated for 10 min at 42°C. Proteins were separated on a 12% SDS-PAGE gel and blotted on to a PVDF membrane. Murine anti-tetra-His monoclonal antibody (Qiagen) was used as a primary antibody and goat anti-murine F(ab')₂ conjugated to horseradish peroxidase (Pierce) as a secondary antibody. Light development using the ECL cocktail (Amersham Pharmacia Biotech) was detected by a CCD camera (ChemImager 5500, Alpha Innotech). No non-specific staining was observed on the western blot, assessed with the lysate from non-transfected cells.

[³⁵S]GTPγS binding assay

The assay was performed similarly to the described procedure (Befort *et al.*, 1996; Zhu *et al.*, 1997). Briefly, ligands were added to membranes from transiently transfected HEK293T cells (2 µg of membrane protein) in 200 µl of incubation buffer containing 5 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% (w/v) BSA, 3 µM GDP and 0.3 nM [³⁵S]GTPγS (Perkin-Elmer Life Sciences) (1250 Ci/mmol) in 50 mM HEPES buffer, pH 7.6, and incubated either for 2 h at 4°C or for 30 min at 30°C. Filtration and a fast wash with 1 ml of ice-cold washing buffer (5 mM MgCl₂, 50 mM NaCl in 50 mM Tris-HCl, pH 7.5) were performed on a filtration plate with glass-fiber filters (MAFBNOB, Millipore) presoaked with water. Radioactivity remaining on the filters was detected as described above. G-protein activation data were fitted to a two-state model by using non-linear least-squares regression in order to obtain best estimates for EC₅₀ (ligand concentration needed for half-maximum activation of G-proteins). Experimental data were normalized by taking the basal activation in the absence of ligand as a minimum (0%) and the activation caused by 10 µM Dynorphin A(1-13) as a maximum (100%).

Adenylyl cyclase inhibition assay

The 24-well plates were coated with poly-L-lysine ($M_r > 300\,000$; 40 $\mu\text{g}/\text{ml}$) in 150 mM borate buffer, pH 8.4, for 30 min at room temperature. Plates were rinsed twice with water and stored at -20°C until used. Stable cell lines were grown in the coated wells in the selection medium for 28 h, then naloxone (30 μM) was added and cells were grown for another 20 h. The cells were then washed twice with PBS. Adenylyl cyclase in the cells was stimulated by forskolin in the presence of IBMX and inhibited by the action of G_i proteins induced by agonist according to the protocol used for metabotropic glutamate receptor type 4 (Flor *et al.*, 1995). Cells were lysed with 0.1 M HCl (400 $\mu\text{l}/\text{well}$) for 20 min at room temperature and the resulting extract was stored at -20°C . Non-acetylated cyclic AMP (cAMP) was assayed with the direct cAMP ELISA kit (Sigma, CA-200). Adenylate cyclase activity data were fitted similarly to the G-protein activation curves above. Experimental data were normalized by taking an adenylate cyclase activity stimulated by forskolin in the absence of ligand as a maximum (100%) and an adenylate cyclase activity in the absence of both forskolin and receptor ligand as a minimum (0%).

Localization of the receptor studied by confocal fluorescence microscopy

Cells were seeded on glass coverslips coated with poly-L-lysine (500 $\mu\text{g}/\text{ml}$). Four hours after transfection, the medium was exchanged and cells were grown for an additional 20 h in the presence or absence of naloxone (30 μM). Cells were washed once with cold HBSS (Hanks' Balanced Salt Solution; Invitrogen) and incubated with primary antibody [anti-FLAG M2 antibody diluted 1:1000 (v/v) in HBSS] at 4°C for 60 min, followed by incubation with HBSS at 4°C for 30 min. Further incubations took place at room temperature. Cells were fixed with 4% (w/v) paraformaldehyde + 4% (w/v) sucrose in PBS, pH 7.6, for 10 min and blocked with a solution of 10% (v/v) FCS and 0.1% (w/v) glycine in PBS for 1 h. Donkey anti-murine antibody conjugate with Cy-3 (contaminant precipitate was routinely removed by centrifugation) diluted 1:300 (v/v) in PBS was applied for 45 min. Cells were washed three times with PBS (15 min incubations) and mounted using Vectashield mounting medium (Vector Laboratories). Fluorescence images were acquired using a confocal microscope (Leica TCS SP) and a $63\times$, 1.32 numerical aperture objective.

Internalization of the receptor studied by confocal fluorescence microscopy

Cells were seeded on coated coverslips, transfected and grown as described above. One day after transfection, cells were washed twice with PBS and incubated with anti-FLAG M2 antibody for 30 min at 37°C , which allowed for antibody internalization. Cells were incubated in HBSS for 30 min at 4°C and then fixed and blocked. Subsequently, cells were incubated with the secondary antibody for 45 min at room temperature in PBS containing 3% FCS and 0.1% saponin. When receptor variants without FLAG tag were studied, goat anti-KOR antibody diluted 1:100 (N-19, Santa Cruz Biotechnology, sc-7494) and donkey anti-goat antibody conjugate with Texas Red diluted 1:200 were used as primary and secondary antibodies, respectively. Cells were washed with PBS, mounted and observed by fluorescence microscopy, as indicated above.

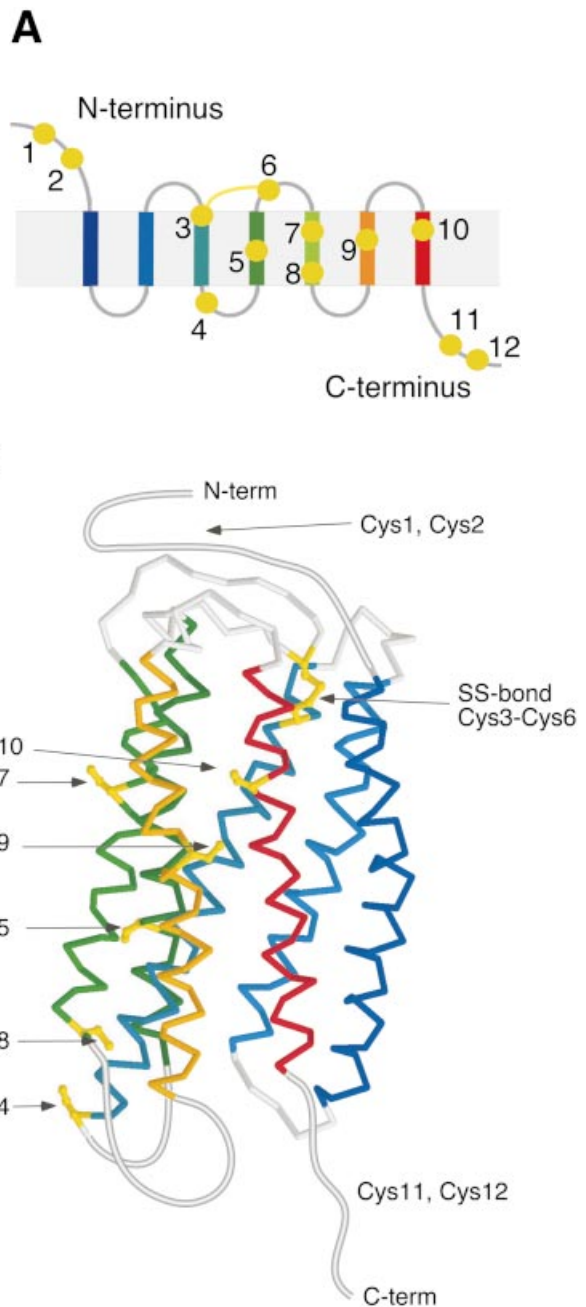


Fig. 1. Schematic representation of cysteines in KOR. Snake-like plot indicating the trans-membrane α -helices (A) and a model of the tertiary structure (B) with the indicated cysteines (Pogozheva *et al.*, 1998). Cysteines are numbered sequentially. For the correspondence to the sequence number, see Figure 2A.

Results

Design of cysteine replacements

Initially, we examined the KOR tertiary structure, which had been modeled based on geometric distance calculations with hydrogen bonding constraints (Pogozheva *et al.*, 1997, 1998). For simplicity, we have numbered the cysteines consecutively (Figure 1A) and give their corresponding sequence numbers in Figure 2A. Their positions in the structure are shown schematically in Figure 1B. It is very likely that Cys131 (C3)

A

Consecutive Cys No. in KOR	1	2	3	4	5	6	7	8	9	10	11	12
Sequence No. in KOR	16	21	131	161	181	210	229	245	286	315	340	345
Peptide subfamily (524 members)	S	A	C	V	A	C	L	C	C	T	Y	L
Most frequent residue												
Conservation in %	33	26	92	63	47	-	30	58	69	56	31	66

Rhodopsin family (1850 members)	Most frequent residue	Conservation in %
-	-	-
-	-	-
-	C	92
-	C	56
-	L	42
-	C	-
-	Y	32
-	C	48
-	C	58
-	T	43
-	C	-
-	C	-

B

	Consecutive cysteine No.:												specific relative binding	
	1	2	3	4	5	6	7	8	9	10	11	12	naloxone in medium	
													-	+
WT	C	C	C	C	C	C	C	C	C	C	C	C	100	130
	C	C	C	C	C	A	C	C	C	C	C	C	0	0
	C	C	A	C	C	C	C	C	C	C	C	C	0	0
	A	A	C	C	C	C	C	C	C	C	C	C	120	120
	C	C	C	C	C	C	C	C	C	C	A	A	50	118
	C	C	C	C	C	C	C	C	A	C	A	A	3	66
	C	C	C	C	C	C	C	C	S	C	C	C	43	103
	C	C	C	C	C	C	C	C	M	C	C	C	60	106
	C	C	C	C	C	C	C	C	T	C	C	C	57	108
	C	C	C	C	C	C	C	C	C	S	C	C	50	83
	C	C	C	C	C	C	C	C	C	T	C	C	44	77
	C	C	C	C	C	C	C	C	C	M	C	C	85	115
	C	C	C	C	C	C	C	C	C	A	C	C	51	106
	C	C	C	C	C	C	C	C	A	S	C	C	4	11
	C	C	C	C	C	C	C	C	S	S	C	C	16	49
	C	C	C	C	C	C	C	C	S	S	C	C	6	89
	C	C	C	C	C	C	C	C	M	C	C	C	73	102
	C	C	C	C	C	C	C	C	S	C	C	C	30	125
	C	C	C	S	C	C	C	C	C	C	C	C	6	103
	C	C	C	A	C	C	C	C	C	C	C	C	13	111
	C	C	C	M	C	C	C	C	C	C	C	C	24	98
	C	C	C	V	C	C	C	C	C	C	A	A	17	121
	C	C	C	C	L	C	C	C	C	C	C	C	89	115
	C	C	C	C	C	L	C	C	C	C	C	C	93	115
	C	C	C	C	A	C	A	C	C	C	C	C	62	108
	C	C	C	C	S	C	S	C	C	C	C	C	40	116
	A	A	C	C	C	C	C	C	S	S	A	A	9	47
	A	A	C	M	A	C	A	M	C	S	A	A	2	22
	C	C	C	S	S	C	S	S	S	S	C	C	0	0
	A	A	C	A	A	C	A	A	A	A	C	C	0	0
A	A	C	C	L	C	L	M	M	M	A	A	24	92	
FCL	A	A	C	M	L	C	L	M	M	M	A	A	4	75

Fig. 2. Variation of residues in cysteine positions of KOR. (A) Consensus residue analysis for GPCRs. The most frequent residues and their degree of conservation are shown for the rhodopsin family and the subfamily of peptide binders. Since the alignment of GPCR sequences does not allow an assessment of consensus residues in less conserved regions (loops, N- and C-termini), no consensus (minus sign) is indicated for these positions. (B) Expression of His-tagged receptor mutants with different cysteine substitutions in HEK293T cells. Transfected cells were grown for 20 h in either the absence or presence of naloxone in the medium. The binding of antagonist [³H]diprenorphine was measured on whole cells. Mock transfections (with the vector without an insert) resulted in only 1% relative specific binding compared with the vector with WT KOR.

and Cys210 (C6) form a disulfide bond. Cys181 (C5) and Cys229 (C7) seem to be fully exposed to the acyl chains of phospholipids. Cys161 (C4) resides in the second intracellular loop. The model suggests several possible interhelical side-chain interactions for the transmembrane Cys245 (C8) and Cys315 (C10) within a distance of 3.7–4.0 Å.

More information was obtained from the alignment of sequences of all known members within the GPCR subfamilies in the GPCR database (<http://www.gpcr.org/seq/001/>

001.html). Inspired by the success of consensus design (Knappik *et al.*, 2000; Lehmann *et al.*, 2002), we have considered the consensus residues in the positions of KOR cysteines (Figure 2A).

Cysteine replacements

When cysteines C3 or C6 were mutated to alanine, [³H]diprenorphine binding, measured on whole cells, was totally lost (Figure 2B). We could not detect any specific

binding or mutant receptor labeling at the cell surface even when naloxone (10 μ M) or naltrexone (10 μ M) were present in the growth medium during receptor expression (data not shown). However, the mutant receptor is expressed in the cell at a similar level as WT, as assessed by western blotting (data not shown) and is probably retained in the endoplasmatic reticulum and/or Golgi apparatus and its size indicates the absence of complex glycosylation. For this reason, we decided to keep these two cysteines, which are probably involved in disulfide bond formation, in the receptor template.

All other cysteine residues could be replaced, resulting in receptor mutants which bind ligand. A replacement by alanine is well tolerated in the N- and C-terminal regions of the receptor (double mutants C1A/C2A and C11A/C12A, respectively). As explained above, other cysteine replacements had to be designed following structural considerations and by using consensus design. From testing single and multiple mutants (Figure 2B), we can conclude that leucine is the best replacement for cysteines C5 and C7, suspected to face the lipid bilayer, whereas methionine is the best substitution among the substitutions tested (Figure 2B) for cysteines C4, C8, C9 and C10, which all may possibly interact with residues in other helices. The successful substitution for methionine indicates that these residues are in an apolar environment and that it is unlikely that the cysteines are involved in S–H bonds, otherwise substitutions for threonine or serine would be expected to be more beneficial than they are. We found that we had to test cysteine substitutions by carrying out cell growth without naloxone, since growth in the presence of naloxone can rescue the expression of some single mutants, which, in combination with other mutations, result in a non-functional receptor. For example, cysteine can be individually replaced at most positions with alanine and even serine, while the total alanine FCL receptor or the multiple serine mutant receptor could not be detected on the cell surface even when the antagonists naloxone or naltrexone were present during the expression. Interestingly, these mutants were expressed, but retained inside the cell, as we had observed for mutants C3A and C6A (data not shown).

However, naloxone (30 μ M) in the growth medium dramatically improves the functional expression of the optimized FCL receptor (C1A/C2A/C4M/C5L/C7L/C8M/C9M/C10M/C11A/C12A) almost 20-fold. In contrast, the expression of the WT in the presence of naloxone was increased by a factor of only 1.3. When cells are grown in the absence of naloxone, the level of specific ligand binding of the optimized FCL mutant is only 4% of WT, but it reaches 58% of WT when cells are grown with naloxone. Clearly, the optimized FCL receptor responds much more strongly to naloxone in the growth medium. The same effect of naloxone on the WT and FCL receptors was observed when the receptors were expressed in COS-1 cells or fused to an additional N-terminal FLAG tag and C-terminal myc-tag and expressed in HEK293T cells (see Table I). We also tested the expression of KOR in cells grown in the presence of Dynorphin A(1–13) (10 μ M) or Peptide I (10 μ M); however, the presence of these ligands in the growth medium did not significantly affect the expression level of either the WT or FCL receptor (data not shown).

For the optimized FCL and WT receptors, the binding assay on whole cells was performed both at room temperature for 45 min (our default set-up) and at 4°C for 4 h to eliminate any possible effects of receptor internalization on the assay. However, the ratio of specific FCL–receptor binding to

Table I. Expression of WT and FCL KOR in mammalian cells^a

Cells	Receptor	Fusion	Naloxone in growth medium ^b	
			–	+
HEK293T	WT	–	100 ^c	106
HEK293T	WT	GFP fusion	77	80
HEK293T	FCL	–	2	46
HEK293T	FCL	GFP fusion	2	36
COS-1	WT	–	100	ND
COS-1	FCL	–	4	51

^aFor expression in HEK293T cells, the receptor was fused on its N-terminus to a FLAG tag and on its C-terminus to a myc-tag followed by a His-tag, and in some cases to eGFP in front of the myc- and His-tags. For expression in COS-1 cells, the receptor was fused on its C-terminus to a His-tag only.

^bTransfected cells were grown for 20 h in the presence or absence of naloxone in the medium. The binding of the antagonist [³H]diprenorphine was measured on whole cells.

^cSpecific relative binding is given in % of WT.

WT–receptor binding did not differ significantly between these two experimental setups (data not shown).

Ligand-binding characteristics of the WT and FCL receptors

Membrane preparations from cells grown in the presence of naloxone were used to determine the expression level of functional receptor (R_T) and to estimate the receptor affinity to various ligands. R_T , determined from different growth experiments, varied for the WT from 2 to 13 pmol/mg and for the FCL from 1 to 7 pmol/mg (moles of active receptor/mass of total membrane protein) in transient transfection experiments. The ratio of R_T of the WT to that of the FCL was typically 2:1, when both receptors were expressed in parallel. Equilibrium dissociation constants for binding of [³H]diprenorphine to the WT and FCL receptors are 0.4 ± 0.1 and 0.6 ± 0.1 nM, respectively (Figure 3A).

Inhibition of the binding of [³H]diprenorphine by other opioid receptor ligands is depicted in Figure 3B and C. Naloxone gave very similar values of 4 ± 2 nM for the WT and 6 ± 2 nM for the FCL receptor, while the values for agonist Dynorphin A(1–13) indicated somewhat higher affinity for WT (-0.02 ± 0.01 nM) than for FCL receptor (0.2 ± 0.1 nM). The affinities of both WT and FCL KOR for the mu opioid receptor agonist DAMGO are weak ($K_i > 1$ μ M). The peptidic agonist Dynorphin A(1–13) competes with radioligand; however, the single-site binding model (see Materials and methods) does not properly describe this inhibition. This apparent discrepancy indicates differential modes for interaction of receptor with distinct ligands involving subtle changes in receptor conformation. It has been postulated (Chidiac, 2002) that, at steady state and in the absence of ligand, a GPCR reversibly isomerizes between the inactive (ground) state and the active state (G-proteins are activated). It has also been suggested that an agonist may have a significantly lower affinity for a receptor in the inactive state. Furthermore, more than two states of the receptor may be needed to describe its function. Such complexities may not be adequately captured by a simple single-site binding model.

WT and FCL receptor-mediated G-protein activation

We used a [³⁵S]GTP γ S binding assay to assess whether the FCL receptor still couples to G-proteins. Agonist binding typically leads to receptor internalization (Kallal *et al.*, 1998; Hasbi *et al.*, 2000; Li *et al.*, 2000) and it was remarkable that

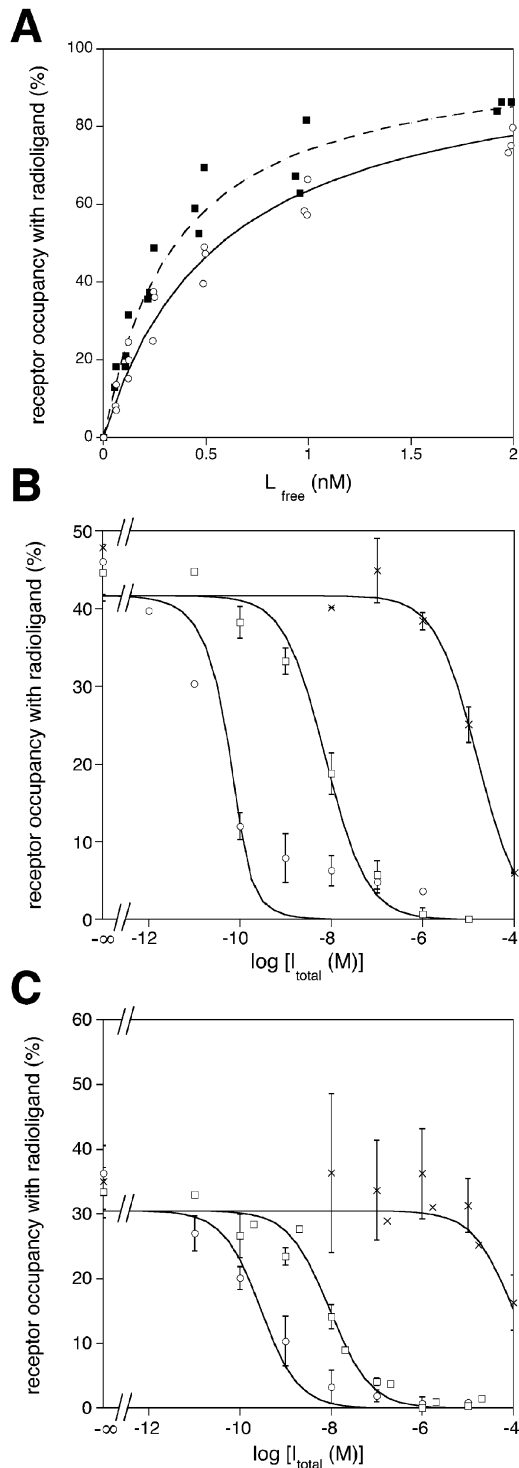


Fig. 3. Ligand binding characteristics of KOR. HEK293T cells were transfected with the receptor fused to the FLAG- (N-terminus) and myc- followed by His-tag (C-terminus). The cells were grown for 20 h in the presence of naloxone in the medium. Binding assays were performed with [3 H]diprenorphine and membrane preparations. (A) Saturation binding assay with WT (filled squares and dashed fitted curve) and FCL (open circles and solid fitted curve). Binding curves for (B) WT and (C) FCL by displacement of the radioligand. A fixed [3 H]diprenorphine concentration of 0.25 nM was used in the presence of various competitors, kappa opioid agonist Dynorphin A(1-13) (circles), antagonist naloxone (squares) and mu opioid agonist DAMGO (crosses).

the FCL receptor was poorly expressed, except when an antagonist was added to the growth medium, in which case

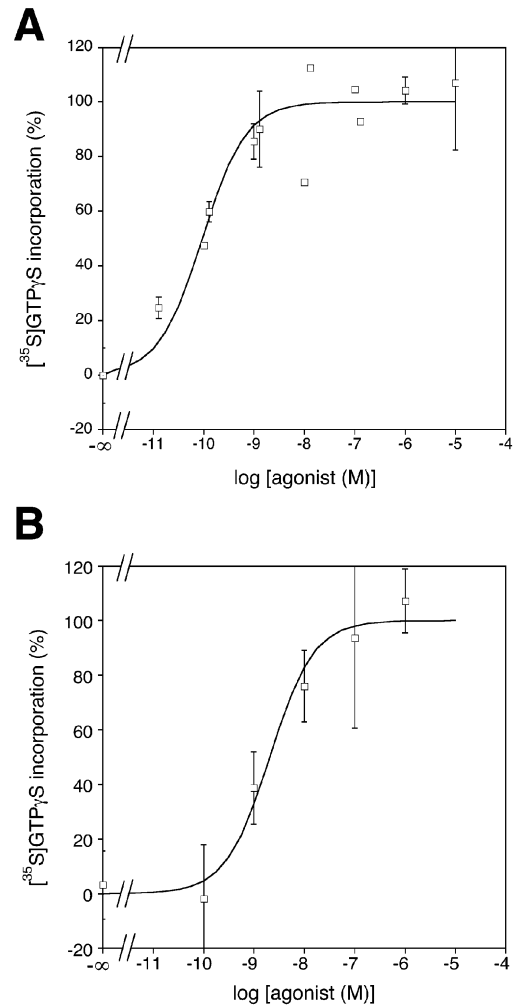


Fig. 4. G-protein coupling for KOR measured by [35 S]GTP γ S binding assays. HEK293T cells were transfected with (A) WT and (B) FCL receptor fused to the FLAG- (N-terminus) and myc- followed by His-tag (C-terminus). The cells were grown for 20 h in the presence of naloxone in the medium. Membranes were prepared and [35 S]GTP γ S binding assays were performed in the presence of various concentrations of the agonist Dynorphin A(1-13) at 30°C for 30 min, as described in Materials and methods.

almost WT levels were reached. In contrast, the effect of the antagonist added to the growth medium was small for the expression of WT KOR. Because of this pronounced effect of naloxone in the growth medium, it was conceivable that the FCL receptor behaves, at least to some degree, as a constitutively active mutant (Li *et al.*, 2001a; McLean *et al.*, 2002). The WT receptor does activate endogenous G-proteins present at the membrane of HEK293T cells in an agonist concentration-dependent manner at 4°C. In contrast, the FCL receptor appears to be totally inactive at 4°C (not shown). However, at higher temperature (30°C), both the WT and FCL receptors activate G-proteins in an agonist concentration-dependent manner (Figure 4). At this temperature, the basal activity of G-proteins is increased, making the relative effect of receptor-mediated activation smaller. However, the basal activity of the G-proteins was at the same level for the WT and FCL receptor, when the assay was performed at 4 or 30°C (data not shown). This indicates that FCL receptor is not a constitutively active mutant.

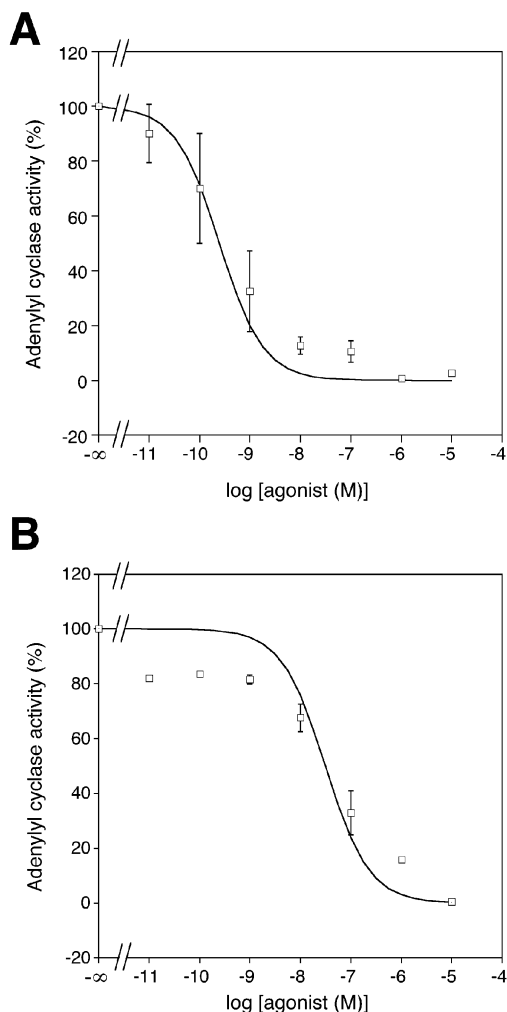


Fig. 5. G-protein coupling for KOR monitored by G_i protein-mediated inhibition of adenylyl cyclase. Stable cell lines expressing (A) WT and (B) FCL receptor in Flp-HEK293TM cells were grown for 20 h in the presence of naloxone and stimulated with forskolin in the presence of various concentrations of agonist Dynorphin A(1–13) at 30°C for 20 min, as described in Materials and methods.

The receptor couples to G_i and therefore an agonist causes an inhibition of adenylyl cyclase activity. Therefore, the accumulation of cAMP in the cell decreases as a function of agonist action. Forskolin is well known to enhance dramatically the activity of adenylyl cyclase and thus greatly facilitates the examination of inhibitory responses. However, forskolin is not suitable for use with transiently transfected cells, because it acts on the entire population of cells, including non-transfected cells (Wong, 1994). We therefore generated isogenic stable cell lines expressing the WT and FCL receptors using the Flp-InTM system (Invitrogen), which is based on the Flp recombinase-catalyzed insertion of the gene of interest into a single integrated Flp recombination target site in isogenic Flp-InTM cells. A polyclonal selection, in which all survivors are pooled for subsequent cell expansion, then becomes possible, because the survivors of the selection can be considered to carry the gene of interest in an identical position in their genome. We selected stable cell lines expressing the receptor from independent transfections and found that these cell lines express the receptor at very similar levels, although at lower levels than observed with transient transfectants (data not shown). A clear

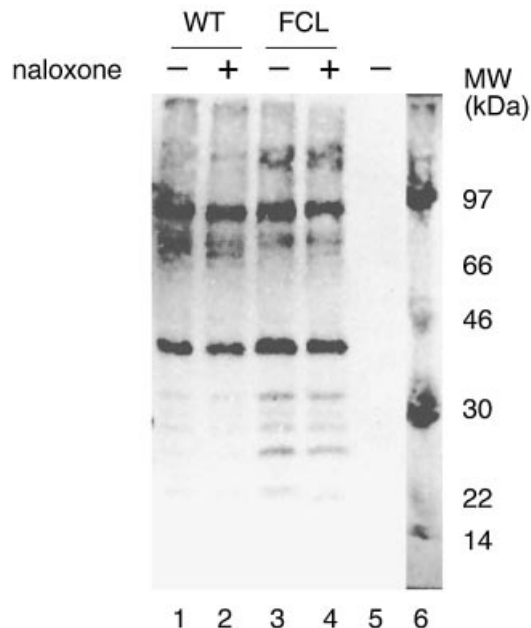


Fig. 6. Expression of KOR in HEK293T cells. HEK293T cells were transfected with WT (lanes 1 and 2) and FCL (lanes 3 and 4) receptor fused to the FLAG- (N-terminus) and myc- followed by His-tag (C-terminus). The cells were grown for 20 h in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of naloxone in the medium. Proteins from whole cell lysates were resolved by SDS-PAGE, blotted on to a PVDF membrane and detected using an anti-His tag antibody. Lane 5 corresponds to cells transfected with the vector without an insert and lane 6 corresponds to High-range Rainbow Molecular Weight Marker (Amersham Biosciences).

decrease in cAMP accumulation with increasing agonist concentration is seen for both WT and FCL (Figure 5). From these experiments, it can be concluded that both the WT and FCL receptors activate inhibitory G-proteins in living cells at 30°C. The EC_{50} of agonist Dynorphin A(1–13) is ~0.1 nM for the WT receptor and 2 nM for the FCL receptor as determined from the [³⁵S]GTP γ S binding assay. The EC_{50} of the agonist is ~0.3 nM for the WT receptor and ~30 nM for the FCL receptor as determined using the inhibition of cAMP accumulation assay. The lower concentration of agonist needed for half-maximum activation of G-proteins by WT, compared with FCL receptor (Figures 4 and 5), correlates well with a stronger agonist binding to WT in radioligand competition assays (Figure 3B and C).

Localization of the WT and FCL receptors

Western blots (Figure 6) show the overall cellular expression levels of the receptors, which are comparable for the WT and FCL receptors and are unaffected by the addition of naloxone into the medium (lanes 2 and 4). The anti-tetra-His antibody is highly selective and when we analyzed a control extract from cells transfected with a vector without an insert, only one very faint band at ~70 kDa was detected, while the receptor bands (43–60 kDa) were absent (lane 5). The major band at an apparent M_r of 43 kDa (lanes 1–4), which corresponds to a full-length receptor without complex glycans, probably indicates receptor retained in the internal cellular stores (endoplasmic reticulum, Golgi apparatus and inclusion bodies). The presence of naloxone in the growth medium also had no significant effect on the expression pattern, e.g. bands corresponding to the full-length receptor, degradation products or any oligomers, as seen on the western blot (lanes 2 and 4). Receptor forms with

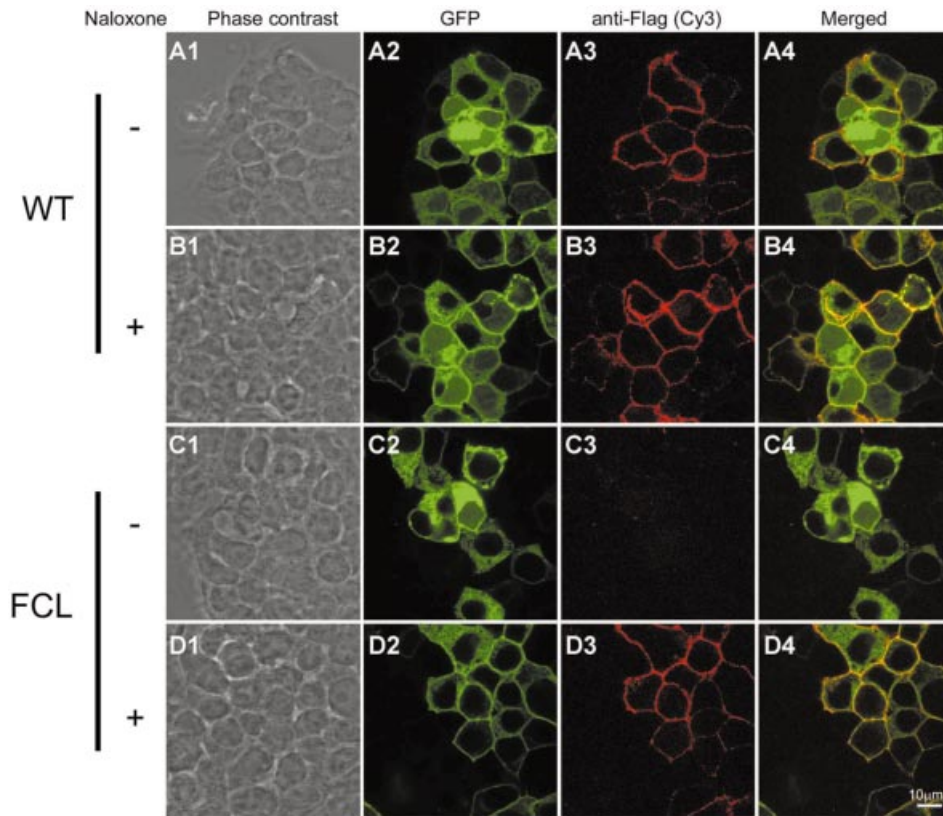


Fig. 7. Localization of KOR in HEK293T cells. FLAG- and eGFP-tagged WT (rows A and B) and FCL (rows C and D) receptors were expressed in HEK293T cells. The cells were stained with an anti-FLAG antibody at 4°C and subsequently with a Cy-3-labeled secondary antibody. Cells were grown either in the absence of naloxone (rows A and C) or in its presence (rows B and D) in the growth medium. Phase contrast (column 1), eGFP fluorescence (column 2), anti-FLAG detection (Cy-3 fluorescence; column 3) and merged images (column 4). Scale bar: 10 μm.

complex glycosylation could not be clearly detected even for the WT, probably owing to the relatively low abundance of receptor correctly inserted into the plasma membrane when compared with the total amount of receptor accumulated inside the cell.

Cytoimmunochemistry provided further information on receptor localization and dynamics. We fused eGFP (Lippincott-Schwartz and Patterson, 2003) to the C-termini of the WT and FCL receptors. Fused eGFP did not significantly affect either [³H]diprenorphine binding (Table I) or inhibition of radioligand binding by Dynorphin A(1–13) or by naloxone or agonist-induced G-protein activation using [³⁵S]GTPγS (data not shown). Similar observations were reported recently for the WT KOR (Ramsay *et al.*, 2002; Schulz *et al.*, 2002). Cells expressing the WT and FCL receptors, each carrying an N-terminal FLAG tag and a C-terminal eGFP moiety, were labeled with anti-FLAG M2 antibody at 4°C for 1 h, conditions under which internalization can be neglected. In these experiments, the WT receptor was present on the plasma membrane (seen both by eGFP fluorescence and by anti-FLAG tag detection) and also in internal stores (seen only by eGFP fluorescence) (images A2 and A3 in Figure 7). Naloxone slightly increased the concentration of WT in the plasma membrane (compare A3 and B3). In contrast, when the FCL receptor was expressed in the absence of naloxone, the antibody did not label the cell surface (image C3). The mutant is, however, detected in the internal cellular stores, by its eGFP fluorescence, at a level similar to WT (image C2). Strikingly, when cells were grown in the presence of naloxone, the mutant

was clearly detectable both on the cell surface and in the internal stores (images D2 and D3). This completely mirrors the effects seen in the ligand-binding experiments (see above) (Figure 2B, Table I). We conclude that naloxone stabilizes the FCL mutant on the cell surface.

A different experimental set-up was also employed (Figure 8), in which cells expressing the N-terminally FLAG-tagged receptor without eGFP were incubated at 37°C for 30 min (promoting internalization) in the presence of anti-FLAG M2 antibody and then fixed. They were later stained with a labeled secondary antibody under conditions where membranes and vesicles were permeabilized. The WT receptor was mainly located on the plasma membrane and there was no remarkable difference between cell growth in the presence or absence of naloxone (Figure 8, images A2 and B2). When naloxone was omitted during the FCL receptor expression, the plasma membrane was not stained. In this case, stained vesicles were observed inside the cells (image C2). However, when these cells were grown in the presence of naloxone, the FCL receptor was also present on the plasma membrane (image D2). Taken together, we infer that the FCL receptor is properly targeted to the plasma membrane, but it does not stay there and is quickly internalized. Naloxone significantly decreases the rate of internalization.

Discussion

We have engineered a mutant of the KOR with 10 exchanged residues. The mutant, devoid of all free cysteines, is functional in agonist and antagonist binding and was shown to activate

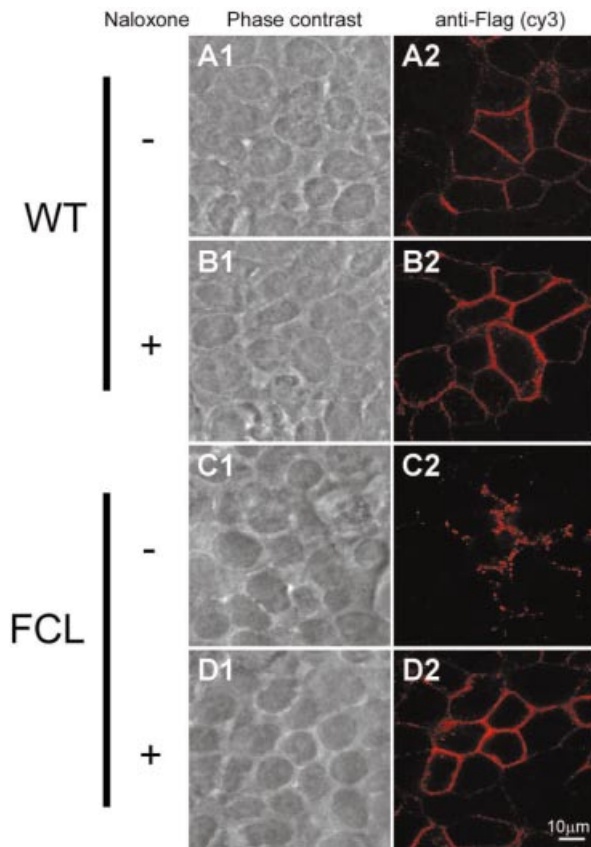


Fig. 8. Internalization of KOR in HEK293T cells. FLAG-tagged WT (rows A and B) and FCL (rows C and D) receptors were expressed in HEK293T cells. The cells were stained with an anti-FLAG antibody at 37°C and subsequently with a Cy-3-labeled secondary antibody in the presence of saponin. Cells were grown either in the absence of naloxone (rows A and C) or in its presence (rows B and D) in the growth medium. Phase contrast (column 1) and anti-FLAG detection (Cy-3 fluorescence; column 2). Scale bar: 10 μ m.

G-proteins for GDP/GTP exchange and to signal by inhibiting adenylyl cyclase. While binding of antagonists was indistinguishable from WT, there are subtle differences in agonist binding, in the temperature dependence of G-protein activation and, most strikingly, in receptor internalization in the absence of agonist. A good correlation was observed for both receptors between radioligand binding and labeling on the cell surface, as detected by fluorescence microscopy, and this provided evidence for pronounced constitutive internalization of the FCL receptor.

When designing the sulfhydryl-free receptor, the first problem was to determine the appropriate substitutions for 10 free cysteines of the WT, which occur in the N-terminal region, the transmembrane helices, the second cytoplasmic loop and the cytoplasmic tail. Typically, cysteine has been replaced by alanine and serine. Cysteine is a hydrophobic residue, which, under certain circumstances, can form a hydrogen bond (Raso *et al.*, 2001), but serine does not seem to be a very suitable replacement because of its high polarity. Nevertheless, many receptors tolerate the replacement of one or even multiple endogenous cysteines with serine, but do not tolerate all substitutions at once (Gaibelet *et al.*, 1997; Gether *et al.*, 1997; Ehrlich *et al.*, 1998; Zeng *et al.*, 1999; Scholl and Wells, 2000; Xu *et al.*, 2000; Ponimaskin *et al.*, 2002). We also found that a

complete cysteine replacement with alanines did not yield a functional molecule, even when the putative disulfide bond is retained. An inspection of the KOR model suggested replacement of cysteines with a hydrophobic residue in the case of C5 and C7, which are likely to be exposed to phospholipids, and our choice was leucine, which is the GPCR family consensus residue in these positions. Threonine is a consensus residue for C10. Consensus residues in the positions of the transmembrane cysteines in KOR are indeed often cysteines (in positions C3, C8 and C9), which indicate the spatial and possibly functional role of cysteine at these transmembrane positions. We cannot conclude that our engineered cysteine substitutions are the optimal ones. Nevertheless, the WT and optimized FCL receptors feature almost the same binding characteristics to the ligands tested and the measured affinities are similar to published binding data for KOR (Goldstein and Naidu, 1989; Meng *et al.*, 1993; Yasuda *et al.*, 1993; Raynor *et al.*, 1994).

However, the FCL receptor has about a 10-fold lower affinity for the agonist Dynorphin A(1–13) compared with WT. Similarly, the EC_{50} of Dynorphin A(1–13) for activation of G-proteins via the FCL receptor is about 20–100 times higher than in the case of the WT. It should be stressed that our aim was to express functionally both WT and FCL receptor at comparable levels for all assays. Hence the [35 S]GTP γ S binding assay was performed with membranes from the cells grown in the presence of naloxone and we also grew the cells prior to the cAMP accumulation assay in the presence of naloxone. We could not characterize FCL receptor that had been expressed in the absence of naloxone in the medium, because there was very little FCL receptor in the plasma membrane under these conditions. There is a dramatic difference in the stabilities of the WT and FCL receptors on the cell surface *in vivo* and this difference can be suppressed by adding naloxone to the growth medium. This phenomenon was observed in both HEK293T and COS-1 cells.

The fact that all of these differences between the WT and FCL receptors are rather subtle suggests that both receptor variants have very similar conformations in both the active and inactive receptor signaling states (Chidiac, 2002), but the inactive conformation of the FCL receptor needs to be stabilized by naloxone. Unstabilized FCL receptor is quickly eliminated from the plasma membrane (Figure 8). It is possible that this is mostly due to a lack of palmitoylation. Note that the C-terminal cysteines C11 and C12, when both substituted to alanine, have only a small effect on receptor expression and that the low expression of FCL receptor and its efficient rescue with naloxone are reflected in the properties of KOR with mutated C4 (Figure 2B). It has been suggested (Chen *et al.*, 1998) that the opioid receptors are not palmitoylated in the C-terminal tail, but instead in the second intracellular loop, where C4 in KOR is located. It is conceivable that this mutation alters the affinity to G-proteins and has important consequences for receptor phosphorylation and internalization, probably involving G protein-coupled receptor kinase and/or β -arrestin, known adaptors of human KOR internalization (Li, J., *et al.*, 2002; Schulz *et al.*, 2002). This behavior is reminiscent of MOR, which has been mutated in the conserved DRY motif, located in the beginning of the second intracellular loop. The mutant MOR was found to be constitutively internalized via G protein-coupled receptor kinase-, β -arrestin-, dynamin-, rab5- and rab7-dependent pathways (Li *et al.*, 2001a). We also cannot completely rule out the possibility that the FCL receptor has a slightly altered oligomerization state

compared with the WT receptor; however, we did not observe any qualitative difference in the receptor expression profiles on western blots. Therefore, we propose that the FCL receptor behaves like the WT receptor with respect to its signaling functions.

Our constructed receptor, devoid of free endogenous cysteines, now opens the door to a number of studies in which these cysteines would have been problematic. For example, the introduction of fluorescent labels at any position in the receptor now becomes possible, to detect conformational changes or local binding events. Many possibilities for directed immobilization become available. Finally, receptor folding is greatly simplified, as the only two remaining cysteines probably form a disulfide bond, but no disulfide scrambling can occur. This strategy may help in the future biophysical characterization of GPCRs.

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