Protein Engineering, Design & Selection vol. 18 no. 3 pp. 153–160, 2005
Published online March 24, 2005 doi:10.1093/protein/gzi012

Engineering and functional immobilization of opioid receptors

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Opioid receptors, like many G protein-coupled receptors (GPCRs), are notoriously unstable in detergents. We have now developed a more stable variant of the μ-opioid receptor (MOR) and also a method for the immobilization of solubilized, functional opioid receptors on a solid phase (magnetic beads). Starting with the intrinsically more stable κ-opioid receptor (KOR), we optimized the conditions (i.e. detergents and stabilizing ligands) for receptor extraction from lipid bilayers of HEK293T cells to obtain maximal amounts of functional, immobilized receptor. After immobilization, the ligand binding profile remains the same as observed for the membrane-embedded receptor. For the immobilized wild-type μ-opioid receptor, however, no conditions were found under which ligand binding capacity was retained. To solve this problem, we engineered the receptor chimera KKM where the N-terminus and the first transmembrane helix (TM1) of wild-type MOR is exchanged for the homologous receptor parts of the wild-type KOR. This hybrid receptor behaves exactly as the wild-type MOR in functional assays. Interestingly, the modified MOR is expressed at six times higher levels than wild-type MOR and is similarly stable as wild-type KOR after immobilization. Hence the immobilized MOR, represented by the chimera KKM, is now also amenable for biophysical characterization. These results are encouraging for future stability engineering of GPCRs.
Keywords: functional immobilization/μ-opioid receptor/opioid receptors

Introduction

Opioid receptors are members of the peptide-binding subfamily of class A of G protein-coupled receptors (GPCRs). Agonists selective for the κ-opioid receptor (KOR) produce an effective analgesia without the substantial side effects (constipation, respiratory depression, vomiting and physical dependence) associated with μ-opioid receptor (MOR)-selective agonists such as morphine (Reece *et al*., 1994). MOR represents a key mediator for a wide range of both beneficial and adverse activities of morphine. Using knockout mice, MOR was shown to be necessary for a reward of and dependence on other drugs of abuse [e.g. Δ^9-tetrahydrocannabinol and ethanol; reviewed elsewhere (Gaveriaux-Ruff and Kieffer, 2002)]. The detailed mechanistic and, ideally, structural understanding of opioid receptors and their interactions with agonists and antagonists are therefore of obvious importance.

Detergent-mediated extraction of receptors from membranes and subsequent purification of the receptor in the presence of detergents are prerequisites for any direct high-resolution study of receptor structure by X-ray crystallography or NMR and even for detailed biochemical characterization of the purified protein (Clark *et al*., 2001; Karlsson and Löfas, 2002; Banères and Parello, 2003; Banères *et al*., 2003; Stenlund *et al*., 2003). Owing to the low stability of most GPCRs in detergent, one could up to now, however, investigate their structure only indirectly and at an unsatisfactory level of detail. The single exception, rhodopsin, may be atypical in some respects and was studied after isolation from its natural source, where it is highly abundant (Palczewski *et al*., 2000). Most antagonistic or agonistic ligands for therapeutic intervention have thus been obtained from whole-cell screening (see, e.g., Conway and Demarest, 2002; Kassack *et al*., 2002; Wise *et al*., 2004).

One possibility is to follow changes of receptor conformation, albeit at low spatial resolution, in real time as a function of ligand-induced receptor activation, e.g. by fluorescence microscopy (Neumann *et al*., 2002). The receptor is first solubilized from the plasma membrane of the expression host and then immobilized on a solid phase. Then a fluorescence label is coupled to a cysteine residue introduced into a desired position in the receptor sequence. However, not even this analysis can be universally applied, as some receptors lose their native structure too fast in detergents.

While detergent extraction and maintenance of activity were possible with KOR, preliminary experiments showed that the homologous MOR was significantly less stable upon solubilization with detergents. We were therefore interested in finding the molecular determinants for their different behavior. We report here the results for hybrid proteins, leading to an engineered MOR with substantially increased expression level and higher stability in detergents. We believe that this approach can be of general utility.

We also present a setup for the functional immobilization of GPCRs and demonstrate it for opioid receptors. Opioid receptors are known to be unstable in detergents [reviewed elsewhere (Simon, 1986, 1991; Smith and Loh, 1991; Li *et al*., 1993)] and we therefore sought an optimal balance between functionality (such as ligand binding) and harsher extraction conditions leading to homogeneity of the immobilized receptor. In order to characterize qualitatively the immobilized receptor, we compared the ligand binding profiles of immobilized and membrane-embedded receptor.

Materials and methods

Materials

Most detergents were obtained from Anatrace. *rac*-2,3-Dihydroxypropyloctyl sulfoxide (OPSO) was purchased from

Bachem, non-detergent sulfobetaines (NDSB series) and Zwittergent 3-14 from Calbiochem, sodium lauroylsarcosine (sarcosyl), sodium cholate, sodium dodecyl sulfate (SDS), Lubrol PX ($C_{12}E_9$) and Nonidet P-40 (NP-40) from Sigma and Triton X-100 and Tween-20 from Fluka. Cholesteryl hemisuccinate (Cat. No. C-6013; CHS; 1% stock solution prepared in 5% CHAPS), the opioid ligands butorphanol (Cat. No. B-9156), naltrexone (Cat. No. N3136), nalbuphine (Cat. No. N-4396) and a protease inhibitor cocktail [Cat. No. P-8340; used at 0.3% (v/v) final concentration] were supplied by Sigma and Dermorphin (Cat. No. H-2565) by Bachem. Sources of all other chemicals used in this study are indicated elsewhere (Ott *et al*., 2004).

Construction of receptor variants

Genes of all opioid receptor variants (wild-types and chimeras) were cloned in frame with an N-terminal FLAG-tag and C-terminal myc- and His-tags. The cloning of the wild-type KOR (Swiss-Prot No. P34975) and FCL KOR (Ott *et al*., 2004) genes into the vector pcDNA3.1a(–)/myc/His was described previously (Ott *et al*., 2004). The gene for the wild-type (wt) MOR (Swiss-Prot No. P33535) was inserted into the vector pcDNA3.1a(–)/myc/His with the N-terminal FLAG-tag (MDYKDDDDK), resulting in a short linker (Gln–Leu) between the FLAG-tag and the receptor. We designed three receptor chimeras for the MOR, where either the N-terminal extracellular part or the first transmembrane helix (TM1) or both parts together were exchanged for the homologous sequences of the KOR. The decisions on the domain boundaries were made based on the predictions in the SWISS-PROT Annotated Protein Sequence Database entry files for the receptors (see Results). For the chimera KKM, residues Pro4–Thr88 of the KOR were N-terminally fused to residues Lys98–Pro398 of the MOR; for the chimera KMM, residues Pro4–Ile58 of KOR were N-terminally fused to residues Met65–Pro398 of the MOR and for the MKM chimera, residues Pro59–Thr88 of KOR replaced residues Met65–Thr97 in MOR.

Cell culture, transient transfection, radioligand binding assays, membrane preparation, western blot analysis and [^{35}S]GTPγS binding assay

These techniques were performed essentially as described previously (Ott *et al*., 2004). We routinely centrifuged a stock solution of [^{35}S]GTPgS (50 nM in Tricine buffer, pH 7.4, with 10 mM DTT) for 1 h at 4°C at 90 000 r.p.m. (400 000 *g*) in a Beckman Optima TLX ultracentrifuge in order to remove any insoluble impurities which might interfere with the assay.

Immobilization of receptor on paramagnetic beads

Cell pellets of transfected HEK293T cells (from one well in a 24-well plate) were resuspended in 100 µl of immunoprecipitation (IP) buffer [TBS with a CHAPS–cholesteryl hemisuccinate (CHS) mixture (8.3/1.7 mM), 10% glycerol and 10 mM $MgCl_2$] and incubated for 10 min on ice. The suspension was centrifuged for 10 min at 3300 r.p.m. (1100 *g*) in an Eppendorf 5417R centrifuge at 4°C. The supernatant was mixed with the anti-myc monoclonal antibody 9B11 [Cell Signaling, Cat. No. 2276; diluted 1:200 (v/v)] and paramagnetic Dynabeads carrying protein G (Dynal, Cat. No. 100.03; 10 µl/sample). The suspension was shaken (1400 r.p.m.) for 30 min at 30°C or for 1 h at 4°C. By using a magnetic rack, beads were washed four times with 300 µl of IP buffer at room temperature (RT) (with 3 min incubations between the washes). For western blot analyses, beads were resuspended in 30 µl of 2.5-fold concentrated SDS–PAGE loading buffer and incubated for 10 min at 42°C. For the binding assay, beads were resuspended in 200 µl of TBS containing CHAPS–CHS (1.7/0.33 mM) and incubated with radioligand in the presence or absence of non-labeled competitor for 1 h at RT. Separation of free and bound ligand was performed on a filtration vacuum manifold (Millipore) as described elsewhere (Ott *et al*., 2004) with the following modifications: after the filtration, beads on glass-fiber filters were transferred into 20 ml scintillation vials (Ratiolab), scintillation cocktail (5 ml) was added and 16 h later the samples were measured in a BETAmatic β-counter (from Kontron).

When various detergents were tested, the complete detergent-containing IP buffer including protease inhibitor cocktail was also used for the binding assay. Extraction of receptor from cells by using sarcosyl (0.5%), SDS (0.15%), sodium deoxycholate (0.5%) and hexyl maltoside (2%) resulted in viscous cell lysates. Addition of DNase I (Roche) and a short incubation for 15 min at 30°C partially helped to reduce the viscosity of the lysate. The viscosity could not be reduced in sodium deoxycholate (0.5%) lysate and therefore we excluded this detergent from screening. Sarcosyl (0.5%) and particularly CHAPSO (0.6%) were prone to precipitation at 4°C. Therefore, we used the IP buffers containing these detergents at RT for the washing steps in the radioligand binding assays. When the receptor was immunoprecipitated in digitonin (1%), IP buffer with 1 M NaCl was used.

The relative stability of receptor variants in detergents was determined as the ratio of specific ligand binding measured on beads with the immobilized receptor and on whole cells. The relative receptor stabilities were determined only for conditions where receptor variants were expressed in the absence of ligand in the growth medium.

Results

Engineering a stabilized μ opioid receptor

We wished to establish a general strategy to solubilize GPCRs in detergent and immobilize them for functional studies. In preliminary experiments we observed that the rat KOR lost its activity in detergent at a much slower rate than the highly homologous MOR. At the sequence level, MOR and KOR show 57% pair-wise sequence identity with the highest divergence at the N-terminus (20% identical amino acid positions). We wished to exploit these observations to find a strategy for stabilizing MOR.

There are several reports that the N-terminus, including the first transmembrane helix (TM1), of opioid receptors is not important for receptor specificity towards subtype-selective ligands (Onogi *et al*., 1995; Wang *et al*., 1995; Ide *et al*., 2000). To test whether the N-terminus is responsible for the higher stability of immobilized KOR in detergent, we engineered a receptor chimera KKM, where the N-terminus and TM1 up to the first cytoloop originates from wt KOR and the rest of the sequence is taken from wt MOR (Figure 1).

We first determined the ligand binding profiles of the constructs MOR, KOR and KKM in membrane preparations of HEK293T cells expressing the receptor variants.

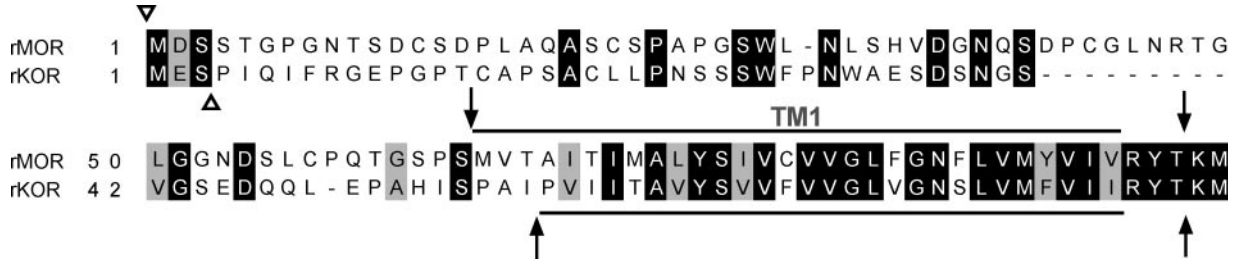

Fig. 1. Sequence alignment of the relevant region in the μ- and κ-opioid receptors from rat. The aligned residues of the N-terminal extracellular domain and transmembrane helix 1 (TM1) up to the first cytoplasmic loop are shown. The overscored and underscored regions represent the predicted TM1 according to the SWISS-PROT Annotated Protein Sequence Database entries for the receptors. For the construction of the chimeric receptors, we used the cutting sites indicated by arrows. The N-terminal FLAG-tag was introduced at the sites marked by an open triangle. The sequences were aligned using Clustal W (version 1.81) followed by manual refinement. SWISS-PROT accession numbers for the sequences are P33535 for rMOR and P34975 for rKOR.

Table I. Ligand binding profiles of membrane-embedded (M) or immobilized (I) receptor variants[a]

Ligand	Receptor variant							
	wt MOR		KKM		wt KOR		FCL KOR	
	M	I	M	I[e]	M	I[f]	M	I[g]
Diprenorphine	0.3 ± 0.2	ND[c]	0.4 ± 0.2	0.07 ± 0.04	0.4 ± 0.1[b]	0.12 ± 0.02	0.6 ± 0.1[b]	0.5 ± 0.3
U-50488	>1000	ND	>1000	>1000	7 ± 7	5.3 ± 0.4	70[d]	70 ± 60
DAMGO	30 ± 10	ND	58 ± 1	70 ± 50	>1000[b]	>1000	>1000[b]	>1000
Dermorphin	40 ± 30	ND	60 ± 30	24 ± 6	>1000	>1000	ND	ND
Naloxone	3 ± 1	ND	2 ± 1	0.9 ± 0.4	4 ± 2[b]	4 ± 1	6 ± 2[b]	10 ± 7
Butorphanol	0.5 ± 0.1	ND	0.6 ± 0.2	1.9 ± 0.4	0.23 ± 0.03	0.7 ± 0.4	9[d]	8 ± 11

[a]Equilibrium dissociation constant for [^{3}H]diprenorphine and equilibrium inhibition constants for all other tested ligands are expressed in nM and were determined as described previously (Ott *et al*., 2004).
[b]Data determined previously (Ott *et al*., 2004).
[c]ND, not determined.
[d]Value obtained from one independent experiment.
[e]Receptor was expressed in the presence of naltrexone (20 μM) in the growth medium.
[f]Receptor was expressed in the presence of naloxone (20 μM) in the growth medium.
[g]Receptor was expressed in the presence of butorphanol (20 μM) in the growth medium.

The equilibrium binding constants are summarized in Table I. Notably, the wt MOR and the chimera KKM show similar pharmacological profiles for the ligands tested (opioid antagonists [^{3}H]diprenorphine and naloxone, the non-selective agonist butorphanol, the κ-selective agonist U-50488 and μ-selective agonists DAMGO and dermorphin). Since all three constructs showed comparable affinities for the radioligand [^{3}H]diprenorphine, we decided to perform all further competitive binding assays at non-saturating concentrations of the radioligand (slightly above K_D), where we measured high signal-to-noise ratios. We could thus avoid working at saturating concentrations, which would give rise to high non-specific binding signals. In this experimental setup, the presence of functional receptor (either on whole cells or in solubilized, immobilized form) is directly determined via its capacity to bind radioligand.

We compared relative expression levels of wt KOR, wt MOR and the KKM chimera by using whole cell binding assays with the radioligand [^{3}H]diprenorphine (1 nM). MOR was functionally expressed at slightly lower levels, i.e. ~50% of the KOR expression. Interestingly, the chimera KKM is expressed at a ~3-fold higher level than KOR and hence at ~6-fold higher levels than MOR (Figure 2).

In order to assess a coupling of the receptor variants to G-proteins, we used the [^{35}S]GTPγS binding assay with the membrane preparations. The potency of the μ-selective agonist dermorphin to activate G-proteins via the receptor is very similar for the wt MOR and the chimera KKM (half-maximal activity ~20 nM; Figure 3). Taking these data together, the chimera KKM behaves like the wt MOR in all functional assays performed.

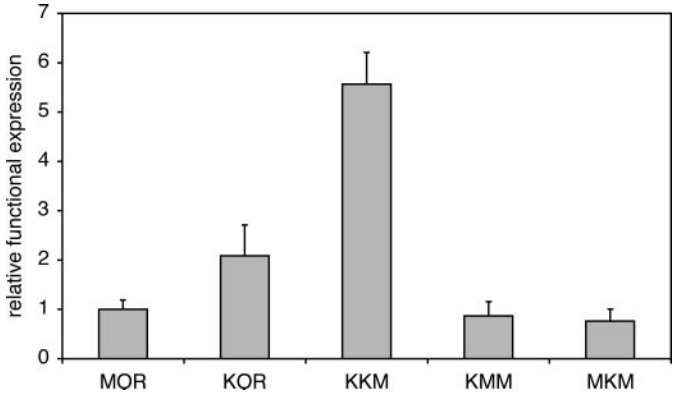

Fig. 2. Functional expression of receptor variants in HEK293T cells. Specific binding of receptor variants is given in relative numbers, with the wt MOR arbitrarily set to 1. Transfected cells were grown in the absence of receptor ligand in the medium. The binding of antagonist [^{3}H]diprenorphine (1 nM) was measured on whole cells. This experiment represents the average of five independent receptor expression experiments for MOR, KOR and KKM and three independent receptor expression experiments for KMM and MKM (duplicate or triplicate samples were analyzed in each independent experiment).

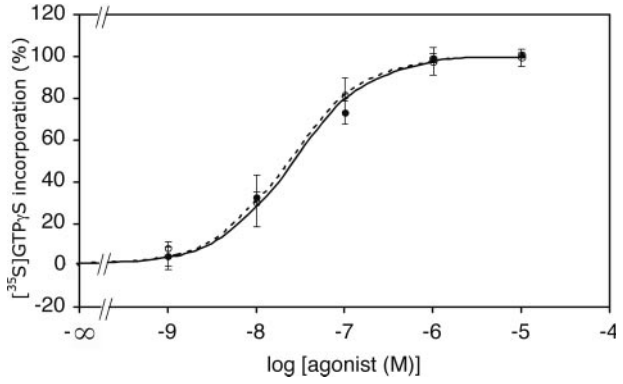

Fig. 3. G-protein coupling for the wt MOR and chimera KKM measured by [^{35}S]GTPγS binding assays. Membranes were prepared from cells expressing receptor in the presence of naloxone and [^{35}S]GTPγS binding assays were performed in the presence of various concentrations of the agonist dermorphin as described in Materials and methods. 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 dermorphin as a maximum (100%). Data are represented for the wt MOR, by filled circles and the solid fitted curve and for the chimera KKM by open circles and the dashed fitted curve.

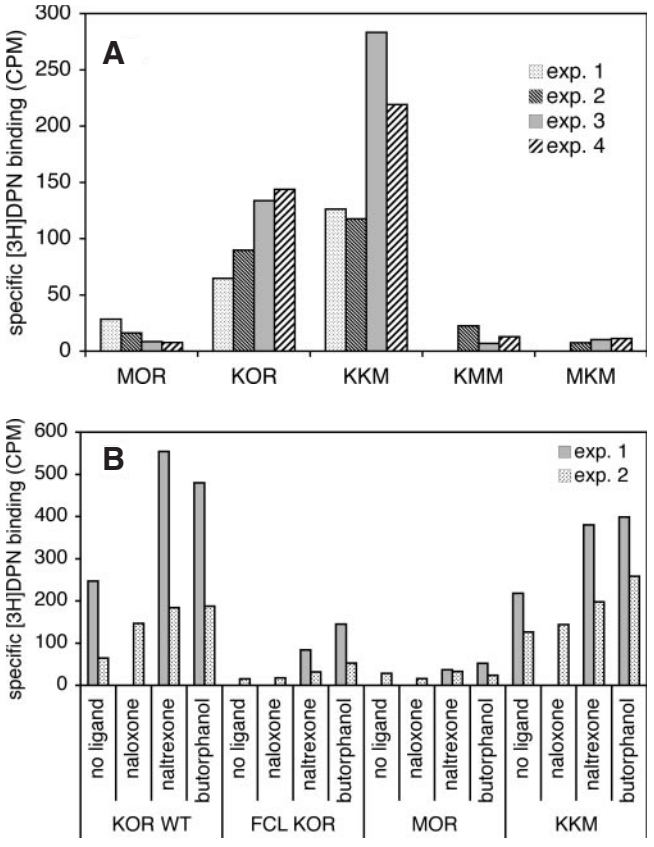

Fig. 4. Functional immobilization of opioid receptors. Receptor, expressed in the absence or presence of various ligands in the growth medium, was extracted from HEK293T cells in CHAPS–CHS, immobilized on paramagnetic beads and tested for specific [^{3}H]diprenorphine binding as described in Materials and methods. Bars of different texture represent independent experiments. (**A**) Receptor variants were expressed in the absence of ligand in the growth medium. (**B**) Receptor variants were expressed in the absence or presence of various ligands in the growth medium. The effect of ligands in the growth medium on the functionality of the immobilized receptor was tested in two independent experiments (each with duplicate samples), with the exception of naloxone, which was tested in one independent experiment with duplicate samples only.

We then investigated the solubilization and immobilization of KOR, MOR and KKM with the strategy described in the Materials and methods section. We found that by using the optimized solubilization and immobilization format, both KOR and MOR can be efficiently immobilized on the beads, as detected by western blots (not shown). KOR was immobilized in an active form, whereas the immobilized MOR was almost non-functional (Figure 4A). In sharp contrast, the KKM chimera was immobilized in an active form at even higher levels than wt KOR (Figure 4A).

The substantial difference in the yield of immobilized receptor between the engineered receptor form KKM and MOR can be attributed to two factors, expression level and amount of functional protein in detergent after solubilization: KKM was expressed in cells at a substantially higher level (~6-fold) than MOR (Figure 2) and the relative stability of KKM (7 ± 2%, n = 4), i.e. the percentage of receptor molecules remaining active after immobilization, was also higher than the relative stability of MOR (4 ± 3%, n = 4). For comparison, the relative stability of KOR was 11 ± 2% (n = 4). These data indicate that it was indeed possible to improve a wild-type GPCR by engineering for both higher cellular expression and stability in detergent.

We then wanted to clarify whether the favorable properties of KKM arise from the N-terminal region and/or TM1 of the receptor. For this purpose, we separately exchanged the N-terminus and TM1 between KOR and MOR. We created the constructs KMM and MKM, where the first, second and third letters denotes the origins of the N-terminal domain, TM1 and the remainder of the protein, respectively (Figure 1).

The equilibrium binding constants for radioligand binding to KMM and MKM (measured with membrane preparations) was comparable to wt MOR ($K_D \approx 0.3$ nM; data not shown), consistent with the expectation that the major part of the receptor determines the binding site. The expression of KMM and MKM was determined in parallel with the receptors MOR, KOR and KKM using binding assays on whole cells. We found that the expression levels of KMM and MKM were as low as for wt MOR and that these chimeras could not equal the expression levels of the KKM construct (Figure 2).

Furthermore, the immobilized chimeras KMM and MKM were almost non-functional, similarly to the wt MOR (Figure 4A). Hence we cannot narrow down the favorable effect seen in KKM to particular residues or receptor domains. However, it appears that the simultaneous exchange of the N-terminal domain and the TM1 resulted in a very favorable situation and only the KKM chimera of the MOR could be functionally immobilized with an appreciable yield.

GPCR solubilization and immobilization

We developed a strategy of GPCR solubilization and immobilization as a basis for functional and mechanistic receptor characterization *in vitro*. In order to characterize the functionality of the solubilized receptor, we found it important to compare the ligand binding affinities of receptor variants determined in the presence of detergent with those determined for the receptor embedded in the native membranes (whole cell binding assay). If both could be done on a solid phase, binding assays would become greatly simplified. Our goal was to immobilize the solubilized opioid receptors in highly enriched

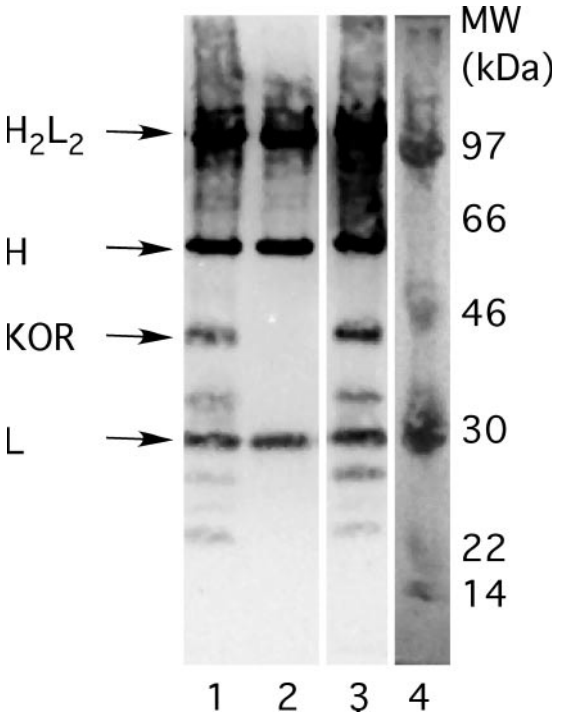

Fig. 5. Immunoprecipitation of KOR variants. KOR fused to FLAG-, myc- and His-tags was extracted from transfected HEK293T cells and immobilized on paramagnetic beads by using the anti-myc antibody in the IP buffer containing CHAPS (10 mM). Residual proteins on the beads were then analyzed by SDS–PAGE and western blots by using the anti-tetra-His antibody as described in Materials and methods. Results obtained with the cells transfected with wt KOR (lane 1), no DNA (lane 2) or KOR FCL (lane 3) are shown. Lane 4 corresponds to Rainbow marker RPN756 (Amersham). Several bands on the blot correspond to various forms of the anti-myc antibody (depicted by arrows), such as reduced and dissociated light (L) or heavy (H) chains or whole IgG (H_2L_2). The anti-myc antibody used for immobilization is of murine origin and is therefore detected on the blot by the secondary (anti-murine) antibody used for detection of the primary murine anti-tetra His antibody. The band, which corresponds to the full-length non-glycosylated form of KOR (~43 kDa), is also indicated by an arrow. All other protein bands in lanes 1 or 3 that are not observed in lane 2 correspond to degradation products of the receptor.

form in an oriented and functional fashion. We carried out immobilization studies at a temperature of 30°C, which is suitable also for fluorescence and signaling studies.

Preliminary experiments had indicated that the anti-myc tag antibody could efficiently immunoprecipitate KOR, carrying FLAG- and myc-tags, in buffer containing the detergent CHAPS (Figure 5), and also under relatively harsh conditions such as the radioimmunoprecipitation assay (RIPA) buffer (not shown). Therefore, we decided to use the anti-myc antibody for further optimization of the IP procedure. It was necessary to test a variety of detergents for maximal receptor extraction and maintenance of activity and to investigate the influence of stabilizing ligands during the solubilization and immobilization steps.

Choice of detergent. First, we expressed wt KOR in HEK293T cells in order to test the suitability of a variety of detergents for the solubilization and immobilization of the receptor in functional form. CHAPS was previously reported as a suitable detergent for the solubilization of opioid receptors when solubilization was followed by receptor reconstitution in liposomes (Simon, 1986; Zukin and Maneckjee, 1986; Smith and Loh, 1991; Gioannini *et al*., 1993; Fan *et al*., 1995). When screening for a suitable detergent, we found that 10 mM CHAPS was the sole condition under which wt KOR was immobilized in a highly enriched and functional form. While the use of other detergents for the IP also resulted in receptor enrichment (Figure 6), specific ligand binding was not detected or was significantly lower than for the conditions with CHAPS (data not shown). These inactivating detergents tested included sarcosyl (0.5%), sodium cholate (0.5%), Cymal-5 and Cymal-6 (both 0.5%), octyl glucoside (1%), Triton X-100 (0.05%), Nonidet P-40 (1%), LDAO (0.1%), octyl and nonyl maltoside (both 1%), sucrose monodecanoate (0.5%), $C_{12}E_9$ (0.2%), HEGA-10 (0.5%), digitonin (1%), Fos-choline-14 (0.14%), Fos-choline-16 (0.2%), dodecyl maltoside (0.3%) and Zwittergent 3-14 (0.2%).

Table II. Ligand binding detected for KOR wt immobilized in various reagents

Detergent	Concentration of detergent (%)	Relative specific binding (%)
CHAPS	0.6	100
None	–	200 ± 60
NDSB-195	0.5	300 ± 60
NDSB-195	2	170[a]
NDSB-201	0.5	270 ± 60
NDSB-211	0.5	320 ± 40
NDSB-211	2	180[a]

[a]Value obtained from one independent experiment.

When we performed receptor IPs in the absence of detergent or in the presence of hexyl maltoside (2%) or non-detergent sulfobetaines (NDSB-195, NDSB-201 or NDSB-211; all at 0.5%), the measured specific ligand binding was higher than what we observed for IP in CHAPS (Table II). However, receptor bands (representing the full-length and degradation products of KOR) were very faint on western blots (Figure 6A, lane 5) and preparations of the immobilized receptor were contaminated with other cellular proteins (Figure 6B, lane 5). These results indicate that membrane fragments were deposited on the beads and that these reagents were not able to solubilize the receptor sufficiently from membranes.

When we used OPSO (1%), dodecyl maltoside (0.03%), Fos-choline-14 (0.014%) or Zwittergent 3-14 (0.02%) at concentrations only 2–3 times higher than their theoretical critical micelle concentration (CMC), receptor bands (representing the full-length and degradation products of KOR) were also very faint on western blots (results were comparable to Figure 6A, lane 5) and preparations of the immobilized receptor were again contaminated with many cellular proteins (results were comparable to Figure 6B, lane 5). Furthermore, no functional receptor was detected in these preparations. Conversely, some detergents result in a prominent receptor band (shown for LDAO as an example, Figure 6B, lane 6), but no activity way found (see enumeration of inactivating detergents above). These results indicate that most detergents were deleterious for receptor function, some even at low concentrations which were not even sufficient for the complete release of receptor from membranes into detergent micelles.

CHS in a mixture with CHAPS and dodecyl maltoside was successfully used for the solubilization of the neurotensin receptor (Tucker and Grisshammer, 1996). Because dodecyl maltoside is deleterious to KOR (see above), we tested a mixture of CHAPS (0.5%) and CHS (0.1%) for the IP of wt KOR.

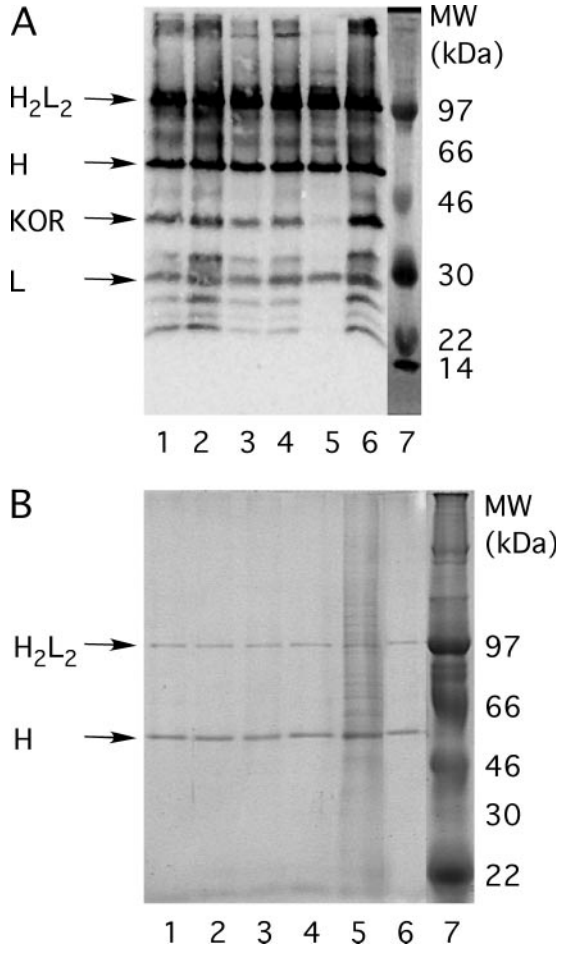

Fig. 6. Immunoprecipitation of wt KOR in various detergents. KOR extracted from transfected HEK293T cells was immobilized on paramagnetic beads by using the anti-myc antibody. Residual proteins on the beads were then analyzed by SDS–PAGE and western blot by using the anti-tetra-His antibody as described in Materials and methods. Various detergents were used for IP of the receptor and only a subset is shown. For a full account, see the text. (**A**) Western blot; (**B**) SDS–PAGE gel stained with Coomassie Brilliant Blue. Lane 1, CHAPS (0.6%); lane 2, CYMAL-5 (0.5%); lane 3, octyl glucoside (1%); lane 4, Nonidet P-40 (1%); lane 5, no detergent; lane 6, LDAO (0.1%); lanes A7 and B7, Rainbow marker RPN756 (Amersham).

Interestingly, the addition of CHS to the CHAPS-containing IP buffer resulted in a 3-fold increase in the yield of functional receptor. The purity of the receptor immobilized in CHAPS–CHS or only CHAPS did not differ (data not shown). We therefore selected the detergent mixture CHAPS–CHS for the setup of the IP procedure also for other opioid receptor variants. By using CHAPS–CHS, we achieved the best compromise between wt KOR homogeneity and functionality.

As described above, MOR was immobilized in CHAPS–CHS only in non-functional form. The same observation was made for the detergents Cymal-5 (0.5%) and dodecyl maltoside (0.3%), where no specific ligand binding to MOR was detected. In contrast, IP of the chimera KKM led to the immobilization of receptor in functional form, and in CHAPS–CHS about 3-fold more functional receptor was obtained than by IP in CHAPS alone. The chimera KKM immobilized in dodecyl maltoside (0.3%) or Cymal-5 (0.5%) was not functional (data not shown).

Choice of ligand. The addition of ligand to the receptor before solubilization can have two effects. First, when it is added during cell growth, it may lead to an improved receptor expression, possibly by preventing receptor internalization. Such an effect was seen for the FCL (free-cysteine-less; a receptor devoid of all free cysteines) mutant of KOR (Ott *et al*., 2004) and also for other receptors (Li *et al*., 2001; McLean *et al*., 2002). Second, it may stabilize the receptor during the solubilization step, when the ligand is added to the cells before detergent addition. Favorable effects were observed for MOR (Weems *et al*., 1996) and endothelin B receptor (Doi *et al*., 1997). Our intention was to keep a maximal amount of receptor occupied with ligand in order to stabilize the receptor during solubilization. For this reason, we routinely used mild washing conditions for the transfected cells during the cell harvest (three washes with PBS at 4°C with ~2 min incubations between the washes). Provided that the ligand has a high (subnanomolar) affinity to its receptor and hence a very slow dissociation rate, it may block to some extent the access of radioligand to the receptor during the following binding assay. Very long incubations would be necessary to remove the bound ligand from the binding site on the receptor. Binding assays performed on whole cells grown in the presence of ligand may therefore underestimate the true receptor expression. Therefore, it is very difficult to make statements about the relative stability of receptor variants that have been exposed to ligands before solubilization. Nevertheless, one can measure the beneficial effect of various ligands on increasing the yield of functional immobilized receptor, being aware that these measurements may underestimate the true contributions of ligands on expression, as the effects are partially masked.

We tested the effect of naloxone and other ligands, present in the growth medium (at a concentration of 10 μM) during receptor expression, on the yield of the functional receptor immobilized in the detergent mixture CHAPS–CHS. We observed that the wt KOR and the chimera KKM can be immobilized in functional form in the CHAPS–CHS mixture, even when expressed in the absence of ligand. Nevertheless, the presence of various ligands during receptor expression leads to an increase in the observed amount of active, solubilized receptor. The ligands naloxone, naltrexone and butorphanol are most beneficial for increasing the amount of functional immobilization of wt KOR. Similarly, the ligands naltrexone, U-50488 and butorphanol improved most significantly the amounts of the functionally immobilized chimera KKM (Figure 4B). The highest amounts of functionally immobilized FCL KOR were observed when the ligands naltrexone or butorphanol were present in the growth medium. Nevertheless, it is obvious that the yield of the functional immobilized FCL KOR is significantly lower than for the wt KOR (Figure 4B). In contrast, none of the tested ligands present during receptor expression significantly stabilized the wt MOR during the immobilization (Figure 4B).

Ligand-binding characteristics of immobilized receptor variants

In order to assess fully the effect of solubilization in CHAPS–CHS on the functionality of immobilized receptor, we determined the ligand binding affinities of the immobilized receptor variants wt KOR, FCL KOR and chimera KKM to various ligands. Remarkably, for all receptor variants, there was no substantial difference in the ligand binding profile between the membrane embedded and the immobilized forms (Table I). Taken together, these results suggest that the receptor variants

wt KOR, FCL KOR and the chimera KKM are functional in all respects tested after immobilization in detergent-solubilized form.

Discussion

Stability engineering for GPCRs

KOR and MOR are highly homologous (about 60% identity), with the highest divergence occurring at the N-terminus of the receptors. However, we observed significant differences in their stability in detergent-solubilized form. Receptor chimeras have been used to elucidate sequence–function relationships for many GPCRs; however, the stability in detergent has not been investigated.

In the present study, we constructed the chimera KKM, where the N-terminus up to the first cytoloop originates from wt KOR and the rest of the sequence is taken from wt MOR (Figure 1), and we observed that KKM shows substantially increased expression levels and enhanced stability in detergent when compared with MOR. The ligand binding profiles of immobilized and membrane-embedded KKM do not differ (see above) and the maintenance of ligand binding affinities was also found for the immobilized KOR. Our study points to a yet unexplored receptor region for possible GPCR engineering. Based on the vast amount of mutagenesis data for opioid receptors (Bot *et al*., 1998; Lu *et al*., 1998; Law *et al*., 1999; Mollereau *et al*., 1999; Ide *et al*., 2000; Feng *et al*., 2001; Scearce-Levie *et al*., 2001; Decaillot *et al*., 2003; Tanowitz and von Zastrow, 2003; Wang *et al*., 2003) and on GPCR structural models (Pogozheva *et al*., 1998; Chavkin *et al*., 2001; McFadyen *et al*., 2002; Visiers *et al*., 2002; Archer *et al*., 2003; Mirzadegan *et al*., 2003), the receptor N-terminal region does not seem to be directly involved in the ligand binding and/or interaction with G-proteins; however, its alteration might result in significantly increased expression and/or stability of the receptor, for instance in detergents. As the chimeras with either only the N-terminal region or only TM1 taken from KOR into MOR did not improve these properties, we have as yet no molecular-level explanation for the beneficial effect of the KKM mutant. Receptor expression can be influenced by the translocation of the N-terminal tail (Andersson *et al*., 2003), which is longer in MOR than in KOR (Figure 1), and one may speculate that the N-terminal region in such receptors, which do not have a cleavable signal sequence, affects their membrane insertion and/or degradation. However, additional effects within the protein must mediate the greater robustness of KKM in detergent after solubilization, especially when compared with MOR. The exchange of only the extracellular N-terminal region did not improve the properties, possibly since the point of junction we chose may have led to molecules with incorrect helical length for both chimeras KMM and MKM. Furthermore, we have engineered an N-terminal FLAG tag in front of all receptor variants studied here. It is possible that this tag enhances the intrinsic difference in the effect of N-terminal regions on the receptor expression. The most important result is, however, that KKM can be immobilized in functional form with ~20-fold higher yield than wt MOR under the same conditions (Figure 4A). This is certainly encouraging for further GPCR engineering.

We do not yet know the true state of the receptor immobilized on the beads. Especially, we cannot state whether the functional, immobilized receptor is monomeric or oligomeric. Even though detergent concentrations above the CMC were used, we also cannot present direct evidence that the immobilized receptor is exclusively in a micellar state. However, the lack of activity of MOR and some other receptor variants in the immobilized state may actually be taken as supporting evidence that the immobilized receptor is probably not embedded in small patches of remaining lipid bilayers. All tested receptor variants (wt MOR, KMM, MKM, KKM, wt KOR, KOR FCL) were clearly functional in the cell or in membrane preparations.

There are reports on the construction of chimeras which resemble our chimeras. However, the stability in detergents has not been investigated for any of them. In two studies, the whole N-terminal region including the first TM helix originated from the δ-opioid receptor (DOR) and the rest of the sequence from MOR and the ligand-binding profile of the chimera is almost identical with the profile of wt MOR (Onogi *et al*., 1995; Wang *et al*., 1995). In another report, two chimeras were constructed where again the whole N-terminal region including the first TM helix originated either from DOR or from KOR and the rest of sequence from MOR (Ide *et al*., 2000). No significant deviation from the ligand-binding profile of the wt MOR was observed for the two chimeras. These reports, together with the present study (Table I), indicate that the protein sequence of the N-terminal part of the MOR is not important for the specificity of ligand binding for the ligands under study.

Solubilization and immobilization of opioid receptors

We also present here an optimized immobilization procedure, which allows biophysical characterizations of opioid receptors in a highly enriched and active form. The investigation of ligand binding to an immobilized receptor has many advantages over other binding assays with solubilized receptors in solution, which require the subsequent separation of bound from unbound ligand. Although this is easily achieved with filtration in the case of membrane fragments, it is much less straightforward in the case of solubilized receptor. Therefore, it had not been easy to troubleshoot the lack of binding signal for unknown receptors with such assays, as this could come either from loss of receptor activity upon solubilization or problems with filter-binding assays. However, the immobilization of solubilized receptors to magnetic beads circumvents this problem and makes it convenient to handle the solubilized receptor for binding assays and thus allows one to test the influence of detergents and other factors on stability.

Most GPCRs are not stable in detergents, but the extraction of receptor from the membrane by using detergent is a prerequisite for any biophysical study of the receptor structure. The solubilized and purified MOR had been reported to have significantly reduced (~1000-fold) affinity for opioid agonists (Simon, 1991; Ofri *et al*., 1992; Gioannini *et al*., 1993). The exact molecular reasons for the loss of GPCR function in detergents are not known and have been attributed to a dissociation of lipids (Lagane *et al*., 2000; Garavito and Ferguson-Miller, 2001; Lee, 2003) and/or G-proteins (Ofri *et al*., 1992; Fan *et al*., 1995; Stanasila *et al*., 1999) from specific sites on the receptor or to the disassembly of receptor oligomers in the presence of detergent or to receptor denaturation and aggregation because of water access to the receptor in detergent micelles. Protein immobilized on a solid phase is much better protected from aggregation (Li *et al*., 2004). We decided to

immobilize opioid receptors on paramagnetic beads, as they were successfully used previously for the efficient immobilization of other GPCRs, such as chemokine receptors CCR5 (Mirzabekov *et al*., 2000) or CXCR4 (Babcock *et al*., 2001) in an active form. In this case, however, supported lipid bilayers were used, rather than the detergent-solubilized form used in our study.

There are reports about immobilization of opioid receptors in functional form on a protein A-coated Sepharose resin by using an anti-MOR antibody (Chalecka-Franaszek *et al*., 2000) or on an artificial phospholipid-monolayer support (Beigi and Wainer, 2003); however, evidence that the receptors were completely extracted from the lipid bilayers prior or during the immobilization was not presented.

We have also shown here that it was possible to immobilize the KOR devoid of all free cysteines (FCL receptor) in functional form. The KOR FCL had been designed for mapping of the conformational changes in KOR molecule by using fluorescence microscopy (Ott *et al*., 2004).

Our optimized method for the oriented immobilization of highly enriched opioid receptors can find application not only in studies of receptor conformational changes (by using site-specific labeled receptor and fluorescence microscopy) and of G-protein coupling (by using surface plasmon resonance), but also especially for testing of various GPCR mutants and conditions under which the detergent-solubilized protein can be retained functional. It also allows drug identification in high-throughput screening. Selection from protein libraries against the immobilized receptor (Ostermeier *et al*., 1995; Padan *et al*., 1998; Grisshammer *et al*., 2002; Röthlisberger *et al*., 2004) may provide stable native-receptor-conformation specific binders, which might further stabilize the receptor during its biophysical and/or structural characterization.

Acknowledgements

The authors thank Drs Stephen F.Marino, Tomoaki Matsuura and Manca Kenig for valuable discussions. This work was supported by the National Center for Competence in Research in Structural Biology and by TopNano21 Project (KTI, Berne, Switzerland).

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Received February 15, 2005; accepted February 18, 2005

Edited by David Eisenberg