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# Expression and pharmacological characterization of the human μ-opioid receptor in the methylotrophic yeast *Pichia pastoris*

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**Abstract The human μ-opioid receptor cDNA from which the 32 amino-terminal codons were substituted by the *Saccharomyces cerevisiae* α-mating factor signal sequence has been expressed in the methylotrophic yeast *Pichia pastoris* using the host promoter of the alcohol oxidase-1 gene. Cell membranes exhibited specific and saturable binding of the opioid antagonist [$^3$H]diprenorphine ($K_d$ = 0.2 nM and $B_{max}$ = 400 fmol/mg protein or 800 sites/cell). Competition studies with non-selective, and μ-, δ- and κ-selective opioid agonists and antagonists revealed a typical μ-opioid receptor binding profile, suggesting proper folding of the protein in yeast membranes.**

*Key words:* μ-Opioid receptor; Heterologous expression; *Pichia pastoris*; Membrane receptor; Methylotrophic yeast

## 1. Introduction

Many compounds of the modern pharmacopoeia exert their effects by binding to G-protein-coupled receptors which share many structural features including seven α-helical membrane-spanning domains. Among these, opioid receptors are of particular importance since they represent the target of the most potent and as yet unequalled central analgesics. G-protein-coupled receptors are perhaps the most widespread pharmacological targets, and thus there is great interest in obtaining structural data that could provide new leads and concepts during the research and development of selective therapeutic agents.

During the past decade, hundreds of genes coding for G-protein-coupled receptors have been isolated. Biochemical and mutagenesis experiments have allowed accumulation of a wealth of information on the structure/function relationships within this family of receptors. However, interpretation of the data relies on substantially speculative three-dimensional models derived from the structure of related but yet poorly homologous molecules such as bacterial and visual opsins [1–3]. Indeed, G-protein-coupled receptors generally occur at very low levels in biological materials and direct structural studies such as neutron or electron diffraction, NMR spectroscopy and X-ray crystallography cannot yet be performed.

The methylotrophic yeast, *Pichia pastoris* has been described as a particularly efficient host for high level expression of heterologous genes when these are placed under control of the host alcohol oxidase-1 (AOX1) promoter [4]. The activity of the AOX1 promoter is tightly regulated. It is repressed in the presence of glucose or glycerol, allowing production of more than 100 g of dry cell weight/l [5]. In the presence of methanol, promoter activity is maximal and AOX1 may then contribute to 30% of the total protein content [6]. Produced proteins are usually properly folded because *P. pastoris* provides an intracellular folding machinery similar to that of mammalian cells. Finally, *P. pastoris* can be grown in minimal medium to obtain $^2$H-, $^{15}$N- and $^{13}$C-labelled proteins [7].

This system has been used for heterologous expression of secreted or intracellular proteins [8] but there is a single report on the expression of a G-protein-coupled receptor, the 5-$HT_{5A}$ serotonin receptor, in *P. pastoris* with expression levels as high as 20 pmol/mg membrane protein [9]. Moreover, despite the cloning of numerous genes for G-protein-coupled receptors, only five of these have been expressed in yeast [9–15]. In this paper, we report on the expression of the human μ-opioid receptor (HuMOR) in the methylotrophic yeast *P. pastoris*. The typical μ-opioid binding profile of the expressed protein suggests that it is folded in the yeast membrane in a conformation close to that occurring in mammalian cells.

## 2. Materials and methods

### 2.1. Vector construction

The cDNA for the HuMOR was isolated by reverse transcription and PCR-amplification from human neuroblastoma SH-SY-5Y RNA (Emorine, L.J., unpublished). The coding region of the cDNA was cloned into the pGEM4z vector, yielding the pGEM4z-HuMOR construct. The sequence of the cDNA was shown to be identical to that already published [16].

Two expression vectors were constructed using the commercially available (Invitrogen, San Diego, USA) *P. pastoris* expression vector pPIC9 (Fig. 1). For one construct, a synthetic oligonucleotide constituted of 25 bp of 5′ non-coding sequences containing a *Bgl*II site and a yeast translation initiation codon, and of 55 bp coding for the amino-terminal part of the *Saccharomyces cerevisiae* $STE_2$ gene [17] was inserted into the *Sac*I and *Mun*I sites of pGEM4z-HuMOR. This resulted in the replacement of the 11 amino-terminal codons of the HuMOR cDNA by the 18 amino-terminal codons of the $STE_2$ gene. A *Bgl*II to *Xba*I fragment was purified from the resulting plasmid and cloned into the *Bam*HI and *Avr*II sites of pPIC9 to yield pPIC9-STE2-HuMOR (Fig. 1A). For the second construct, pPIC9-αMF-HuMOR, a *Hin*cII to *Eco*RI fragment was purified from pGEM4z-HuMOR and ligated into the *Sna*BI and *Eco*RI sites of pPIC9 (Fig. 1B). This resulted in the replacement of the 32 amino-terminal codons of the HuMOR cDNA by the *S. cerevisiae* α-mating factor signal sequence (89 amino acids) contained in pPIC9.

*E. coli* strain DH5α [F$^-$ ϕ80dlacZΔM15, recA1, endA1, gyrA96,

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*Abbreviations:* HuMOR, human μ-opioid receptor; AOX1, gene for alcohol oxidase-1; STE2, gene for α-mating factor receptor; MD, minimal dextrose; MM, minimal methanol; BMGY, buffered minimal glycerol-complex medium; BMMY, buffered minimal methanol-complex medium; PMSF, phenylmethylsulfonyl fluoride; 5-HT, 5-hydroxytryptamine; DPN, diprenorphine; DAGO, [D-Ala$^2$,*N*-Me-Phe$^4$,Gly-ol$^5$]enkephalin; DTLET, Tyr-D-Thr-Gly-Phe-Leu-Thr; U-50,488, *trans*-(−)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzene acetamide; β-FNA, β-funaltrexamine; ICI-174,846, *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu; NorBNI, nor-binaltorphimine


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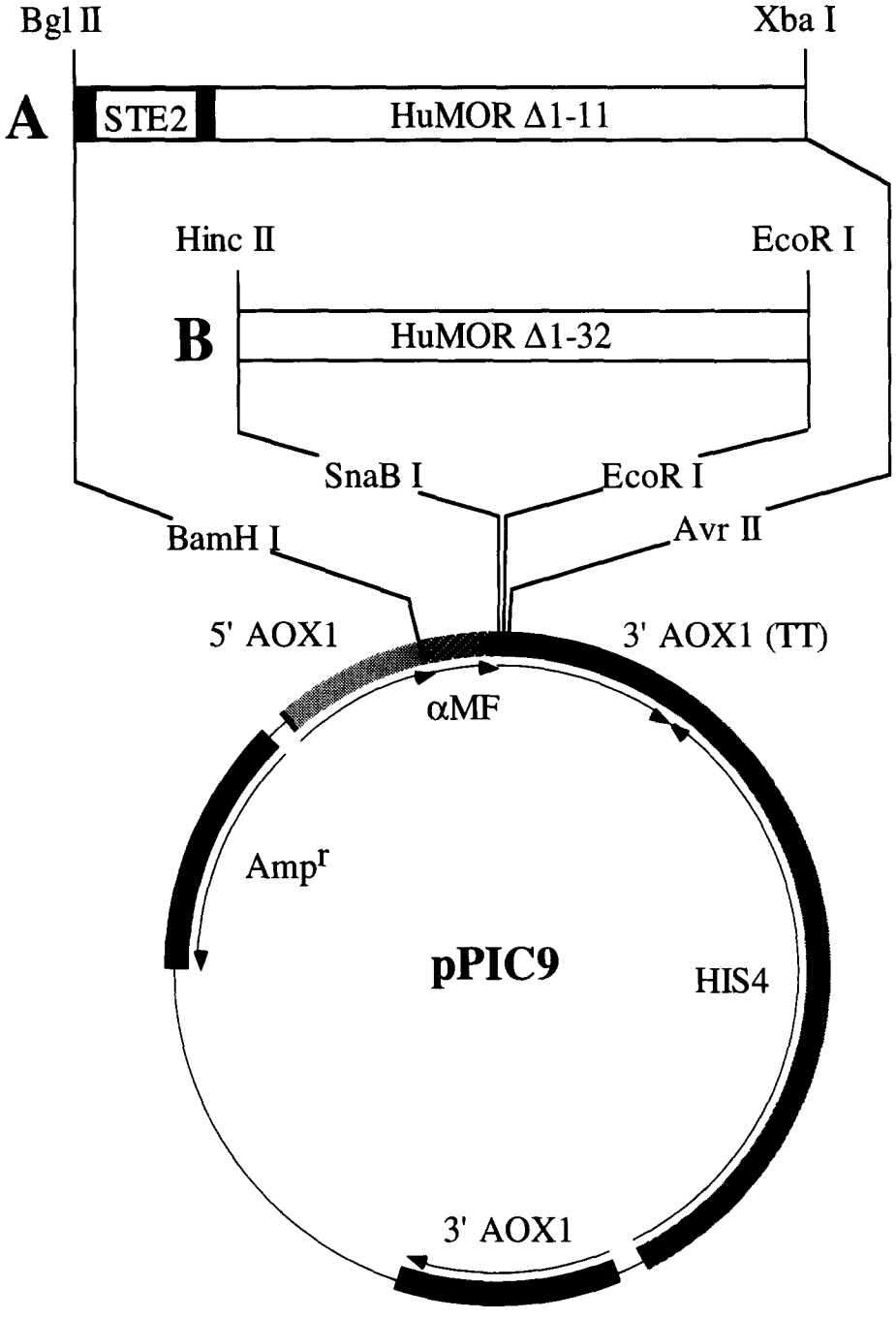


Fig. 1. Schematic representation of the vectors used for expression of the human μ-opioid receptor in *Pichia pastoris*. pPIC9-αMF-HuMOR plasmid (A) was constructed by inserting the HuMOR cDNA deleted from its 32 amino-terminal codons (HuMOR Δ1–32) in frame with the sequence coding for the α-mating factor signal sequence (αMF) present in the pPIC9 vector. pPIC9-STE2-HuMOR (B) contains the HuMOR coding region from which the 11 amino-terminal codons (HuMOR Δ1–11) were replaced by the 18 amino-terminal codons of the STE2 gene. The 5′ AOX1 and 3′ AOX1 (TT) correspond to the promoter and the transcription termination regions of the AOX1 gene, respectively. The histidinol dehydrogenase (HIS4) and the β-lactamase ($Amp^r$) genes were used for transformant selection in yeast and *E. coli*, respectively.

thi-1, hsdR17($rk^-$, $mk^+$), supE44, relA1, deoR, Δ(lacZYA-argF)-U169] was used for propagation of recombinant plasmids.

For electrotransformation, a 6.6 kb fragment was isolated from pPIC9-STE2-HuMOR and a 6.8 kb fragment from pPIC9-αMF-HuMOR by partial digestion of the plasmids with *Bgl*II.

*2.2. Yeast transformation and culture*

Two *P. pastoris* strains (Invitrogen, San Diego) were used in this study; GS115 (his4) and SMD1168 (his4, pep4). Cells were electrotransformed with DNA by application of a square wave electric pulse of 2.7 kV/cm for 15 ms [18]. For determination of histidine auxotrophy ($His^+$), cells were platted on minimal dextrose (MD) plates (0.67% yeast nitrogen base without amino acids, 2% glucose, $4\times10^{-5}$% biotin) and grown at 30°C for 48–72 h. Controls were performed with linearized pPIC9 DNA and with non-transformed cells. For determination of the methanol utilization phenotype, single colonies of $His^+$ transformants were duplicated on MD plates and on minimal methanol (MM) plates (same as MD with 0.5% methanol instead of glucose) and grown for 2 days at 30°C.

For receptor expression, $His^+$ recombinants with slow methanol utilization ($Mut^s$) were first grown for 48 h at 30°C in repressive BMGY medium (1% yeast extract, 2% bacto-peptone, 0.67% yeast

nitrogen base without amino acids, $4\times10^{-5}$% biotin, 1% glycerol (v/v), 0.1 M phosphate buffer pH 6). After centrifugation (10 min, 3000×*g*) cells were re-suspended in the same volume of inducing BMMY medium (same as BMGY with glycerol replaced by 0.5% methanol) and grown for 40 h at 30°C.

*2.3. Spheroplast preparation*

Spheroplasts were prepared essentially as described (*P. pastoris* expression kit, Invitrogen, San Diego). After induction, cells were harvested by centrifugation (10 min, 3000×*g*) and sequentially washed with water and with 1 M sorbitol, 25 mM EDTA, 50 mM DTT. Cells were suspended ($10^9$ cells/ml) in 1 M sorbitol, 10 mM citrate buffer pH 5.8, 1 mM EDTA, supplemented with 30 μg/ml of zymolyase (Seikagaku Corp., Japan, $10^5$ U/g) and were incubated for 30 min at 30°C. Spheroplasts were harvested by centrifugation (20 min, 1000×*g*) washed with 1 M sorbitol and suspended in 50 mM sodium phosphate buffer pH 7.4, 1 mM EDTA, 5% glycerol, 100 mM PMSF.

*2.4. Membranes isolation and radio-ligand binding assays*

All further manipulations were performed on ice. Cells were lysed using a Potter-Elvehjem apparatus and the lysate was centrifuged for 10 min at 10000×*g* to remove particulate matter. The supernatant was centrifuged at 100000×*g* for 35 min. The crude membranes pellet was suspended (80 mg/ml) in binding buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA), aliquoted and stored at −70°C. Protein concentration was measured [19] in the presence of 1% SDS and was between 4 and 15 mg/ml.

Saturation binding assays were performed for 1 h at 25°C in 500 μl of binding buffer containing either whole cells ($10^8$) or isolated membranes (50 μl) and varying concentrations (0.05 to 3 nM) of [$^3$H]diprenorphine ([$^3$H]DPN) (Amersham, spec. act. 41 Ci/mmol). Non-specific binding was determined in parallel test tubes in the presence of non-labelled diprenorphine (0.05–3 μM). Bound and free ligand were separated by rapid filtration (Whatman GF/B filters, soaked in 0.3% polyethylenimine) and, after 3 washes with 10 mM Tris-HCl pH 7.5, filter-bound radioactivity was determined. For displacement studies, various concentrations of unlabelled opioid ligand and 1 nM [$^3$H]DPN were used. When indicated 100 μM Gpp(NH)p was added to test tubes. Data were analyzed with the INPLOT program (GraphPad software Inc., San Diego, USA) and the results are presented as the mean ± S.E.M. of 3 independent experiments performed in duplicate.

## 3. Results and discussion

The methylotrophic yeast *P. pastoris* has been successfully used for the expression of intracellular or secreted proteins [8] but there is only one report on the expression of an integral membrane protein [9]. The heterologous expression of the HuMOR was examined here using integrative plasmids allowing expression from the endogenous AOX1 promoter which upon induction with methanol usually warrants the expression of large amounts of methanol oxidase (up to 30% of cell proteins) [6]. To increase the possibility of achieving membrane expression of the receptor, two fusion genes were tested. In pPIC9-STE2-HuMOR (Fig. 1A), the hybrid protein comprised the 18 amino-terminal residues of the *S. cerevisiae* α-mating factor receptor which is also a 7 α-helical membrane receptor. In pPIC9-αMF-HuMOR (Fig. 1B), the secretion signal of the *S. cerevisiae* α-mating factor was used to facilitate membrane translocation of the receptor. In both cases, part of the amino-terminus of the receptor was deleted since it has been reported that this had no effect on ligand binding [20–22].

DNA fragments comprising the fusion genes flanked by 5′ and 3′ AOX1 sequences were electro-transformed into the GS115 (his4) and the protease-deficient SMD1168 (his4, pep4) *P. pastoris* strains and His$^+$ transformants were selected. The Mut$^s$ phenotype of the transformants was considered as indicative of homologous recombination at the AOX1 locus but this was not verified further.

Clones with the His$^+$/Mut$^s$ phenotype were then tested for HuMOR expression. After biomass production in repressive

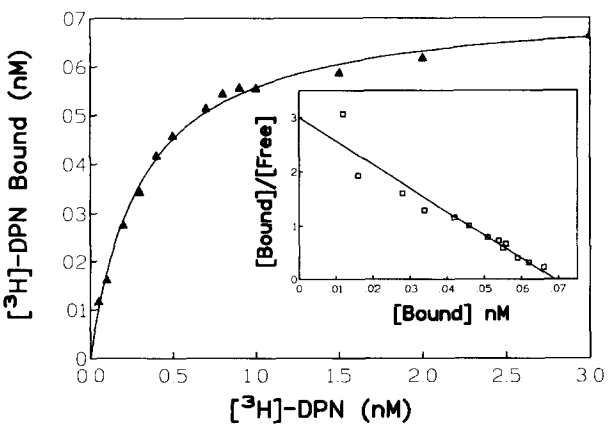


Fig. 2. Binding of [$^3$H]diprenorphine to membranes of *Pichia pastoris* yeast strain GS115 transformed with pPIC9-αMF-HuMOR. The saturation isotherm and Scatchard plot thereof (inset) are representative of one experiment.

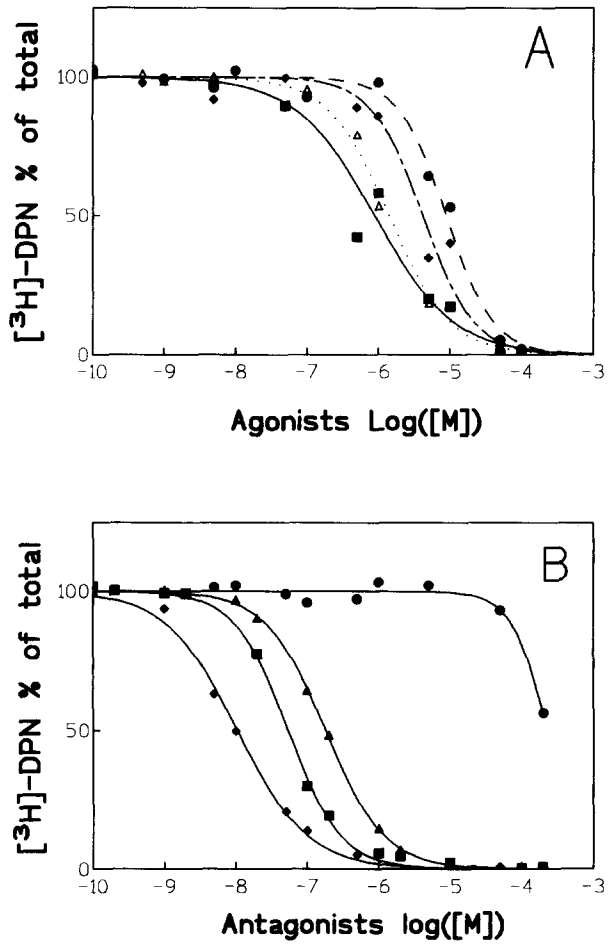


Fig. 3. Pharmacological profiles of the μ-opioid receptor expressed in *Pichia pastoris*. Inhibition of [$^3$H]DPN binding (1 nM) to recombinant yeast membranes was performed using agonists (A) DAGO (■), morphine (△), DTLET (◆), U-50,488 (●) and antagonists (B) β-FNA (◆), naloxone (■), NorBNI (▲) and ICI-174,486 (●).

Table 1
Ligand binding properties of μ-opioid receptors expressed in *Pichia pastoris*

| | Ligands | $K_i$ (nM) |
|---|---|---|
| Antagonists | | |
| non-selective | [$^3$H]DPN ($K_d$) | 0.26 ± 0.03 |
| | naloxone | 5 ± 2 |
| μ-selective | β-FNA | 2.0 ± 0.5 |
| δ-selective | ICI-174,486 | > 15000 |
| κ-selective | NorBNI | 30 ± 3 |
| Agonists | | |
| μ-selective | morphine | 240 ± 10 |
| | morphine+Gpp(NH)p | 260 ± 15 |
| | DAGO | 95 ± 20 |
| | DAGO+Gpp(NH)p | 160 ± 10 |
| δ-selective | DTLET | 950 ± 95 |
| κ-selective | U-50,488 | 2300 ± 100 |

$K_i$ values were calculated from competition experiments presented in Fig. 3.

glycerol-containing medium, AOX1 promoter activity was induced in methanol-containing medium. Intact transformant cells were then screened for the presence of opioid binding sites by using the antagonist [$^3$H]DPN. Following transformation of the GS115 strain with pPIC9-αMF-HuMOR, 35 [$^3$H]DPN binding clones were obtained out of 40 tested. No binding occurred before induction, or on cells transformed with the pPIC9 vector. For pPIC9-STE2-HuMOR, no significant binding was detected on intact cells or on membranes from 10 His$^+$/Mut$^s$ clones.

One of the GS115 clones displaying high and reproducible [$^3$H]DPN binding was further characterized. Saturation isotherms and Scatchard analyses thereof indicated a single class of specific and saturable binding sites with a $K_d$ of 0.26 ± 0.03 nM and a $B_{max}$ of 420 fmol/mg membrane protein (Fig. 2). In similar experiments performed on whole cells the $K_d$ was 0.17 ± 0.02 nM and the receptor density 810 sites/cell (not shown). Such receptor densities are in the same range as those obtained for expression of M1- and M5-muscarinic receptors [10,12] in yeast but lower than for β2-adrenergic, D2-dopaminergic and $5HT_{5A}$-serotoninergic receptors [11,13–15]. Utilization of protease-deficient strains has been suggested to increase expression levels of G-protein-coupled receptors in *S. cerevisiae* [11] and *P. pastoris* [9]. The SMD1168 strain was thus tested here but yielded a single positive clone (out of 10) with an expression level similar to that of the GS115 clones. Since it has been shown for oxytocin receptors heterologously expressed in *Sf9* cells that a low membrane cholesterol/phospholipid ratio may shift receptors into a low affinity state for ligands [23], this possibility was investigated here for the μ-opioid receptor. However, no low affinity binding was detected in competition experiments using concentrations up to 1 mM of unlabelled DPN.

The apparent affinity of [$^3$H]DPN for the μ-opioid receptor expressed in *P. pastoris* is similar to that obtained in mammalian brains (0.1 nM) [24] or cells transfected with the HuMOR cDNA (0.2 nM) [16]. The binding properties of the μ-opioid receptor expressed in *P. pastoris* membranes were further assessed by determining the inhibition constants ($K_i$) of [$^3$H]DPN binding by a series of opioid ligands (Fig. 3, Table 1). These included naloxone, a non-selective antagonist, β-FNA, ICI-174,864 and NorBNI, respectively μ-, δ- and κ-selective antagonists as well as morphine, DAGO, DTLET and U-50,488 which are two μ-, a δ- and a κ-selective agonists, respectively.

The rank order of potency for antagonist binding, [$^3$H]DPN > β-FNA ≥ naloxone > NorBNI >> ICI-174,864, was typical of μ-opioid receptors [24–27]. As expected, non-selective and μ-selective antagonists bound to μ-opioid receptors with $K_i$ values in the low nanomolar range, whereas the κ-selective antagonist NorBNI bound with an affinity in the 10–100 nM range and the δ-selective antagonist ICI-174,864 did not readily displace [$^3$H]DPN binding at concentrations as high as 15 μM. Similarly, the rank order of potency for agonists binding, DAGO≥Morphine ≫ DTLET > U50.488, was the same as that for μ-opioid receptors present in mammalian tissues or cells [24–26]. The corresponding $K_i$ values (Table 1) were, however, low as compared to those of 'native' μ-opioid receptors.

It has been reported that the 64 amino-terminal extracellular residues of the μ-opioid receptor could be deleted with little effect on ligand binding [20–22]. Our data obtained by expressing a wild-type and a truncated (61 amino-terminal residues) HuMOR in COS-M6 cells (Emorine L.J., unpublished) showed that this was indeed true for antagonists but that $K_i$ values were increased from 7 to 60 nM for DAGO and from 10 to 80 nM for morphine in wild-type and truncated receptors, respectively. Additionally, in mammalian cells, Gpp(NH)p a non-hydrolysable analogue of GTP, is able to shift receptors from a high-affinity to a low-affinity state for agonists by uncoupling the receptors and G-proteins [24,28]. We showed here that 100 μM Gpp(NH)p had no such effects on agonist binding (Table 1), certainly reflecting the lack of functional interactions of the HuMOR with host G-proteins. Such a situation has already been reported for muscarinic and adrenergic receptors expressed in yeast [10–12]. Thus, the deletion of the 32 amino-terminal residues of the receptor as well as the lack of coupling to G-proteins may both contribute to the low-affinity binding of agonists to μ-opioid receptors expressed in *P. pastoris*.

The receptor densities determined in this work are somewhat lower than those reported for other G-protein-coupled receptors expressed in various species of yeast [9,11,13,15]. Several studies have documented the utility of signal sequences for the expression of such receptors in yeast. In an initial study it was shown that with no modifications of its amino-terminus, the human M1-muscarinic receptor was poorly expressed in *S. cerevisiae* (20 fmol/mg protein) [10]. Subsequently, it was suggested that higher expression levels could be reached by replacing the amino-terminal part of the receptors by the corresponding region of the $STE_2$ gene [11,13,15]. Since then, other amino-terminal fusion partners, such as the signal sequences for the α-mating factor [9,12] or the acid phosphatase [9] have been successfully used. It should be noted, however, that high levels of unmodified dopamine receptor were obtained in both *S. cerevisiae* (3 pmol/mg) and *Schizosaccharomyces pombe* (15 pmol/mg) [14]. Together with our results, these observations suggest that the need for a signal sequence as well as its nature will have to be empirically determined in order to obtain higher expression levels of the μ-opioid receptor. This could also be achieved by utilizing multi-integrative vectors and multi-protease deficient *P. pastoris* strains instead of the SMD1168 tested here.

μ-Opioid receptors have been expressed in the *P. pastoris* methylotrophic yeast. Their pharmacological profile is similar

to that of receptors naturally occurring in membranes of mammalian brains or cells. Expression of δ- and κ-opioid receptors which are highly homologous to μ-opioid receptors will certainly follow. These results pave the way for large-scale production of μ-opioid receptors, stable isotope labelling [7] for NMR or neutron diffraction experiments, and for the determination of the conformations of peptidic ligands bound to the receptor and eventually of the receptor itself.

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