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Human μ-opioid receptor overexpressed in baculovirus system and its pharmacological characterizations[1]

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KEY WORDS mu opioid receptors; Baculoviridae; Sf9 insect cells; diprenorphine; radioligand assay; cyclic AMP; fentanyl; enkephalins; morphine; naloxone

AIM: To overexpress human mu-opioid receptor (μOR) with characteristics similar to those of mammalian origin. **METHODS**: Human μOR with a tag of 6 consecutive histidines at its carboxyl terminus was expressed in recombinant baculovirus infected Sf9 insect cells. Then the pharmacological characterizations of the product were studied by receptor binding assay and cAMP assay. **RESULTS**: The maximal binding capacity for the [^{3}H]diprenorphine and [^{3}H]ohmefentanyl (Ohm) were 9.1 ± 0.7 and 6.52 ± 0.23 nmol/g protein, respectively. The [^{3}H]diprenorphine or [^{3}H]Ohm binding to the receptor expressed in Sf9 cells was strongly inhibited by μ-selective agonists [*D*-Ala^2, *N*-methyl-Phe^4, glyol^5] enkephalin (DAGO), Ohm, and morphine, but neither by the δ-selective agonist [*D*-Pen^2, *D*-Pen^5] enkephalin (DPDPE) nor by the κ-selective agonist {*trans*-(±)-3, 4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl]} benzacetamide (U50488). NaCl 100 mmol·L^{-1} and guanosine triphosphate (GTP) 50 μmol·L^{-1} could reduce μ agonists Ohm and etorphine affinity binding to the expressed μOR. DAGO and Ohm effectively inhibited forskolin-stimulated cAMP accumulation. This agonist-dependent effect was blocked by opioid antagonist naloxone. **CONCLUSION**: The overexpression of human μOR with a tag of six consecutive histidines at its carboxyl terminus in Sf9 insect cells retained the characteristics of wild-type human μOR.

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Opioid receptors are members in the family of G-protein-coupled receptors which negatively regulate the activity of adenylyl cyclase. They are considered to mediate pharmacological effects, including analgesia, sedation, euphoria, and respiratory depression. Three major subtypes of opioid receptors, designated as μ, κ, and δ, have been well characterized by pharmacological studies[1-2], and the cDNA for each has been cloned[3]. However, opioid receptors are not expressed naturally in great abundance, they are relatively labile and frequently difficult to solubilize as monomers. The availability of their cDNA makes them good candidates for overexpression in heterologous cells, which offers possibilities for the purification of membrane receptors for biochemical and structural studies. The baculovirus expression system has been proved to be a convenient and powerful method of obtaining high level expression of many signal transduction components, including cell surface receptors[4-5]. The present experiments were designed to express human μOR in baculovirus system, and to verify if the recombinant human μOR displayed characteristics similar to those of mammalian origin.

MATERIALS AND METHODS

pBluescript II KS (+/−) was purchased from Stratagene. Sequenase T7 kit was purchased from Pharmacia. pVL1393 and linearized baculovirus were purchased from PharMingen. Sf9 insect cells were obtained from Shanghai Institute of Cell Biology. [^{3}H]Diprenorphine (1.44 PBq·mol^{-1}) purchased from Amersham. [^{3}H]Ohmefentanyl (Ohm, 1.10 PBq·mol^{-1}) and Ohm were synthesized in our institute. The δ-selective agonist [*D*-Pen^2, *D*-Pen^5] enkephalin (DPDPE) and the κ-selective agonist {*trans*-(±)-3, 4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl]} benzacetamide (U50488) were purchased from Sigma. TNM-

FH medium and Sf-900 Ⅱ were purchased from Gibco BRL. Fetal bovine serum was purchased from Sijiqing Institute of Biomaterials, Hangzhou, China.

Construction of recombinant vector All recombinant DNA procedures were conducted according to standard protocols[6]. The codons encoding a tag of 6 consecutive histidines were added to 3′-end of human μOR cDNA[7] by polymerase chain reaction (PCR) with primer pair 5′-AATACGACTCACTATAG-3′ and 5′-AAAGGTCGACTAATTATCATTAGTGATGGTGATGGTGATGGGGCAACGGAGCAGTTTCTGC-3′. The 1.5 kilobase PCR product containing the open reading frame of μOR cDNA was subcloned into pBluescript Ⅱ KS (+/−) in the Hind Ⅲ and Sal Ⅰ sites, and the fidelity of cDNA amplification was verified with dideoxy DNA sequencing. The plasmid vector containing μOR cDNA was digested with Nco Ⅰ and Sal Ⅰ, and the 1236 bp fragment was filled in with Klenow and deoxynucleotides. The fragment was then cloned into the unique Xba Ⅰ site of the transfer vector pVL1393 by blunt-ended ligation. The correct subcloning orientation of μOR cDNA was identified by restriction mapping.

Cell culture Sf9 cells were routinely grown in monolayer at 27 ℃ in TNM-FH medium supplemented with 10 % heat-inactivated fetal bovine serum or a defined nonserum supplemented medium (Sf-900 Ⅱ).

Infection of Sf9 with recombinant baculovirus To obtain recombinant viruses, Sf9 cells were cotransfected with the pVL1393 transfer vector 2 μg, containing the encoding regions of the μOR cDNA, and the linearized baculovirus DNA 0.5 μg. The technique followed the instructions of the manufacturer (PharMingen). Individual recombinant viral clones were isolated ("plaque assay"), grown, and tested for the highest level of [^{3}H]diprenorphine binding.

Receptor binding assay The plasma membranes were prepared as described previously[8]. Briefly, the cells were harvested in Tris-HCl 50 mmol·L^{-1} (pH 7.4) and spun at 1000 × *g* for 10 min. The precipitation was resuspended in TE buffer (pH 7.4), containing leupeptin 10 mg·L^{-1}, benzamidine 10 mg·L^{-1}, apretimim 10 mg·L^{-1}, phenylmethylsulfonyl fluoride 0.2 mg·L^{-1}, pepstatin A 10 mg·L^{-1}, and spun at 12 000 × *g* for 10 min. The pellet was homogenized in Tris-HCl 50 mmol·L^{-1} (pH 7.4) for binding assay. For saturation binding experiments, membranes containing 20 μg protein were incubated with increasing concentration of [^{3}H] diprenorphine 0.1 – 6.0 nmol·L^{-1} or [^{3}H]Ohm 0.1 – 6.6 nmol·L^{-1} in a final volume of 210 μL at 27 °C for 30 min. For competition binding experiments, the membranes were incubated with [^{3}H]diprenorphine 1.8 nmol·L^{-1} or [^{3}H] Ohm 2.0 nmol·L^{-1} in the presence of increasing amount of competing ligands. The nonspecific binding was determined by the addition of etorphine 1 μmol·L^{-1}. The cell membranes were filtered onto Whatman GF/C filters and the bound radioactivities measured by scintillation. Data from binding assay of 3 determinations were analyzed using ACCUFIT and LIGAND programs developed by London Software Inc. The inhibitory binding constant (K_i) was calculated by Cheng-Prusoff equation.

Cyclic AMP assay Cells were challenged with Ohm or DAGO in the presence of forskolin 10 μmol·L^{-1} and 3-isobutyl-1-methylxanthine (IBMX) 500 μmol·L^{-1} at 27 °C for 10 min. The reactions were terminated with perchloric acid 1 mol·L^{-1} and neutralized with K_2CO_3 1 mol·L^{-1}. The cAMP level of each sample was determined using radioimmunoassay as described previously[9]. Data were presented as a percentage of control (in the presence of forskolin alone).

RESULTS

Receptor binding characterization After cotransfection and plaque assay, 10 positive clones were tested by ligand binding experiments using the nonselective antagonist [^{3}H]diprenorphine. The clone exhibiting the highest ligand binding capacity was served for multiplication of the recombinant virus.

Receptor density expressed per mg membrane protein in recombinant baculovirus infected Sf9 insect cells increased progressively and reached a maximum at 36 h post infection. Noninfected cells failed to display opioid binding sites. The bindings of [^{3}H]diprenorphine and [^{3}H] Ohm[10] to membranes prepared from Sf9 cells expressing human μOR were saturable. Scatchard analyses of [^{3}H] diprenorphine and [^{3}H]Ohm saturation binding were most consistent

with a single population of high-affinity binding sites for each ligand with the K_d values of 1.6 ± 0.2 nmol·L^{-1} and 2.1 ± 0.1 nmol·L^{-1}, and the B_{max} values of 9.1 ± 0.7 and 6.52 ± 0.23 nmol/g protein, respectively (Fig 1). Therefore, the baculovirus expression system was very useful for large scale production of human μOR.

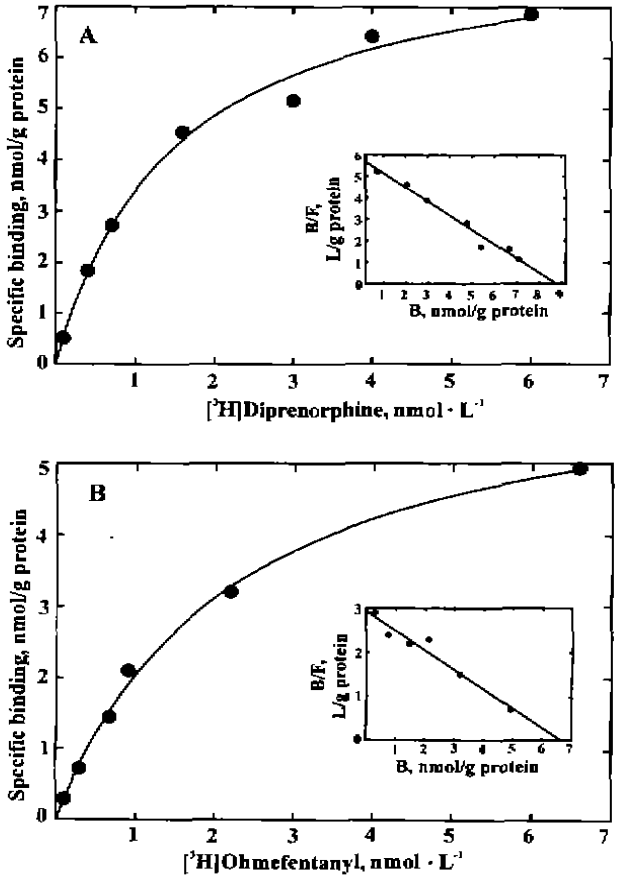

Fig 1. Saturation analyses of binding of [³H]diprenorphine (A) and [³H]ohmefentanyl (B) to membranes prepared from Sf9 insect cells expressing human μOR. (inset) Scatchard plot analyses of tritiated ligands saturable binding to the expressed μOR.

To characterize the pharmacological features of μOR in Sf9 cells, we used various ligands to displace [³H]diprenorphine or [³H]Ohm binding. K_i values were obtained from three binding experiments for each ligand and listed in Tab 1.

Both the μ-selective agonists (Ohm, morphine, DAGO) and antagonist (naloxone) displaced [³H]diprenorphine or [³H]Ohm binding to the receptors expressed in Sf9 insect cells displaying high affinities with K_i valuesin the nanomolar or subnanomolar range,

Tab 1. K_i (nmol·L^{-1}) values from competition by opioid ligands against [³H] diprenorphine and [³H] ohmefentanyl (Ohm) binding to Sf9 insect cells expressing μOR.

Ligands	Potency in displacing [³H]diprenorphine binding K_i	Potency in displacing [³H]Ohm binding K_i
Ohm	0.71 ± 0.06	0.54 ± 0.10
DAGO	5.1 ± 0.9	4.5 ± 1.2
Morphine	4.18 ± 0.11	4.1 ± 0.7
Naloxone	5.4 ± 0.3	5.2 ± 0.4
DPDPE	> 1 000	> 1 000
U50488	> 1 000	> 1 000

whereas the δ-selective agonist DPDPE and the κ-selective agonist U50488 showed low affinities with K_i values in the micromolar range. These results demonstrated that opioid receptors expressed in Sf9 cells displayed the characteristics of μOR similar to those of mammalian origin[7].

The μOR expressed in Sf9 cell lines could be demonstrated to be functionally coupled to G proteins. Two of the criteria that established opioid receptor G-protein coupling are the absolute dependence of opioid receptor activities on NaCl and GTP as reflected by the ability of both NaCl and GTP to attenuate opioid agonist binding affinity[11]. By means of displacement studies, we clearly showed an inhibitory effect of NaCl 100 mmol·L^{-1} and GTP 50 μmol·L^{-1} on displacement potencies of agonists Ohm and etorphine at μOR in Sf9 cells (Fig 2).

The μOR expressed in Sf9 existing in multiple affinity states indicated the coupling between receptor and G proteins.

cAMP assay Agonists DAGO 0.1 μmol·L^{-1} and Ohm 0.1 μmol·L^{-1} inhibited 10 μmol·L^{-1} forskolin-stimulated increase in intracellular cAMP production. The inhibition of cAMP accumulation was antagonized by opioid receptor antagonist naloxone, indicating functionality of receptors with respect to their signaling properties (Tab 2).

DISCUSSION

The baculovirus expression system, utilizing the strong polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV), has become an important tool for protein production in the field of molecular

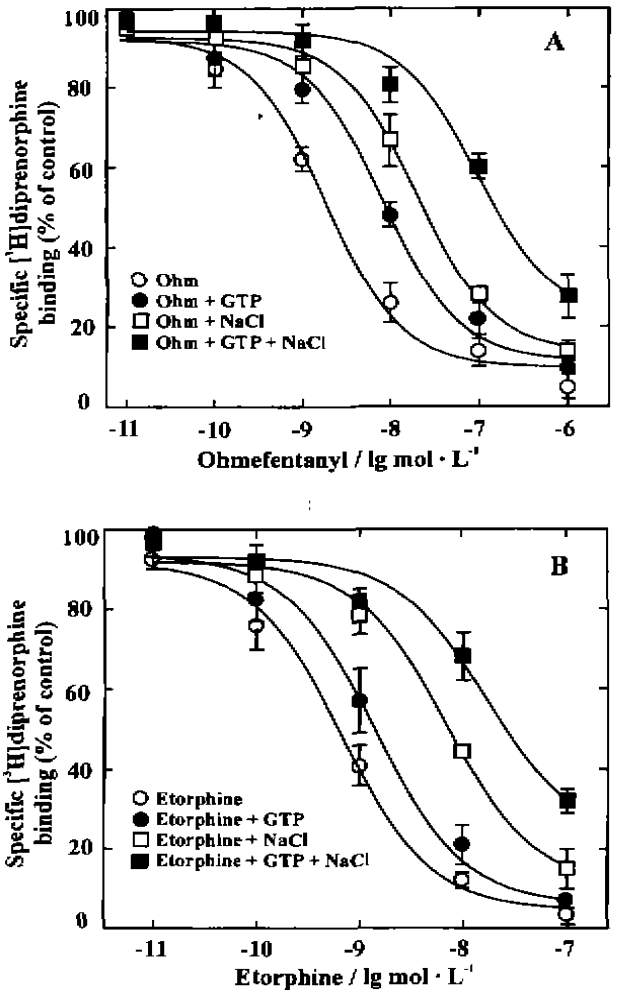

Fig 2. Regulation of μ opioid agonists ohmefentanyl (Ohm, A) and etorphine (B) against [³H]diprenorphine binding to Sf9 cells expressing μOR by NaCl and GTP.

Tab 2. Effect of opioid on forskolin-stimulated cAMP accumulation in Sf9 insect cells overexpressing human μOR.

Ligands	cAMP (% of control)
DAGO	30 ± 6
Ohm	14.52 ± 0.24
DAGO + naloxone	112 ± 7
Ohm + naloxone	104 ± 9

biology. Since polyhedrin is non-essential for viral replication, the promoter of the corresponding gene is highly useful for the abundant expression of a variety of both pro- and eukaryotic genes[12]. The cDNA of human μOR with a tag of six consecutive histidines at its carboxyl terminus had been introduced in the baculovirus genome under the control of the polyhedrin promoter.

In Sf9 insect cells infected with the recombinant baculovirus bearing the μOR cDNA, the production of μOR was approximately 100-fold higher as compared to endogenous opioid receptor levels in neuronal cells, where levels generally ranged between 50 – 100 pmol/g membrane protein[13]. Further, the simplicity by which the insect cells can be cultured and infected in conjunction with high levels of receptor expression, provides a practical system for a variety of pharmacological studies.

By displacement experiment, regulation of opioid agonists Ohm and etorphine against [³H]diprenorphine binding to Sf9 insect cells expressing human μOR by NaCl and GTP, and cAMP assay, we concluded that the overexpression of human μOR with a tag of six consecutive histidines at its carboxyl terminus in Sf9 insect cells retained the characteristics of wild-type human μOR.

In conclusion, our data indicated that the baculovirus expression system was applicable for functional studies of human μOR. The advantages of baculovirus system include the quantitative and qualitative reproducibility of expression. The μOR overexpressed in this study, with a tag of six consecutive histidines at its carboxyl terminus, will facilitate the future studies on exploration of the relationships between phosphorylation and desensitization, reconstitution, and structural basis of ligand recognition of human μOR.

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高效表达在杆状病毒系统的人 μ 阿片受体及其药理学特征[1]

R971.2 R966

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关键词 μ 阿片受体; 杆状病毒; Sf9 昆虫细胞; 二丙诺啡; 放射配位体测定; 环腺苷一磷酸; 芬太尼; 脑啡呔类; 吗啡; 纳洛酮

目的: 高效表达具有类似哺乳动物特性的人 μ 阿片受体. **方法**: 人 μ 阿片受体表达在重组杆状病毒感染的 Sf9 昆虫细胞中, 用受体结合分析和 cAMP 分析研究表达产物的药理学特征. **结果**: [^{3}H]二丙诺啡及[^{3}H]羟甲芬太尼(Ohm)的最大结合能力分别是 9.1 ± 0.7, 6.52 ± 0.23 nmol/g 蛋白. μ 选择性激动剂对[^{3}H]二丙诺啡或[^{3}H]Ohm与表达受体的结合均有很强的抑制作用, 而 δ 及 κ 激动剂则均无抑制作用. μ 选择性激动剂有效抑制 forskolin 刺激的 cAMP 聚集, 这种作用能被拮抗剂纳洛酮阻断. **结论**: 在 Sf9 昆虫细胞中高效表达的人 μ 阿片受体保持着野生型人 μ 阿片受体的特征.