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Human μ-opioid receptor overexpressed in Sf9 insect cells functionally coupled to endogenous $G_{i/o}$ proteins

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ABSTRACT

Human m-opioid receptor (H μ OR) with a tag of six consecutive histidines at its carboxyl terminus had been expressed in recombinant baculovirus infected Sf9 insect cells. The maximal binding capacity for the [^{3}H] diprenorphine and [^{3}H]ohmefentanyl (Ohm) were 9.1 $\pm$ 0.7 and 6.52 $\pm$ 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 m-selective agonists [D-Ala^2, N-methyl-Phe^4, glyol^5] enkephalin (DAGO), Ohm, and morphine, but neither by δ nor by κ selective agonist. Na^+ (100 m *M*) and GTP (50 μ *M*) could reduce HμOR agonists etorphine and Ohm affinity binding to the overexpressed Hμ OR. m-selective agonists DAGO and Ohm effectively stimulated [^{35}S]GTP γS binding (EC_{50} = 2.7 n M and 6.9 n M) and inhibited forskolin- stimulated cAMP accumulation (IC_{50} = 0.9 n M and 0.3 n M). The agonist-dependent effects could be blocked by opioid antagonist naloxone or by pretreatment of cells with pertussis toxin (PTX). These results demonstrated that HμOR overexpressed in Sf9 insect cells functionally coupled to endogenous $G_{i/o}$ proteins.

Key words: *Human μ-opioid receptor (HμOR), Sf9 insect cells, pertussis toxin (PTX), endogenous $G_{i/o}$ proteins.*

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μ-opioid receptor overexpressed in Sf9 cell coupled to G protein

INTRODUCTION

Opioid receptors are members of 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 -opioid receptor overexpressed in Sf9 cell coupled to G protein, κ, and d, have been well characterized by pharmacological studies[1], and cDNAs for each have been cloned[2]. However, opioid receptors are not expressed naturally in great abundance, they are relatively labile and rather difficult to solubilize as monomers. The availability of their cDNAs makes them good candidates for overexpression in heterologous cells, which offers hope for the purification of membrane receptors for biochemical and structural studies.

The baculovirus expression system has been proved to be a convenient and powerful means of obtaining high level expression of many signal transduction components, including cell surface receptors[3],[4] and G-proteins[5],[6]. In addition to providing a large quantity of receptors, the baculovirus system expresses the receptors with characteristics similar to those of mammalian origin. Using the recombinant baculovirus system, Wehmeyer et al[7],[8] reported the overexpression of opioid receptors in insect cells derived from Trichoplusia ni ("High 5" cells). However, coupling of mammalian receptors to PTX-sensitive G proteins in insect cells remains controversial[3],[7],[9-12]. The present experiments were designed to express HmOR in baculovirus system, and our results provide evidence that the HmOR can functionally couple to endogenous PTX-sensitive G proteins in Sf9 cells.

MATERIALS AND METHODS

Construction of recombinant vector

All recombinant DNA procedures were conducted according to standard protocols[13]. The codons encoding a tag of six consecutive histidines was added to 3' -end of HmOR cDNA[14] by polymerase chain reaction (PCR) with primer pair 5' AATACGACTCACTATAG 3' and 5' AAAGGTCGACTAATTATCATTAGTGAT GGTGATGGTGATGGGGCAACGGAGCAGTTTCTGC 3'. The ~1.5 kilobase PCR product containing the open reading frame for HmOR cDNA was subcloned into pBluescript II KS (+/-) (Stratagene) in the Hind III and Sal I sites, and the fidelity of cDNA amplification was verified with dideoxy DNA sequencing (Sequenase T7 kit, Pharmacia). The plasmid vector containing HmOR 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 (PharMingen) by blunt-ended ligation. The correct subcloning orientation of Hm OR cDNA was identified by restriction mapping.

Cell culture

Sf9 cells were routinely grown in monolayer at 27 °C in TNM-FH medium (Gibco BRL) supplemented with 10 % heat-inactivated fetal bovine serum (Evergreen, Hangzhou, China) or a defined non-serum supplemented medium (Sf-900 II, Gibco BRL).

Infection of Sf9 with recombinant baculovirus

In order to obtain recombinant viruses, Sf9 cells were cotransfected with the pVL1393 transfer vector (2μg), containing the encoding regions of the HmOR 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 (Amersham) binding. The clone exhibiting the highest ligand binding capacity served for multiplication of the recombinant virus.

Binding assay

The plasma membranes were prepared as described previously[15]. Briefly, the cells were harvested in Tris-HCl 50 mM (pH 7.4) and spun at 1000 $\times$ g for 10 min. The precipitation was resuspended in TE buffer (pH 7.4), containing leupeptin 10 mg/L, benzamidine 10 mg/L, apretimim 10 mg/L, phenylmethylsulfonyl fluoride 0.2 mg/L, pepstatin A 10 mg/L, and spun at 12000 $\times$ g for 10 min. The pellet was resuspended and homogenized in Tris-HCl 50 mM (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 n M) or [^{3}H]Ohm (0.1-6.6 nM) in a final volume of 210μl at 27 ^{o}C for 30 min. For competition binding experiments, the membranes were incubated with [3H]diprenorphine (1.8 nM) or [^{3}H]Ohm (2.0 nM) in the presence of increasing amount of competing ligands, 50 μM GTP and 100mM NaCl. The non-specific binding was determined by the addition of1μM etorphine. The cell membranes were filtered onto whatman GF/C filters and the bound radioactivity measured by scintillation. Each assay was done triplicate. Three independent experiments were performed. Then data were calculated as means $plusmn; standard errors.

[^{35}S]GTPγS binding assay

The assay was carried out as described previously[16]. Cells were lysed in 5 mM Tris-HCl (pH 7.5), 5 m M EDTA, 5mM EGTA at 4^{o}C and the lysate was centrifuged at 30,000 $\times$ g for 10 min. The membrane pellet (containing 15 mg protein) was resuspended in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, 40 mM GDP, 0.5 nM [^{35}S]GTPgS (DuPont-New England Nuclear) in the presence of agonist in a total volume of 100 ml and incubated at 27 ^{o}C for 1 h. The reaction was terminated by adding 4 ml phosphate-buffered saline and then immediately filtered through GF/C filters under vacuum. The filters were washed and counted by scintillation. Basal binding was determined in the absence of agonist and non-specific binding was obtained in the presence of 10 μM GTPγS. The percentage of stimulated [^{35}S]GTPγS was calculated as 100 $\times$ $(cpm_{sample}\text{-}cpm_{non\text{-}specific})/(cpm_{basal}\text{-}cpm_{non\text{-}specific})$.

Cyclic AMP assay

Cells were challenged with Ohm or DAGO (Sigma) in the presence of 10 μM forskolin and 500 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma) at 27 ^{o}C for 10 min. The reactions were terminated with 1 M perchloric acid and neutralized with 1M K_2CO_3. The cAMP level of each sample was determined using radioimmunoassay as described previously[17]. The percentage of forskolin-stimulated cAMP production in the presence of different concentrations of agonists was calculated as $[cAMP_{(forskolin+agonist)}\text{-}cAMP_{(basal)}]/[cAMP_{(forskolin)}\text{-}cAMP_{(basal)}] \times 100$. Data presented as a percentage of control (in the presence of forskolin alone).

RESULTS AND DISCUSSION

HmOR binding characterization

The baculovirus expression system, utilizing the strong polyhedrin gene promoter of the Autographa californica nuclear polyhedrosis virus (AcNPV), has become an impor-

tant tool for protein production in the field of molecular 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[10]. The cDNA of HμOR with a six consecutive histidines tag 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 HμOR cDNA, expression of HmOR was detected by ligand binding experiments using the non-selective antagonist [^{3}H]diprenorphine and m-selective agonist [^{3}H]Ohm[8,19]. Receptor density expressed per mg membrane protein increased progressively and reached a maximum at 36 h post infection. Noninfected cells failed to display opioid binding sites. The binding of [^{3}H] diprenorphine and [^{3}H]Ohm to membranes prepared from Sf9 cells expressing HmOR was saturable. Scatchard analyses of [3H]-diprenorphine and [^{3}H]Ohm saturation binding were most consistent with a single population of high affinity binding sites for each ligand with Kd values of 1.6 $\pm$ 0.2 n M and 2.1 $\pm$ 0.1 n M, and the Bmax values of 9.1 $\pm$ 0.7 and 6.52 $\pm$ 0.23 nmol/g protein, respectively (Fig 1). The production of HμOR expressed in Sf9 cells 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[20]. 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. Therefore, the baculovirus expression system was very useful for large scale production of HμOR.

To characterize the pharmacological features of HmOR in Sf9 cells, we used various ligands to displace [^{3}H] diprenorphine or [^{3}H]Ohm binding. gi values were obtained from three binding experiments for each ligand and listed in Tab 1. Both the m-selective agonists (Ohm, DAGO, morphine) and antagonist (naloxone) displaced [^{3}H] diprenorphine or [^{3}H]Ohm binding with high affinity with ki values in the nanomolar or subnanomolar range, whereas the d-selective agonist [D-Pen2, D-Pen5]enkephalin (DPDPE) and the k-selective agonist { trans-($\pm$)-3, 4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]} benzacetamide (U50488) showed low affinities with ki values in the micromolar range. These results demonstrated that opioid receptors expressed in Sf9 cells displayed the

Tab 1. Ki (n M) values from competition by opioid ligands against [^{3}H]diprenorphine and [^{3}H] Ohm binding to Sf9 insect cells expressing HmOR calculated by Cheng-Prusoff equation. Data were calculated as means $\pm$ standard errors from three independent experiments.

Ligands	Potency in displacing [^{3}H] diprenorphine binding K*i*	Potency in displacing [^{3}H] Ohm binding K*i*
Ohm	0.71 $\pm$ 0.06	0.54 $\pm$ 0.10
DAGO	5.1 $\pm$ 0.9	4.5 $\pm$ 1.2
Morphine	4.18 $\pm$ 0.11	4.1 $\pm$ 0.7
Naloxone	5.4 $\pm$ 0.3	5.2 $\pm$ 0.4
DPDPE	> 1000	> 1000
U50488	> 1000	> 1000

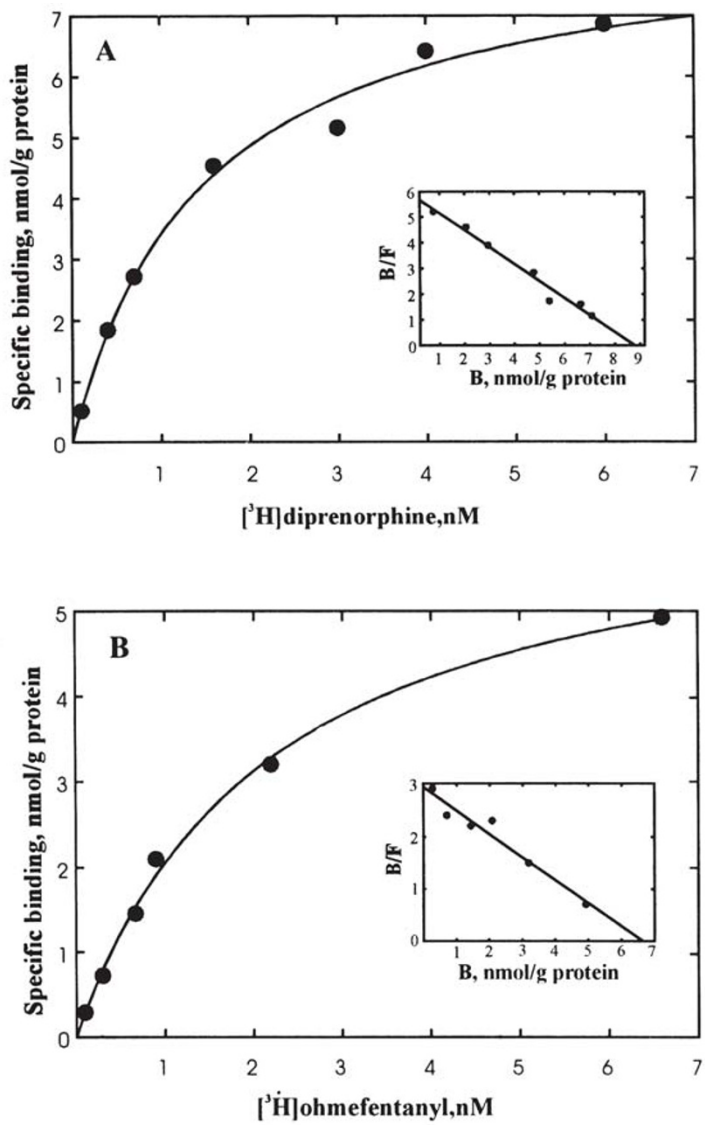

Fig 1.
Saturation analyses of binding of [^{3}H]diprenorphine (A) and [^{3}H]Ohm (B) to membranes prepared from Sf9 insect cells expressing HmOR. (inset) Scatchard plot analyses of [^{3}H]-ligands saturable binding to the expressed μmOR.}

characteristics of values in the micromolar range. These results demonstrated that opioid receptors expressed in Sf9 cells displayed the characteristics of mOR similar to those of mammalian origin[14].

HμOR functionally coupled to endogenous Gi/o proteins

The HμOR expressed in Sf9 cell line could be demonstrated to be functionallycoupled

μ-opioid receptor overexpressed in Sf9 cell coupled to G protein

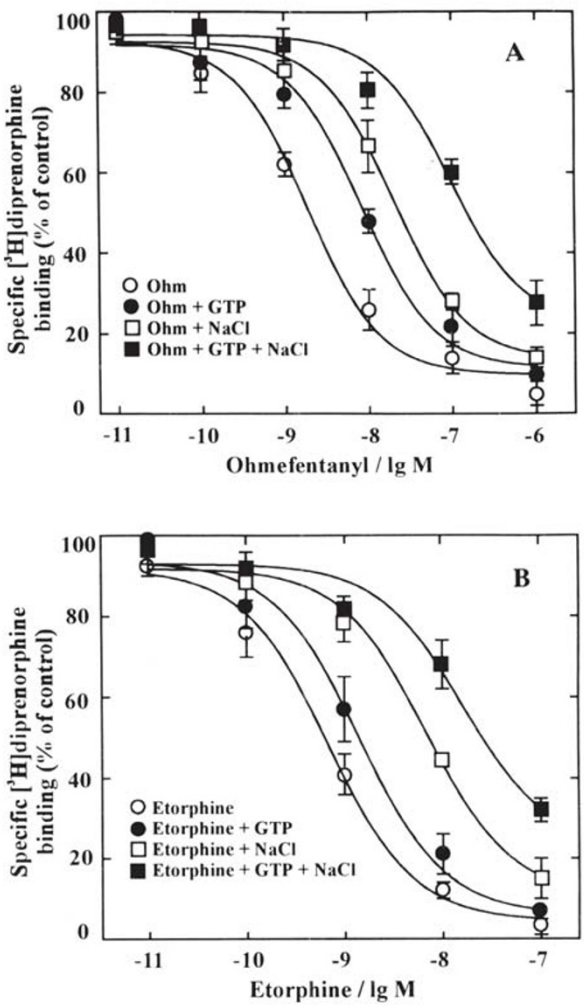

Fig 2.
Regulation of opioid agonists Ohm (A) and etorphine (B) against [^{3}H] diprenorphine binding to Sf9 cells expressing HμOR by Na^+ (100 mM NaCl) and GTP (50μM). Data were calculated as means &plismn; standard errors from three independent experiments.

to G proteins. Two of the criteria that established opioid receptor G-protein coupling are the absolute dependence of opioid receptor activities on Na^+ and GTP as reflected by the ability of both Na^+ and GTP to attenuate opioid agonist binding affinity[21]. By means of displacement studies, we clearly showed an inhibitory effect of Na^+ and GTP on displacement potencies of agonists Ohm and etorphine at HmOR in Sf9 cells (Fig 2). The HmOR expressed in Sf9 existing in multiple affinity states indicated the coupling between receptor and G proteins.

In order to further confirm the notion that H μOR expressed in Sf9 cells coupled to G proteins, the ability of agonist-occupied opioid receptor to activate G proteins was measured in membranes from Sf9 cells expressing the cloned receptor. The binding of [^{35}S] GTP γS to Sf9 cell membranes containing H μ OR was stimulated by m-selective agonists DAGO and Ohm in a concentration-dependent manner with EC_{50} values of 2.7 nM and 6. 9 nM, respectively (Fig 3A). Ohm (0.1 μM) and DAGO (0.1 μM) stimulated [^{35}S]GTPg S bindings were blocked by antagonist naloxone (10 μM, Fig 3B). When the expressed Sf9 cells were pretreated with 300 ng/ml of PTX (Sigma) at 27 °C for 30 h, Ohm (0.1 μM) or

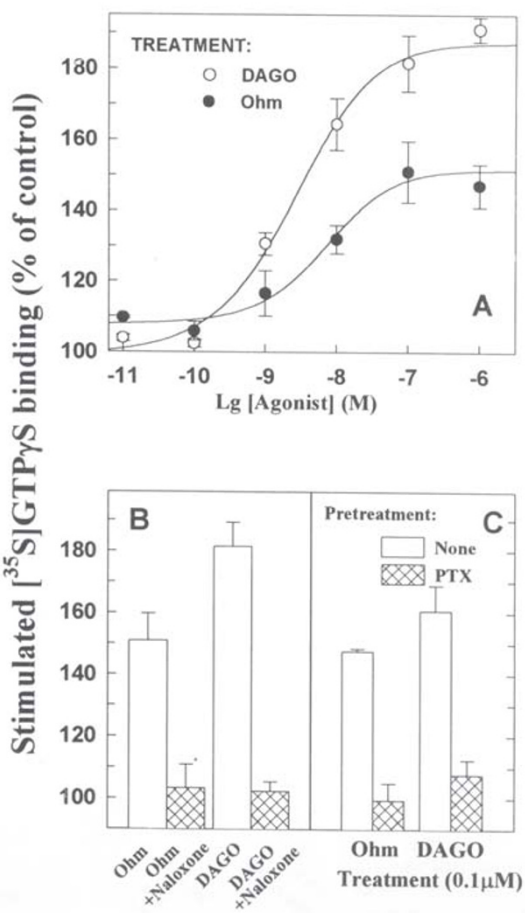

Fig 3.
The agonist-stimulated [^{35}S]GTPgS binding to membranes of Sf9 insect cells expressing HmOR.
(A) Dose-response curves of μ-selective agonists Ohm or DAGO. (B) Agonist Ohm and DAGO stimulated [^{35}S]GTPgS bindings were blocked by antagonist naloxone. (C) When the expressed Sf9 cells were pretreated with PTX, the agonists Ohm (0.1μM) and DAGO (0.1 μM) stimulated [^{35}S] GTPgS bindings were abolished.

DAGO (0.1 m M)-stimulated[^{35}S]GTPgS binding was abolished (Fig 3C), showing this effect in Sf9 cells was mediated via endogenous PTX substrates G_i or G_o.

Furthermore, the ability of agonists to regulate intracellular cAMP level was investigated. In mammalian cells, opioid receptors couple to inhibitory G proteins of the $G_{i/o}$ family and inhibit the adenylyl cyclase[22]. Agonists DAGO and Ohm inhibited 10 μ *M* forskolin-stimulated increase in intracellular cAMP production in a dose-dependent manner with IC_{50} values of 0.9 n*M* and 0.3 n*M*, respectively (Fig 4A). The inhibition of cAMP accumulation was antagonized by opioid receptor antagonist naloxone (Fig 4B), indicating functionality of receptors with re spect to their signaling properties.

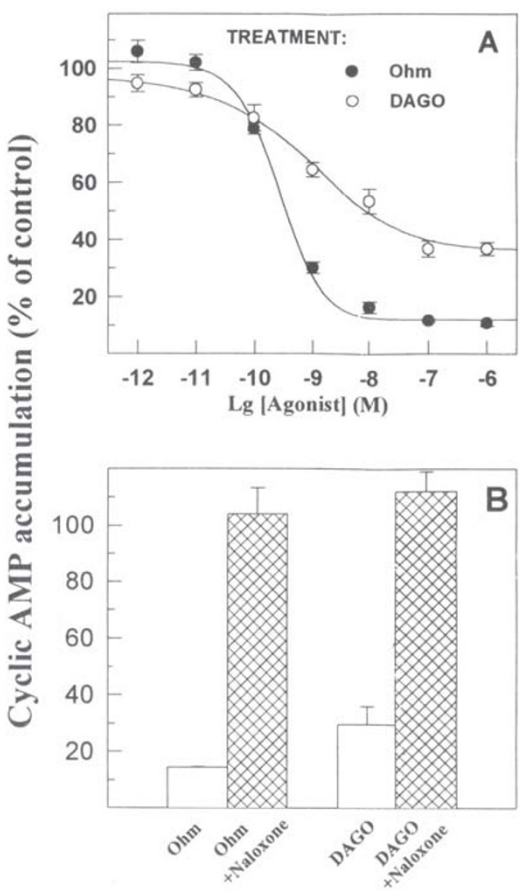

Fig 4.
Effect of agonist on forskolin-stimulated cAMP accumulation in Sf9 insect cells overexpressing HμOR.
(A) Dose response curves of agonist Ohm or DAGO. (B) Agonist Ohm (0.1 μM) or DAGO (0.1μM) inhibited the forskolin-stimulated cAMP accumulation, which could be reversed in the presence of antagonist naloxone (10 μM). Data were calculated as means $\pm$ standard errors from three independent experiments.

Massotte et al[9] reported that opioid receptors were overexpressed in baculovirus-infected Sf9 insect cell, however functional coupling to endogenous G proteins was not detected. Using baculovirus ～ "High 5" insect cell system[7], the functional studies revealed a dose-dependent inhibition by opioid on forskolin-stimulated cAMP synthesis, and this effect was potentiated by exogenous G protein.

In conclusion, our results showed the overexpression of HμOR in Sf9 insect cells retained the characteristics of HμOR and could functionally couple to endogenous PTX-sensitive $G_{i/o}$ proteins. Consequently, the baculovirus expression system is applicable for functional studies of HμOR. The advantages of baculovirus system include the quantitative and qualitative reproducibility of expression. In addition, Sf9 cells are easily maintained in suspension to large densities for biochemical studies without interference with cell growth. It has a potential for further research at both pharmacological and molecular levels. The HmμR 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 H μ OR.

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