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Analysis of binding domain and function of chimeric μ/κ opioid receptors to ohmefentanyl stereoisomers[1]

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KEY WORDS mu opioid receptors; kappa opioid receptors; COS-1 cell line; ohmefentanyl; stereoisomerism; U50488; binding sites; cyclic AMP

ABSTRACT

AIM: To investigate specific domains in μ opioid receptors that accounted for selective binding of three stereoisomers of ohmefentanyl (Ohm9204, Ohm9202, and Ohm9203) and study the function of chimera Ⅱ. **METHODS**: Rat μ and κ opioid receptors (RMOR, RKOR) and four μ/κ chimeric receptors (chimeras) Ⅰ, Ⅱ, Ⅲ, and Ⅳ were transiently expressed in COS-1 cells. The binding ability and binding domain of receptor to ligands were determined by radioactive ligand and receptor binding experiments. Through measuring cellular cAMP levels, we studied the function of chimera Ⅱ in mediating signal transduction. **RESULTS**: Binding affinities of four chimeric receptors were similar to wild type opioid receptors (RMOR and RKOR). The binding affinities of Ohm9204 and Ohm9202 to chimera Ⅱ were similar to that of RMOR. The binding affinities of Ohm9203 to all six receptors were low. U50488 possessed high binding affinity to chimera Ⅰ, however dynorphie A(1 - 9) had some binding affinity to chimera Ⅱ that was similar to RKOR, which indicated the domains of RKOR accounting for selectively binding to peptide ligand dynorphie A(1 - 9) and nonpeptide ligand U50488 were different. The efficacy of Ohm9204 and Ohm9203 on inhibiting forskolin-stimulated cAMP accumulation in cells transfected with chimera Ⅱ was similar to that in cells transfected with RMOR. **CONCLUSION**: Replacing 194 - 268 residues of RMOR with 185 - 262 residues of RKOR does not influence the ability of μ opioid receptor to bind Ohm9204 and Ohm9202 and the receptor mediated inhibition of cellular cAMP level.

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INTRODUCTION

The opioid receptors (OR), classified pharmacologically to μ (MOR), κ (KOR), and δ (DOR), are members of the G protein-coupled receptors (GPCR) family and consisted of 398, 380, and 372 amino acids respectively[1]. Stimulation of opioid receptors activates the pertussis toxin-sensitive $G_{i/o}$ proteins, which lead to an acute inhibition and subsequently to a reduction in cAMP levels in the cell[2,3]. All three opioid receptors are homologous in sequence comparison, but they are very different in the binding of type-selective ligands. Chimeric receptors of closely related receptors have been very useful in delineation of ligand binding domain of receptors. Studies on the ligand-binding characteristics of chimeric μ/κ receptors to selective ligands of κ opioid receptor led to the conclusion that the κ opioid receptor has differential binding domain for peptide and nonpeptide ligands[4]. But the binding domain to ohmefentanyl (Ohm) stereoisomers and the function of chimeric μ/κ receptor were not investigated. So in this study, we transiently expressed RMOR, RKOR, and four chimeric μ/κ opioid receptors in COS-1 cells, then investigated the regions of RMOR binding to Ohm stereoisomers and the effects of Ohm stereoisomers on inhibiting foskolin-stimulated cAMP accumulation mediated by RMOR and chimera Ⅱ.

MATERIALS AND METHODS

Chemicals Ohmefentanyl stereoisomers were synthesized by Shanghai Institute of Materia Medica,

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Chinese Academy of Sciences. [^{3}H] Diprenorphine ([^{3}H] Dip) (1.44 pBq/mol), naloxone, foskolin, dynorphine A(1 – 9) [DynA(1 – 9)], and U50488 were purchased from Sigma. The phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (IBMX) was purchased from CalBiochem. cAMP assay kit was purchased from Shanghai Second Medical University.

Cell culture and receptor expression COS-1 cells (gifts provided by Prof SHEN Qin-Xiang, Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % calf serum, benzylpenicillin 100 kU/L, and streptomycin 100 mg/L. Cells were grown in flasks at 37 ℃ in a humid atmosphere of 5 % CO_2. cDNA of RMOR, RKOR, and chimeras constructed in plasmid pcDNA3, gifts from Prof LIU-CHEN Lee-Yuan in Temple University (USA), were transfected into COS-1 cells as described[5], using DEAE-dextran method. Cells were harvested at 55 – 65 h following transfection and lysed in Tris-HCl 5 mmol/L, edetic acide 5 mmol/L, and egtazic acid 5 mmol/L, then spun at 4000 × g for 5 min. The pellet was stored at – 80 ℃.

Receptor binding assay Receptor binding was conducted with [^{3}H] Dip according to our published procedures[6]. Competitive inhibition of [^{3}H] Dip binding was performed with [^{3}H] Dip 0.2 nmol/L and 4 – 7 concentrations (from 0.01 nmol/L to 50 mmol/L) of the unlabelled ligands [Ohm9204, Ohm9202, Ohm9203, U50488, and DynA (1 – 9)]. The non-specific binding was determined by etorphine (Sigma) 1 μmol/L. Binding experiments were performed in 30 ℃ water-bathing for 30 min.

cAMP assay Cells transfected with RMOR and chimera Ⅱ for 55 – 65 h in flasks were washed with PBS buffer containing 1 % edetic acid, then incubated in PBS containing IBMX 0.5 mmol/L, forskolin 10 μmol/L, with or without Ohm9204, Ohm9202, Ohm9203, or naloxone 1 μmol/L, respectively. Then the cellular cAMP concentration was measured using cAMP assay kits as described[6].

Data statistical analysis Data from 3 – 4 independent experiments were analyzed with Gpip and Sigma Plot 4.0 programs. K_i values were calculated in terms of formula: $K_i = IC_{50}/(1 + [L]/K_d)$. L is the concentration of radioactive ligand and K_d is a constant.

RESULTS

Binding of [^{3}H] Dip to RMOR, RKOR, and μ/κ chimeras Binding of [^{3}H] Dip to chimeras Ⅰ, Ⅱ, Ⅲ, and Ⅳ was performed and compared with RMOR and RKOR. All the six receptors show binding activity to [^{3}H]Dip, which indicates all the six receptors are successfully expressed in COS-1 cells (Tab 1).

Binding of Ohm stereoisomers to RMOR, RKOR, and μ/κ chimeras None of Ohm9204, Ohm9202, and Ohm9203 showed evident binding activity to chimeras Ⅰ and Ⅲ, however Ohm9204 and Ohm9202 exhibited high binding activity to chimera Ⅱ and kept some but very low binding activity to chimera Ⅳ. This indicates that chimera Ⅱ, which is from RMOR in the region of 194 – 268 residues replaced by residues 185 – 262 of RKOR, does not change its binding activity to Ohm9204 and Ohm9202. However, chimera Ⅰ keeping the region of 194 – 268 residues of RMOR did not bind to Ohm9204 and Ohm9202 (Tab 2). This result accords with that of computer molecular simulation, which indicates that the interaction sites of Ohm and RMOR localized at Asp147 in transmembrane helix 3 (TMH3) and His319 in transmembrane helix 7 (TMH7)[7].

Tab 1. [^{3}H]Diprenorphine binding to RMOR, RKOR, chimera Ⅰ (κ1 – 148/μ194 – 268/κ380), chimera Ⅱ (μ1 – 193/κ185 – 262/μ269 – 398), chimera Ⅲ (κ1 – 141/μ151 – 398), and chimera Ⅳ (μ1 – 150/κ142 – 380). n = 3. $\bar{x} \pm s$.

	RMOR	RKOR	Ⅰ	Ⅱ	Ⅲ	Ⅳ
Binding of [^{3}H]Dip/pmol·g^{-1} protein	69 ± 6	49 ± 5	42 ± 5	55.0 ± 1.0	55.0 ± 2.0	34 ± 6

Tab 2. K_i values of ligands for RMOR, RKOR, and the four chimeric μ/κ receptors expressed in COS-1 cells. $n = 3$. $\bar{x} \pm s$.

Ligands	K_i/nmol·L^{-1}					
	RMOR	RKOR	Ⅰ	Ⅱ	Ⅲ	Ⅳ
Ohm9204	1.01±0.18	173±11	>1000	1.0±0.4	>1000	188±51
Ohm9202	3.18±0.18	>1000	>1000	28.3±1.8	>1000	40±5
Ohm9203	269±13	415±141	>1000	>1000	930±310	772±223
U50488	>1000	1.20±0.22	11±5	>1000	>1000	>1000
DynA(1-9)	>1000	176±3	>1000	147±55	>1000	>1000

Analyzing binding affinity of κ opioid receptor agonists κ Opioid receptor agonists, peptide agonist DynA(1 - 9) and non-peptide agonist U50488, show different binding domains to chimeric μ/κ receptors. U50488 maintained high binding affinity for chimera Ⅰ, however DynA (1 - 9) showed similar binding affinity to RKOR and chimera Ⅱ (Tab 2).

Acute effects of Ohm stereoisomers on cellular cAMP levels in COS-1 cells transfected with RMOR and chimera Ⅱ Both mediation of RMOR and chimera Ⅱ stimulated by Ohm9204, Ohm9203, and Ohm9202 inhibited foskolin-stimulated cAMP accumulation in COS-1 cells transfected with RMOR or chimera Ⅱ. In the COS-1 cells expressing RMOR, the inhibition percentage of Ohm9204 was the highest, secondly Ohm9202, thirdly Ohm9203. This order is correlated to their binding affinity to μ opioid receptor. In COS-1 cells expressing chimera Ⅱ, the inhibition percentage of Ohm9204 is 66 % ± 4 %, which is not greatly decreased compared with RMOR. Ohm9203 has similar result. Only the inhibition percentage of Ohm9202 (37 % ± 6 %) is evidently lower than 58 % ± 3 % ($P < 0.01$) which is obtained from cells expressing RMOR. Naloxone blocked the inhibitory effects of Ohm stereoisomers on foskolin-stimulated cAMP accumulation (Fig 1). It suggests that chimera Ⅱ functions similar to RMOR in mediating signal transduction.

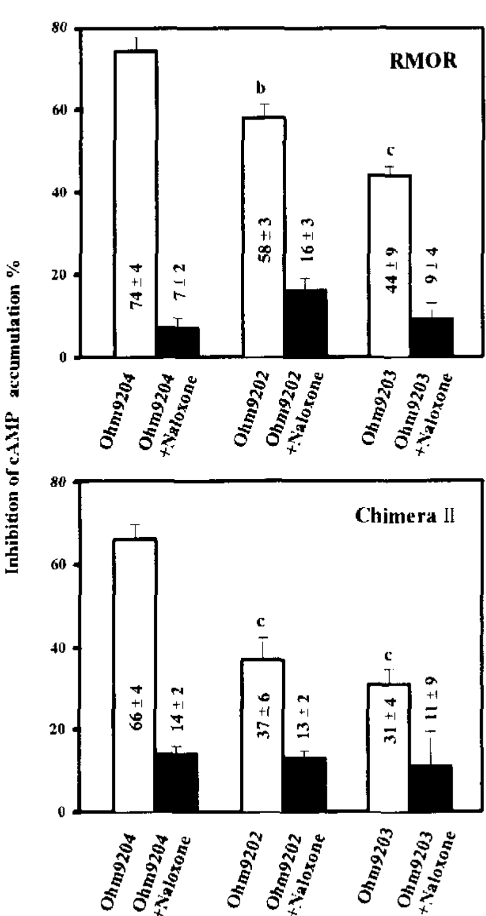

Fig 1. Acute inhititory effects of ohmefentanyl stereoisomers on forsklin stimulated cAMP accumulation in COS-1 cells expressing RMOR and chimera Ⅱ. $n = 4$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ vs Ohm9204.

DISCUSSION

Ohm has eight stereoisomers (Ohm9201 - Ohm9208). Ohm9204 (+)-*cis*-(3*R*,4*S*,2′*S*) and Ohm9202 (+)-*cis*-(3*R*,4*S*,2′*R*) are one pair of stereoisomers. They have similar binding affinities to μ opioid receptor and similar high analgesic activities. Ohm9203 shows the lowest binding affinity to μ opioid receptor and the lowest analgesic activity in Ohm eight stereoisomers[8]. In this study, K_i values of Ohm9204 and Ohm9202 to chimeras show that the region of μ194 - 268 in RMOR is not critical for high binding affinity of Ohm9204 and Ohm9202. Compared with Ohm9202 and Ohm9204, Ohm9203 has great difference in binding affinity.

Ohm9203 does not show very good specificity to μ opioid receptor with the K_i value for RMOR only half of that for RKOR, however the K_i value of Ohm9204 for RKOR is about 173 times higher than that for RMOR and the K_i value of Ohm9202 for RKOR is more than 314 times higher than that for RMOR. Ohm9204 and Ohm9202 have much stronger binding affinity for RMOR and chimera Ⅱ than Ohm9203 for RMOR and chimera Ⅱ. So this stereo-structure of ohmefentanyl, (3*R*, 4*S*) is more potent and matching for interacting with RMOR.

U50488 maintains high binding affinity to chimera Ⅰ, similar to RKOR. This result indicates that all the region of RKOR except the region of aa κ185 – 262 is important for high binding affinity of U50488 to RKOR. DynA(1 – 9) has similar binding affinity to RKOR and chimera Ⅱ, which may suggest the regions of RKOR accounting for selective binding to U50488 is different from that to DynA (1 – 9). Dynorphine (Dyn) is selective peptide agonist of κ opioid receptor with high binding affinity. However DynA (1 – 9) shows weak binding affinity to all the six receptors, which indicates that DynA(1 – 9) may be unique in all the congeners of DynA. The interaction of the ninth residue of DynA with other residues perhaps plays an important role in determining its affinity and selectivity to opioid receptors. DynA(1 – 9) has relatively stronger affinity to RKOR and chimera Ⅱ than that to other four receptors. This suggests that the region of aa κ185 – 262 is important for DynA(1 – 9) binding to RKOR.

The extracellular loops and transmembrane helices are essential for the interaction of opioid receptors and ligands, however intracellular loops (IL) especially IL3 and N-portion of C-terminal is important for receptors to interact with G protein[1]. RMOR and RKOR are highly homologous in IL3 with difference only in two residues. Chimera Ⅱ, compared with RMOR, has only one different residue in IL3, it was Leu in place of Met264[4]. The function of chimera Ⅱ is similar to RMOR in mediating acute effects of Ohm9202 on inhibiting forskolin-stimulated cAMP accumulation. This result indicates that μ194 – 268 replaced by κ185 – 262 does not influence signal transduction and Met264 is not important for RMOR to couple with G protein. The acute effect of Ohm9202 on inhibiting foskolin-stimulated cAMP accumulation mediated by chimera Ⅱ is much lower than that mediated by RMOR. This may result from the lower binding affinity of Ohm9202 to chimera Ⅱ than to RMOR. These results may lead to this conclusion that the effect of agonists on inhibiting foskolin-stimulated cAMP accumulation is related to the binding affinity of agonists to opioid receptor.

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嵌合 μ/κ 阿片受体与羟甲芬太尼异构体结合部位和功能分析[1]

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关键词 μ 阿片受体；κ 阿片受体；COS-1 细胞系；羟甲芬太尼；立体异构；U50488；结合部位；腺苷环磷酸

目的：研究嵌合 μ/κ 阿片受体与羟甲芬太尼异构体的结合部位及 chimera Ⅱ的功能. **方法**：在 COS-1

细胞中瞬时表达大鼠 μ、κ 阿片受体(RMOR、RKOR)以及四种嵌合 μ/κ 阿片受体 chimera Ⅰ、Ⅱ、Ⅲ、Ⅳ. 用放射性受体结合实验进行受体活性的测定和结合区域的分析. 通过测定胞内 cAMP 的水平，研究了 chimera Ⅱ 介导信号转导的特征. **结果**：四种嵌合受体对[^{3}H]Dip 的结合值类似于野生型 RMOR 和 RKOR. Ohm9204 和 Ohm9202 对 chimera Ⅱ的亲和力类似于 RMOR，Ohm9203 对表达的六个受体的亲和力均很弱. U50488 对 chimera Ⅰ 有较高的亲和力，而 dynorphine A(1－9)对 chimera Ⅱ 有类似于对 RKOR 的亲和力，表明肽类和非肽类配体结合 RKOR 的区域不同. 在表达 chimera Ⅱ 的细胞中，Ohm9204 和 Ohm9203 抑制 foskolin 刺激的 cAMP 水平增加的能力和在表达 RMOR 细胞中的相似. **结论**：用 RKOR 中的 185－262 氨基酸序列取代 RMOR 中194－268 氨基酸序列(即 chimera Ⅱ)对结合 Ohm9204 和 Ohm9202 及其介导的信号转导没有影响.

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