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Leucine-2-alanine enkephalin induced δ opioid receptors internalization expressed stably in CHO cells[1]

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KEY WORDS leucine-2-alanine enkephalin; CHO cells; radioligand assay; delta opioid receptors; fluorescence microscopy; diprenorphine; pertussis toxins; staurosporine

ABSTRACT

AIM: To characterize the internalization of δ opioid receptors (DOR) stably expressed in Chinese hamster ovary (CHO) cells and the role of the C-terminal in this process. **METHODS**: Receptor membrane anchoring was shown by immunofluorescence microscopy. Receptor internalization was assessed by measuring the radioligand binding resistant to the acid-buffer wash. **RESULTS**: Originally, all the wild-type (CHO-W) and C-truncated (CHO-T) DOR expressed were localized to the membrane. Agonist [^{3}H] leucine-2-alanine enkephalin (LAE) but not the antagonist [^{3}H]diprenorphine (Dip) induced rapid receptor internalization. The internalization of C-truncated DOR in CHO-T was similar to that of the wild-type in maximal level, but climbed up more slowly. DOR internalization was extracellular osmolarity- and temperature-sensitive. Pertussis toxin and universal protein kinase inhibitor staurosporine had no effect on it. **CONCLUSION**: DOR internalization is an agonist and clathrin-coated pits dependent, but post-receptor cellular signal transduction independent process; moreover, the C-terminal of DOR, not engaged in membrane anchoring, affects the initialization of DOR internalization.

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INTRODUCTION

Opioid receptors (OR), to which opioid compounds bind specifically, were characterized pharmacologically as G protein-coupled receptors (GPCR)[1]. Based on extensive studies of members in this receptor superfamily, agonist-dependent regulation of receptor on cellular level, such as functional desensitization, internalization, and down-regulation, is regarded as important molecular mechanism under tolerance[2]. Regulation studies of OR had ever been hampered by the absence of receptor clones until recently the three main kinds of opioid receptors, namely μ (MOR), δ (DOR), and κ (KOR), were cloned by some different groups[3]. After that, many protocols, working efficiently on other cloned GPCR, were copied to opioid research field. The story seems not so straightforward yet, for inconsistent data have been provided regarding opioid receptor regulation, which was shown to be closely associated with the receptor type, expression system, and some other unknown factors[4-9]. Although the main role of receptor internalization is still in debate (desensitization/resensitization), it is suspected to contribute greatly to the development of opioid tolerance, positively or negatively. We have formerly reported[10] the functional desensitization of the cloned DOR, and now we provide some characterizations of its internalization.

MATERIALS AND METHODS

[^{3}H] leucine-2-alanine enkephalin ([^{3}H]LAE, 1.20 PBq · mol^{-1}) was purchased from DuPont. [^{3}H]diprenorphine ([^{3}H]Dip, 1.44 PBq · mol^{-1}) was purchased from Amersham. Antibiotic G_{418} (G_{418}) was purchased from Life Technologies Inc. Monoclonal antibody 12CA5 was the product of Boehringer

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Mannheim. FITC-conjugated sheep anti-mouse IgG antibody was purchased from Sino-America Bio-engineering Inc. The cDNA of mouse DOR with the influenza hemagglutinin epitope tag (YPYDVPDYA, HA-tag) on its N-terminal recognized by 12CA5 in plasmid pcDNA 1/AMP was provided by Dr PEI Gang (Shanghai Institute of Cell Biology, Chinese Academy of Sciences).

Plasmid construction and transfection Plasmid construction and transfection were performed as described [10]. The cDNA of mouse DOR was subcloned to plasmid pcDNA3 and transfected into Chinese hamster ovary (CHO) cells by coprecipitation with calcium phosphate. To truncate the last 31 amino acids of the C-terminal of DOR, primer pair 5′-CGCG GGGA ATTC ACCA TGTA CCCC TACG ACGT CCCC GACT ACGC CGAG CTGG TGCC CTCT GCC-3′/5′-CATA ATCT CGAG TCAT TCTG GCGG CCGC AGGG GCGT-3′ was used to perform polymerase chain reaction (PCR). The product was cloned and transfected in the same way as the wild-type.

Cell culture and selection Transfected CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % calf serum. Positive clones were selected by addition of G_{418} 1 g·L^{-1} into medium and subsequently maintained in G_{418} 100 mg·L^{-1}.

Binding to intact cells and internalization Receptor internalization was assayed as described [11]. Cells harvested by PBS-0.1 % edetic acid were suspended in DMEM containing 0.1 % bovine serum albumin and [^{3}H]LAE (about 10 nmol·L^{-1}) or [^{3}H]Dip (about 10 nmol·L^{-1}), chilled on ice-water bath for 10 min, then moved to 37 ℃ or 4 ℃ for various period of time. Reactions were terminated by chilling cells again on ice for 10 min, and then, cells were immediately washed three times using ice-cold PBS buffer (pH 7.0 or 2.5) on GF/C filters and counted. The percentage of acid-resistant ligand binding ($binding_{pH\,2.5}/binding_{pH\,7.0}$) was regarded as the percentage of receptor internalized.

Immunofluorescence microscopy CHO cells were suspended in PBS containing 1 % BSA and fixed in cold methanol for 10 min. Fixed cells were immuno-stained by 12CA5 and FITC-conjugated sheep anti-mouse IgG antibody as described [12]. Samples were mounted in 50 % of PBS and 50 % of glycerol. Images were recorded using a confocal microscope (Bio-Rad, MRC1000-UV).

RESULTS

Receptor expression and localization in *CHO* cells After the transfection and selection, a typical CHO clone was obtained and designated CHO-W, which stably expressed DOR with an HA-epitope tag on its N-terminal as we had reported formerly [13]. The K_d value of [^{3}H]LAE binding to CHO-W was (2.39 ± 0.16) nmol·L^{-1} similar to that of the native DOR in the brain [14]. The maximal binding (B_{max}) was (4.3 ± 0.3) nmol·g^{-1} (protein). Meanwhile, the expressed receptor could agonist-dependently activate the endogenous G protein [10]. To determine the cellular distribution of the receptor expressed, it was immuno-stained by 12CA5 and FITC-conjugated anti-IgG antibodies, and visualized by confocal microscope. In the image recorded, all the DOR in CHO-W were localized correctly to the cell membrane (Fig 1A) without any resided in the cytoplasmic compartments.

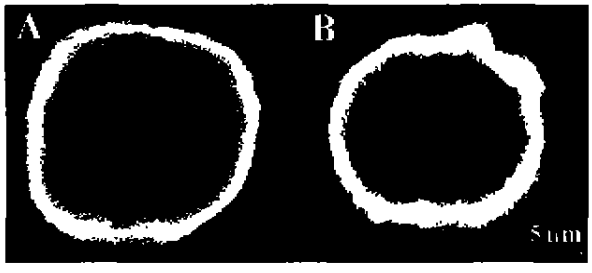

Fig 1. Analysis of surface expression of DOR in CHO-W (panel A) and CHO-T (panel B) by immunofluorescence confocal microsocopy.

LAE-induced DOR internalization Ligand binding to receptor is more endurable to acid wash when ligand-receptor complexes are sequestrated into the cytoplasm, than those dwell on the membrane. Measuring the ratio of the acid-resistant ligand binding to the total binding is a convenient and widely utilized method to assess the receptor internalization [15]. As plotted (Fig 2, CHO-W), when incubated with agonist [^{3}H]LAE, DOR expressed in CHO-W were internalized dramatically. This process was time-dependent and climbed to its maximal level in about 30

min. Contrasted sharply to [^{3}H]LAE, [^{3}H]Dip, a universal opioid antagonist, could hardly induce any receptor internalization at the same concentration. It suggested that internalization of DOR we observed could only be triggered by agonists.

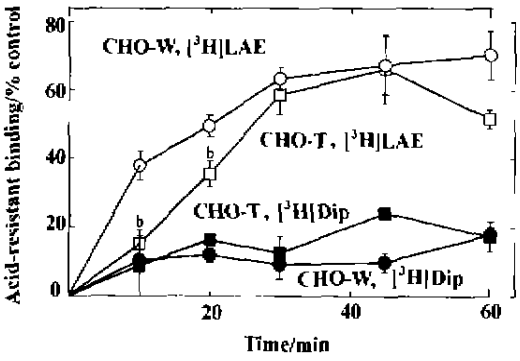

Fig 2. [^{3}H]LAE and [^{3}H]Dip induced DOR (CHO-W) and C-truncated DOR (CHO-T) internalization at 37 ℃ as measuring the percentage of acid-resistant ligand binding in total binding. $\bar{x} \pm s$. $^bP < 0.05$, CHO-T *vs* CHO-W.

Internalization of C-truncated DOR To address the role of C-terminal in DOR internalization, we created a C-truncated DOR (without the last 31 amino acids) and expressed it also in CHO cells. A typical clone, designated CHO-T, bound [^{3}H]LAE with K_d of (2.1 ± 0.6) nmol·L^{-1} and B_{max} of (1.13 ± 0.24) nmol·g^{-1}(protein). The same as CHO-W, all receptors expressed in CHO-T were localized on the cytoplasmic membrane despite the C-terminal truncation (Fig 1B). The truncated receptor underwent rapid internalization while incubated with [^{3}H]LAE but not with [^{3}H]Dip (Fig 2, CHO-T). The maximal levels of the internalization of the two type receptors were similar. C-terminal was not critical to the receptor internalization; however, the time-dependent course of it was slightly changed. For CHO-T, the acid-resistant binding of [^{3}H]LAE increased much slowly than CHO-W and climbed to the top at about 45 min.

Characterizations of DOR internalization Facts have been accumulated in the last several years that GPCR were internalized via clathrin-coated pits[15]. Clathrin-mediated internalization was sensitive to high extracellular hyperosmolarity and low temperature. In our experiments (Fig 3), when pretreated cells with sucrose 0.45 mol·L^{-1}, DOR internalization in CHO cell line was almost entirely abolished. At the same time, receptors were internalized poorly at 4 ℃. Although internalization of DOR could only be triggered by agonist, blockage of G protein activation by treating cells with pertussis toxins (PT) had no effect on it. Additionally, the nonspecific phosphorylation inhibitor, staurosporine (Stau), which was potent in spoiling the agonist-stimulated functional desensitization of DOR in the same clone[10], was inefficient in this study. It seems that G protein activation and receptor phosphorylation contribute little to DOR internalization.

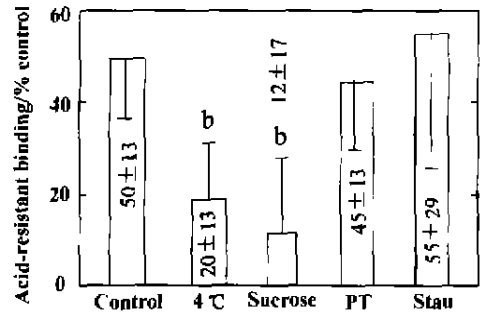

Fig 3. Effects of temperature and various agents on the LAE-induced internalization of DOR in CHO-W. $\bar{x} \pm s$. $^bP < 0.05$ *vs* control.

DISCUSSION

Though it is now a common view that GPCR are internalized via clathrin-coated pits, many points in this regulatory process are to be elucidated. The internalization of DOR could only be triggered by agonist; while pertussis toxins and Stau experiments reveal that the post-receptor signal introduction was not involved. A possible interpretation is that internalization of DOR in CHO cells just recognizes the activated conformation of receptor directly to occur.

Studies that revealed the C-terminal was dispensable and indispensable have both emerged regarding the agonist-induced GPCR internalization[16,17]. On DOR, this issue is also perplexing. An original report strongly suggested that receptor internalization depended on the phosphorylation of the C-terminal of DOR[6]. Most recently, internalization of the C-truncated DOR was observed in HEK-293 cells[7]. Here, we provided firstly a case in CHO stable expression system that the C-terminal truncation did not affect the maximal level of DOR but the

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initialization of DOR internalization.

Briefly, we conclude that internalization of DOR is a clathrin and agonist dependent but post-receptor cellular signal transduction independent process; moreover, the C-terminal of DOR, not involved in the membrane anchoring, was engaged in the initialization of receptor internalization.

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亮氨酸-2-丙氨酸脑啡肽诱导在中国仓鼠卵巢细胞中稳定表达的δ阿片受体内吞[1]

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关键词 亮氨酸-2-丙氨酸脑啡肽；中国仓鼠卵巢细胞；放射配位体测定；δ阿片受体；荧光显微镜检查；二丙诺啡；百日咳毒素类；星形孢菌素

目的：研究在中国仓鼠卵巢（CHO）细胞中稳定表达的δ阿片受体（DOR）内吞现象及其C-末端在受体内吞中的作用。**方法**：用免疫荧光共聚焦显微镜显示受体的着膜；以耐酸性缓冲液洗涤法测定放射性配体-受体结合量确定受体内吞程度。**结果**：表达的野生（CHO-W）和C-末端截短（CHO-T）的DOR都被正确转运到细胞膜上。激动剂[^{3}H]亮氨酸-2-丙氨酸脑啡肽（LAE）孵育诱导DOR快速内吞，拮抗剂[^{3}H]二丙诺啡（Dip）则不能；DOR内吞对胞外高渗透压和温度敏感，但不受百日咳毒素和广谱蛋白激酶抑制剂星形孢菌素的影响；C-末端截短的DOR在CHO-T中的最大内吞不变，但增加较缓慢。**结论**：DOR内吞是一种衣被小泡和激动剂依赖的过程，但不需受体后信号传导。C-末端与受体的胞膜锚定无关，但可影响受体内吞的初始化。

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