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Binding properties of C-truncated delta opioid receptors[1]

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KEY WORDS delta opioid receptors; mutation; CHO cells; diprenorphine, leucine-2-alanine enkephalin, binding sites; radioligand assay

AIM: To study the role of C-terminal delta opioid receptor involved in ligand binding affinity and selectivity. **METHODS:** The 31 amino acid residues of C-terminal truncated delta opioid receptors and the wild-type were expressed stably in Chinese hamster ovary (CHO) cells, respectively. Then the ligand binding properties of the products were studied by receptor binding assay. **RESULTS:** A typical mutated receptor clone CHO-T and a wild-type receptor clone CHO-W were obtained. The K_d values of [^{3}H] diprenorphine (Dip) and [^{3}H]leucine-2-alanine enkephalin (DADLE) bound to CHO-T were similar to CHO-W. Both the specific [^{3}H]Dip bindings of CHO-T and CHO-W were strongly inhibited by delta selective agonists with similar K_i, but neither by mu nor kappa selective agonists. **CONCLUSION:** The C-terminal of the delta opioid receptor is not involved in the ligands binding affinity and selectivity.

Opioid receptors (OR), classified pharmacologically to delta, mu, and kappa subtypes (DOR, MOR, and KOR), are members in the G-protein-coupled receptor family[1]. The successful cDNA clonings[2] and the availability of multiple agonists and antagonists[3] make it feasible to study the structure-function relationship of OR. Extensive studies carried on the G-protein-coupled receptor indicated that their C-terminal played important roles on the agonist-mediated cellular and molecular events[4-7]. As regards to OR, there was less work done on their C-terminal though sites conserved in the C-terminal of other G-protein-coupled receptors are conserved in the C-terminal of OR too[1,2]. Therefore, we truncated the C-terminal of the delta opioid receptor and investigate its role involved in the opioid binding affinity and selectivity.

MATERIALS AND METHODS

Plasmid pcDNA3 was purchased from Invitrigen. CHO cells were obtained from Shanghai Institute of Cell Biology. Antibiotic G_{418} (Geneticin) was purchased from Gibco BRL. [^{3}H]diprenorphine (Dip, 1.44 PBq·mol^{-1}) was purchased from Amersham. [^{3}H][*D*-Ala^2, *D*-Leu^5]enkephalin (DADLE, 1.20 PBq·mol^{-1}) purchased from DuPont. Unlabeled drugs were purchased from Sigma. Calf serum was purchased from Sijiqing Institute of Biomaterials, Hangzhou, China.

Plasmids construction All recombinant DNA procedures were conducted according to standard protocol[8]. Polymerase chain reaction (PCR) was performed on the cDNA of mouse DOR using primer pair 5'-CGCG GGGA ATTC ACCA TGTA CCCC TACG ACGT CCCC GACT ACGC CGAG CTGG TGCC CTCT GCC-3' and 5'-CATA ATCT CGAG TCAT TCTG GCGG CCGC AGGG GCGT-3' to truncate 31 amino acid residues of DOR's C-terminal. The product was cloned into pcDNA3 carrying an antibiotic G_{418}-resistant gene to generate pcDNA3-T which expressed the truncated DOR when transfected into CHO cells, while the wild type DOR was cloned into pcDNA3 as well to generate pcDNA3-W which expressed the wild type DOR when transfected to CHO cells. Both pcDNA3-T and pcDNA3-W were sequenced.

Cell culture CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % calf serum and antibiotic G_{418} 100 mg·L^{-1}. Cultures were maintained at 37 ℃ in a humidified atmosphere of 5 % CO_2 + 95 % air.

Transfection and screening The constructed plasmid pcDNA3-T and pcDNA3-R were transfected respectively into CHO cells by coprecipitation with calcium phosphate[8]. The

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positive clones in media containing antibiotic G_{418} 1 g·L^{-1} were isolated and transferred to the 24-well cell culture plate. After about 3 wk, cells were harvested in Tris-HCl 50 mmol·L^{-1} (pH 7.4) containing 0.2 % edetic acid and spun at 1000 × g for 10 min. The precipitation was resuspended in TE buffer (pH 7.4), containing leupepton 10 mg·L^{-1}, benzamidine 10 mg·L^{-1}, apretinin 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 resuspended and homogenized in Tris-HCl 50 mmol·L^{-1} (pH 7.4), containing 0.1 % bovine serum albumin. This membrane preparation was used for radioligand binding assay using [^{3}H]Dip at 4.2 nmol·L^{-1}. The clones were selected according to the ratio of cpm value to amount of protein.

Receptor binding assay The plasma membrane fragments of CHO cells were prepared as above. Receptor binding assays were performed in a final volume of 210 μL Tris-HCl 50 mmol·L^{-1} (pH 7.4) containing 20 – 30 μg of protein at 30 ℃ for 20 min. The nonspecific bindings were determined by the addition of unlabeled etorphine 1 μmol·L^{-1}. For saturation experiments, cell membrane was incubated with increasing concentration of [^{3}H]Dip 0.2 – 6.0 nmol·L^{-1} or [^{3}H]DADLE 0.2 – 7.0 nmol·L^{-1}. For competition experiments, cell membrane was incubated with [^{3}H]Dip 1.8 nmol·L^{-1} in the presence or absence of increasing concentration of competing ligands. The reaction was terminated by the rapid filtration over Whatman GF/C glass fiber filters pretreated with 0.5 % polyethyleneimine containing 0.1 % bovine serum albumin and washed with 4 mL of ice-cold Tris-HCl 50 mmol·L^{-1} (pH 7.4) for 3 times. The bound radioactivity was counted in a liquid scintillation counter. 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) were calculated from the IC_{50} and the K_d of [^{3}H]Dip using the Cheng-prusoff (1973) equation.

Each experiment was repeated 2 – 3 times with triplicated tubes.

RESULTS

Receptor binding characterization After transfection and screening, tens of positive clones that stably expressed the truncated receptors and tens that expressed the wild-type of high level were obtained. From them, two typical clones, one of which expressed the C-truncated DOR (CHO-T) and the other expressed the wild-type (CHO-W) as the control were chosen. The result of Scatchard analysis performed on them demonstrated that both of the specific [^{3}H]Dip and [^{3}H]DADLE binding to CHO-T and CHO-W were saturably and with a single binding site (Fig 1).

Neither [^{3}H]Dip nor [^{3}H]DADLE specifically bound to the native CHO cells (Data not shown). Two independent experiments yielded the B_{max} and K_d of [^{3}H]Dip and [^{3}H]DADLE bound to CHO-T and CHO-W (Tab 1).

Tab 1. Scatchard analysis of [^{3}H]Dip and [^{3}H]DADLE bound to CHO-T and CHO-W.

	Ligands	CHO-T	CHO-W
B_{max}(nmol/g protein)	[^{3}H]Dip	2.36±0.82	4.15±1.18
K_d(nmol·L^{-1})		1.35±0.05	1.67±0.65
B_{max}(nmol/g protein)	[^{3}H]DADLE	1.13±0.24	4.33±0.30
K_d(nmol·L^{-1})		2.11±0.60	2.39±0.16

According to the B_{max} of CHO-T and CHO-W, and our experiment that one microgram of membrane protein of transfected CHO cells was corresponding to about 2.5 millions of cells, the receptor numbers of CHO-T and CHO-W were estimated to be about 2×10^5 and 4×10^5 molecules per cell respectively.

Despite the C-truncated mutation of the receptor in CHO-T, it displayed similar K_d values to CHO-W when bound by [^{3}H]Dip and [^{3}H]DADLE. This result demonstrated that the deletion of the DOR's C-terminal had no effect on its affinity with opioid.

Ligand competition Competition experiments with [^{3}H]Dip by a variety of opioid ligands were done to investigate the binding selectivity of CHO-T and CHO-W (Fig 2).

The δ-selective agonists cyclic [*D*-Phe^2, *D*-Phe^5]enkephalin (DPDPE) and [*D*-Ser^2, *D*-Leu^5]enkephalin-Thr (DSLET) strongly inhibited the specific binding of [^{3}H]Dip to both CHO-T and CHO-W with similar K_i values of low nanomolar range; on the other hand, μ-selective agonist [*D*-Ala^2, *N*-MePhe^4, Gly-ol^5]enkephalin (DAGO) and κ-selective agonist *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl] benzeneacetamide methane-sulfonate (U-50 488) hardly inhibited even in high concentration and with K_i of high micromolar range (Tab 2). It revealed that the deletion of DOR's C-terminal did not change the

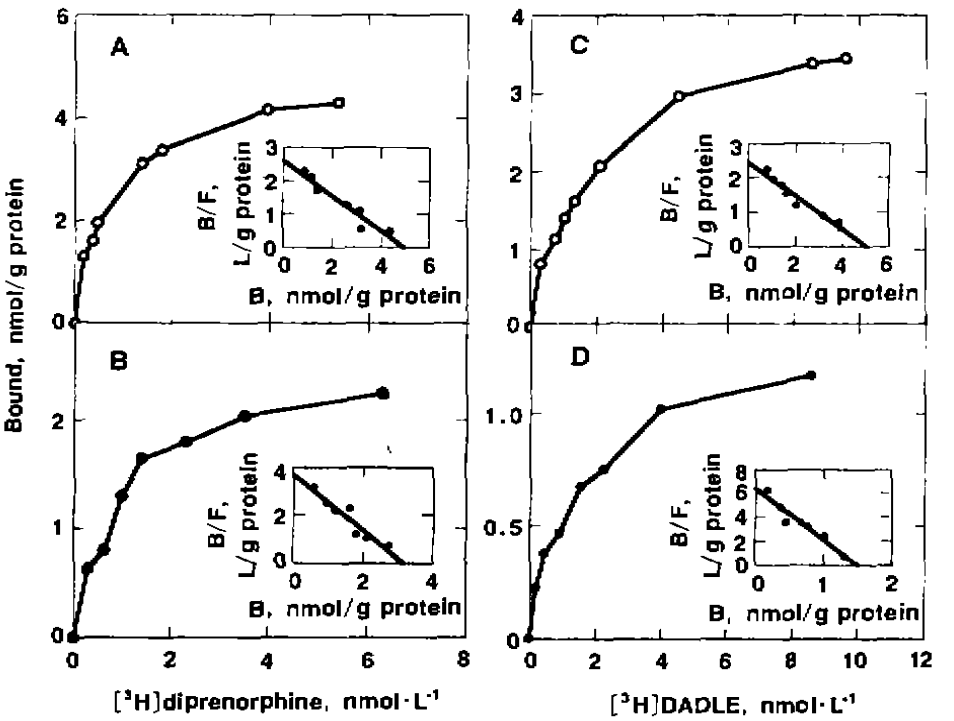

Fig 1. Saturable binding of [³H]Dip to CHO-T (A), CHO-W (B), and [³H]DADLE to CHO-T (C) and CHO-W (D). (Inset) Scatchard analysis of [³H]Dip saturable binding to CHO-T (A), CHO-W (B), and [³H]DADLE to CHO-T (C) and CHO-W (D).

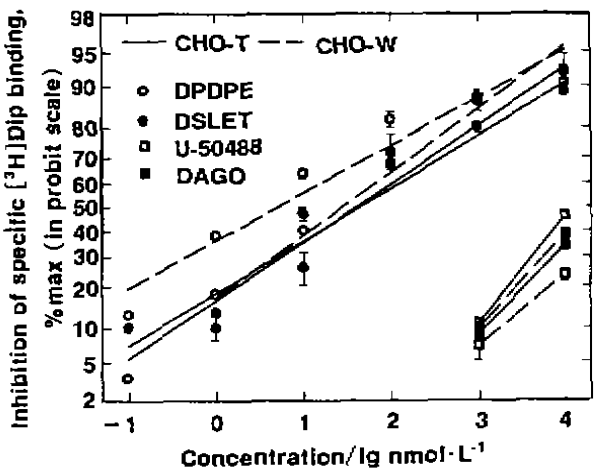

Fig 2. Dose response curves of inhibition of opioid ligands on specific [³H]Dip bindings to CHO-T and CHO-W.

ligand selectivity of receptor either.

DISCUSSION

The structure-function relationship studies of

Tab 2. The K_i (μmol·L⁻¹) of opioid ligands for CHO-T and CHO-W calculated by Cheng-Prusoff equation.

	K_i of CHO-T	K_i of CHO-W
DPDPE	0.0303±0.0052	0.027±0.0011
DSLET	0.0230±0.0034	0.0140±0.0011
DAGO	>1	>1
U-50488	>1	>1

OR were efficacious tools to understand the mechanism underlies the action of opioids. Here, we used the stable expression system of the C-truncated and wild-type DOR in CHO cells to investigate the role played by the 31 amino acid residues of DOR's C-terminal in opioid binding activity. The reason we chose the stable expression system was that it was much more homogeneous than the transient expression system.

From our experiments done on the system of CHO-T and CHO-W, we concluded that the C-terminal truncated mutation did not affect the

binding properties of the DOR. It was to say that the C-terminal of DOR was not a domain involved in ligands recognition and binding activity. This is a meaningful work for that none has reported it before.

However, data obtained from other G-protein-coupled receptors demonstrated that their C-terminals were important to the agonist-induced receptor desensitization and down-regulation. Recently, a report on DOR's C-terminal revealed that it did take a part in the chronic DADLE induced receptor down-regulation[10]. The comprehensive role of DOR's C-terminal are being further studied now in our laboratory.

At the same time, it may be too early to state that the C-terminal of DOR is completely dispensable in opioid ligand binding activities, for that to obtain two absolutely paralleling clones expressing the mutated and the wild-type receptors respectively is impossible in practice due to the difference of receptor numbers, copy numbers, physiological states of cells, and genes destroyed by the target cDNA fragments insertion. So, to provide more convincing evidences, more CHO cell clones expressing the mutated and wild-type DOR are to be investigated and more detailed researches should be performed.

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C-末端截短 δ 阿片受体的结合特征[1]

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关键词 δ阿片受体；突变；中国苍鼠卵母细胞；二丙诺啡；亮氨酸-2-丙氨酸脑啡肽；结合位点；放射配体分析

目的：研究δ阿片受体C-末端在受体结合配体的亲和力及选择性中的作用. **方法**：在中国苍鼠卵巢细胞(CHO细胞)中分别稳定表达C末端截短31个氨基酸残基及野生型δ阿片受体，用受体结合分析法研究表达产物与配体的结合特征. **结果**：表达得到典型突变受体克隆CHO-T及野生型受体克隆CHO-W. CHO-T结合[^{3}H]diprenorphine (Dip)及[^{3}H][*D*-Ala^2, *D*-Leu^5]enkephalin (DADLE)的K_d值与CHO-W一致，δ选择性激动剂对二者与[^{3}H]Dip的结合均有很强的抑制作用，且K_i相似；而μ及κ选择性激动剂则对二者均无抑制作用. **结论**：δ阿片受体的C-末端与受体结合配体的亲和力及选择性无关.